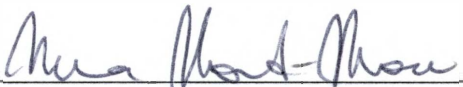
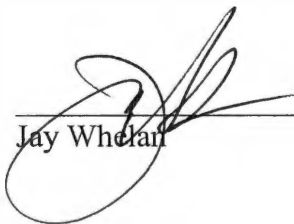


To the Graduate Council:

I am submitting herewith a thesis by Patrick Allen Wortman entitled "Prostaglandin E₂ secretion and effect on adipocyte lipogenesis". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

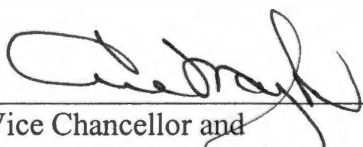

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Thesis
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Prostaglandin E₂ secretion
and effect on adipocyte lipogenesis

A Thesis
Presented for the
Masters of Science
Degree
The University of Tennessee, Knoxville

Patrick Allen Wortman
December 2004

DEDICATION

This thesis is dedicated to my wife, Leslie J. Wortman, whose support and dedication were essential for me to achieve this milestone in my life. She encouraged me to return to school, was there for me when the journey got difficult, and shared equally in the celebrations and disappointments. Thank you for your unconditional love and the many sacrifices you have made to allow me to reach this goal.

Thank you also to my mother, Sara Lawson, for the values she instilled in me and the love and support she has provided over the years.

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ABSTRACT

With the current focus on the role of n-3 PUFAs as beneficial dietary interventions in the fields of cardiovascular disease and cancer, there has been a vastly increased awareness and focus on the mechanism of action for these essential fatty acids.

Many of the effects of PUFAs are mediated via changes in prostaglandin (PG) levels. One primary substrate for PGs is arachidonic acid (AA) (20:4 n-6), which results in the production of 2-series PGs. Increased concentrations of AA have been shown to increase levels of PGE₂ in vitro and in vivo. The omega-3 fatty acid EPA (20:5 n-3) is of particular interest due to its incorporation into cell membrane phospholipids in competition with AA. EPA can also serve as a substrate for prostaglandin production when present in tissue and results in the production of 3-series PGs, which do not appear to produce the same effects as 2-series PGs, although data is extremely limited. The secretion of prostaglandins (PGs), and specifically PGE₂ has been demonstrated in human and rodent adipocytes, and the presence of PGE₂ receptors in these tissues has also been demonstrated. PGE₂ can act in an autocrine/paracrine fashion in adipocytes to decrease lipolysis via G-protein coupled receptors. Preliminary data from our lab on *Apc*^{Min/+} mice showed that cyclooxygenase (COX) inhibition significantly reduced the activity of fatty acid synthase (FAS) in adipose tissue, and PGE₂ receptor agonists partially but significantly reversed this effect.

Our overall objective was to determine whether treatment with EPA or selective COX-2 inhibitors would lead to a reduction in measured PGE₂ that would in turn regulate markers of lipogenesis. We hypothesized that increased levels of EPA would competitively reduce tissue AA levels, and consequently PGE₂ production, leading to decreased activity of markers specific to adipogenesis, specifically the FAS enzyme. Selective COX-2 inhibition was expected to produce similar effects via inhibition of COX metabolism of AA to PGs. Studies were conducted on 3T3-L1 adipocytes. Dose-response studies demonstrated a clear relationship between increasing doses of EPA or AA and measured PGE₂/PGE₃ levels. Manipulation of PGE₂ levels via addition of EPA to culture media led to significant changes in measured PGE₂ levels versus AA, but did not result in a significant decrease in FAS enzyme activity. Addition of celecoxib, a

selective COX-2 inhibitor (CI), to culture media resulted in a significant decrease in PGE₂ levels and FAS activity. Addition of exogenous PGE₂ to CI treatment did not reverse the decreased FAS enzyme activity. These results indicate that addition of EPA can displace AA in cell membrane phospholipids and reduce PGE₂ production, but does not impact FAS activity. Suppression of PGE₂ by selective COX-2 inhibition does lead to decreased FAS activity, which is not reversed by exogenous PGE₂ addition.

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ABREVIATIONS

AA	Arachidonic Acid
ACC	Acetyl CoA Carboxylase
ALA	Alpha-Linolenic Acid
BMI	Body Mass Index
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complimentary Deoxyribonucleic Acid
COX	Cyclooxygenase
cPLA ₂	Cytosolic Phospholipase A ₂
Dex	Dexamethasone
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl Sulfoxide
EIA	Enzyme Linked Immunoassay
EP	Prostaglandin E Receptor
EPA	Eicosapentaenoic Acid
EPR-A	Prostaglandin E Receptor Agonist
EST	Expressed Sequence Tag
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
LA	Linoleic Acid
Mix	Isobutyl Methylxanthine
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NIH	National Institutes of Health
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs

OA	Oleic Acid
PG	Prostaglandin
PGE ₂	Prostaglandin E ₂
PGE ₃	Prostaglandin E ₃
PPAR	Peroxisome Proliferator Activated Receptor
PUFA	Polyunsaturated Fatty Acid
RIA	Radio Immunoassay
RNA	Ribonucleic Acid
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SREBP	Sterol Regulatory Element Binding Protein
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Obesity rates in the United States continue to increase at an alarming rate despite increasing attention from medical professionals, government officials and the media regarding the seriousness of this problem. The most current statistics indicate that 64.5% of Americans are overweight ($\text{BMI} \geq 25$), and that 30.5% of those who are overweight can be classified as obese ($\text{BMI} \geq 30$) [1]. The direct impact of this problem is related to the increased risk of disease and mortality since obesity is a known risk factor for heart disease, hypertension, diabetes and some cancers [2]. The indirect implications of this epidemic of excess weight have manifested as a crippling financial burden to our healthcare system. For example, obesity-attributable medical expenditures were estimated to be \$75 billion in 2003 dollars with approximately one-half of these expenses paid by Medicare and Medicaid [3].

Recent work in the fields of cardiovascular disease and cancer has demonstrated a possible role for omega-3 fatty acids as beneficial dietary interventions, and as a result has greatly increased scientific and public awareness of these essential polyunsaturated fatty acids (PUFAs) [4-7]. One of the cornerstones for decreasing adiposity has been the recommendation to decrease caloric intake by lowering fat intake due to its high calorie density [2, 8]. This emphasis on low fat diets, although sound from a calorie intake standpoint, may have led to an inadvertent decrease in the consumption of essential fats, such as omega-3 fatty acids, in the typical American diet.

Many of the effects of these omega-3 PUFAs are mediated via changes in prostaglandin (PG) levels [4, 9-11]. PGs are chemical messengers derived from PUFAs selectively cleaved from the sn-2 position of cell membrane phospholipids [9] (see fig. 4 on page 12). Prostaglandin E_2 (PGE_2) is of particular interest in the area of energy metabolism and obesity, since it has been shown to decrease lipolysis, or triacylglycerol breakdown [12, 13], and potentially increase the activity of the fatty acid synthase (FAS) enzyme resulting in increased endogenous fatty acid production. One primary substrate for PGs is arachidonic acid (AA) (20:4 n-6), which results in the production of 2-series

PGs [4] such as PGE₂. Increased concentrations of AA have been shown to increase levels of PGE₂ in vitro and in vivo [5, 14].

The omega-3 fatty acid eicosapentaenoic acid (EPA) (20:5 n-3) is also incorporated into cell membrane phospholipids in direct competition with AA [15]. EPA can serve as a substrate for prostaglandin production when present in tissue and results in the production of 3-series PGs, which appear to produce weaker effects when compared to the 2-series PGs, although data remain extremely limited [16].

In addition to competitive inhibition with EPA, PG formation can also be inhibited via inhibition of the COX pathway [17-20]. The use of both non-selective COX inhibitors and selective COX-2 inhibitors has been shown to decrease PGE₂ secretion in adipocytes [12, 21-23]. This pharmacological intervention represents a means to precisely target PGE₂ metabolism to assist in understanding the role it plays in adipocyte metabolism.

Available data suggests the possibility of impacting aspects of lipid metabolism by manipulating PGE₂ levels. Accordingly, the goal of this research is to utilize EPA or celecoxib (selective COX-2 inhibitor) to inhibit AA metabolism to PGE₂ and determine the effect on markers of adipocyte lipogenesis.

More specifically our aims are:

1. To determine if the endogenous production of PGE₂ can be decreased by the addition of exogenous EPA or selective COX-2 inhibitor to cultured adipocytes.
2. To determine if a decrease in endogenous PGE₂ production by adipocytes results in a decrease in markers of lipogenesis, specifically the activity of the FAS enzyme.

Findings from this project may provide a foundation for further research that will help clarify: 1) The role for interventions aimed at decreasing PGE₂ synthesis in reducing lipogenic activity in adipocytes and 2) The public health recommendations for omega-3 fatty acids, and specifically for EPA, in the diet of Americans, including the potential roles of EPA in weight management.

CHAPTER II

LITERATURE REVIEW

I. OBESITY

A. Incidence and Implications

Body mass index (BMI) is the most consistently used measure to indicate excess weight, and is defined as weight in kilograms divided by height in meters squared {weight in kg / (height in meters)²} [2]. BMI is now universally used since the World Health Organization (WHO) recommendations and National Institutes of Health (NIH) report in 1997 and 1999, respectively [24, 25]. Although less accurate in certain populations, such as athletes, children and the elderly [26], BMI correlates well with both morbidity and mortality for most adults and provides a convenient, non-invasive and affordable tool in the clinical setting to classify overweight and obesity (Table 1) [2].

In recent years the incidence of overweight and obesity has received tremendous attention as these conditions have reached epidemic proportions in the United States as well as other countries. This is evidenced by a large volume of resources being invested into understanding the processes involved as well as the interventions that can most effectively halt and/or reverse these conditions. The most current statistics indicate that 64.5% of Americans are overweight (BMI $\geq$ 25), and that 30.5% of those who are overweight can be classified as obese (BMI $\geq$ 30) [1].

With increased weight comes increased risk of death and disease. For the average middle-aged adult, an excess of 10-20 pounds can increase the risk of mortality [27]. Younger adults are more impacted by excess weight than older adults, as indicated by data showing greater years of life lost to obesity than older adults [28]. Being overweight or obese is also implicated as a risk factor in several disease states, including diabetes, heart disease, hypertension, some cancers, and stroke [26], all of which contribute to the increased risk of death (Table 2) [29]. Recently it has been reported that approximately 400,000 deaths per year can be attributed to obesity related causes [30]. This makes obesity second only to smoking as a cause of preventable death in the U.S.

Table 1. Classification of Overweight and Obesity by BMI

Classification	Obesity Class	BMI (kg/m ²)
Underweight	---	<18.5
Normal	---	18.5-24.9
Overweight	---	25.0-29.9
Obesity	I	30.0-34.9
	II	35.0-39.9
Extreme Obesity	III	≥40

National Heart, Lung, and Blood Institute. Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults: the evidence report

Table 2. Rank of Selected Conditions in 2001 Leading Causes of U.S. Deaths

Rank	Condition	Number of Deaths	Percent of Total
1	Heart Disease	700,142	29
2	Cancer	553,768	22.9
3	Stroke	163,538	6.8
6	Diabetes	71,372	3.0
14	Essential Hypertension	19,250	0.8

Anderson, R.N., A.M. Minino, L.A. Fingerhut, M. Warner, and M.A. Heinen. *Deaths: injuries, 2001*. Natl Vital Stat Rep., 2004. 52(21)

Research has shown that obese individuals have 36% higher average medical expenditures when compared to normal weight individuals [31]. Estimates of U.S. obesity-attributable medical expenses were \$75 billion for 2003, with approximately one-half of these financed by Medicare and Medicaid [3]. As the number of overweight and obese individuals continues to climb, so will the burden placed on financial and medical resources of the U.S.

B. Causative Factors

The etiology of obesity is complex and multi-factorial including genetic, metabolic, environmental and behavioral factors [32]. Since genetic factors are predetermined, the primary factors that are controllable, and thus the target for intervention, are environmental and behavioral factors. Lifestyle modifications, and more specifically increasing activity while maintaining or decreasing caloric intake, are the cornerstones of the current effort to reduce the incidence of overweight and obesity [2, 26]. In 1998 it was estimated that 29% of adult Americans were sedentary, 44% participated in some physical activity but still did not meet the recommended 30 minutes of activity on most days, and only 27% of adults actually achieved the recommended amount of activity [33]. In addition to the limited activity prevalent in our society, caloric intake has also increased. During the time period from 1971-2000, average energy intake for men increased from 2,450 calories to 2,618 calories, and energy intake for women increased from 1,542 calories to 1,877 calories, with more recent data indicating that this trend has continued [34]. These specific lifestyle factors, coupled with other environmental influences on the genetics of obesity, have been the driving force behind the epidemic that now faces our nation [35].

II. POLYUNSATURATED FATTY ACIDS

A. Description and Metabolism

Fatty acids (FAs) are molecules that are composed of hydrocarbons with a methyl end (C-CH₃) and a carboxylic acid end (C-COOH). One means of classification of these fatty acids is relative to the number of double bonds. Polyunsaturated fatty acids (PUFAs) are FAs that contain two or more double bonds. From a nutrition standpoint, there are two sub-classes of PUFAs that are considered essential due to the proximity of the double bond to the methyl end, also called the omega end. Omega-3 fatty acids have the first double bond 3 carbons away from the omega end, thus the designation omega-3 and often written n-3. Omega-6 fatty acids have the first double bond 6 carbons away from the omega end and are thus termed omega-6 (n-6) (Fig. 1).

These two classes of FAs are essential for humans and must be obtained from the diet due to the lack of the enzymes necessary to add these double bonds to fatty acids without them. The two primary essential FAs in the American diet include the n-6 linoleic acid (LA) and the n-3 alpha-linolenic acid (ALA) since they can be metabolized into the longer bioactive arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). In the United States, PUFAs contribute approximately 7% of total energy intake and 19-22% of the energy intake from fat. LA, the primary n-6 PUFA consumed, comprises 84-89% of the total energy from PUFAs [36]. ALA is the primary n-3 fatty acid consumed, and makes up approximately 0.7% of total energy from PUFAs [36].

LA is converted to γ -linolenic acid (GLA, 18:3 n-6) via the Δ -6 desaturase enzyme, and subsequently converted to AA through the intermediate dihomo- γ -linolenic acid (DGLA, 20:3 n-6) via successive steps involving the elongase and Δ -5 desaturase enzymes. Following the same pathway, ALA is converted to EPA via the intermediates stearidonic acid (18:4 n-3) and eicosatetraenoic acid (20:4 n-3). This pathway then continues through further elongation, desaturation, and peroxisomal β -oxidation to produce docosahexaenoic acid (22:6 n-3) as a final end product (Fig. 2).

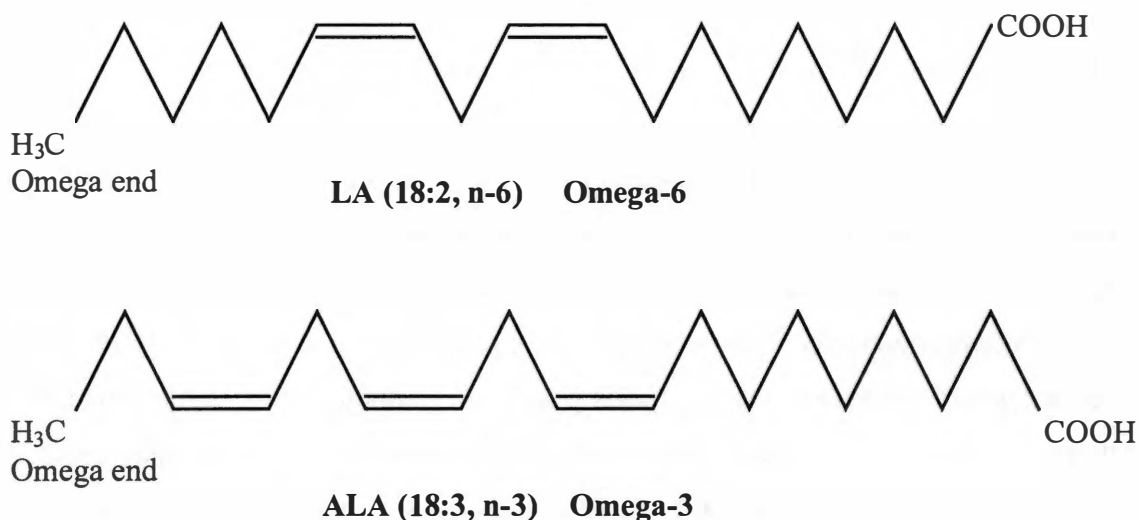


Figure 1. Linoleic Acid and Alpha-Linolenic Acid

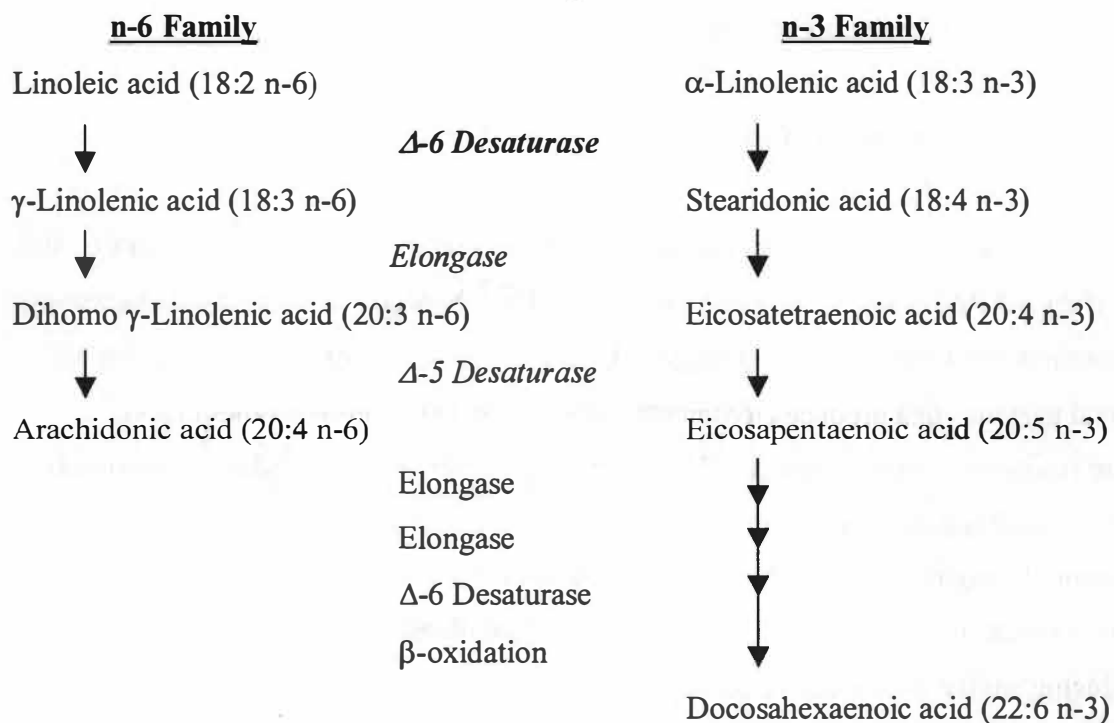


Figure 2. Arachidonic Acid and Eicosapentaenoic Acid Synthesis

The rate-limiting enzyme for this pathway is Δ -6 desaturase [37], and this aspect is critical from a nutritional standpoint. Since LA and ALA compete for this enzyme, and since the majority of the PUFA intake in the U.S. is made up of the n-6 fatty acid LA, the fatty acids produced in this pathway are skewed towards members of the n-6 family, with AA the predominate end product [38, 39]. Increasing intake of the n-3 fatty acid ALA is often recommended to competitively inhibit the production of AA, and has been shown to be moderately successful in some studies [15, 40, 41], although there is some evidence that even when consumed in a 1:1 ratio the tissue levels of n-6 fatty acids may exceed the tissue levels of n-3 fatty acids [42]. A second, and possibly more effective, means of reducing tissue levels of AA is to consume the preformed n-3 fatty acid EPA, which has been shown to compete with AA for incorporation into cell membrane phospholipids leading to increased levels of EPA [43-45]. The effectiveness of this strategy may depend on the foods from animal sources that make up an individual's diet, as studies in mice have shown that when preformed AA and EPA were fed in equal amounts, tissue phospholipids contained higher concentrations of AA [14].

B. Role In Weight Loss

Replacement of AA with fatty acids of the n-3 family in tissue phospholipids, and specifically EPA, is one of the mechanisms on which the recommendations for increasing consumption of these PUFAs are based [6]. AA serves as a substrate for the action of several enzymes that produce eicosanoids, specifically PGs, thromboxanes (TXs), hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (LTs) [4]. These eicosanoids serve as chemical messengers in intracellular and intercellular signaling, and many of the eicosanoids produced from AA have been implicated in inflammation and diseases states such as cancer [5, 10, 43, 46-48]. EPA can be metabolized by the same enzymes as AA producing similar but distinct eicosanoids which appear to have weaker action with regards to inflammation [16, 43, 49, 50]. Although the impact on inflammation is a major focus for the role of n-3 fatty acids, it is not the only means by which these PUFAs may impact disease conditions.

There is an increasing body of evidence that PUFAs themselves may impact disease conditions, and specifically the conditions of overweight and obesity. This impact is most likely a combination of the physiological, biochemical, and genetic effects of these fatty acids. From a physiological standpoint, dietary n-3 fatty acids increase cell membrane permeability [51], possibly influencing the selectivity and rate of substances crossing the membrane. In addition, PUFAs can reduce the triglyceride accumulation in skeletal muscle [52], and may impact accumulation in other tissues as well, which is associated with improvements in insulin sensitivity [52, 53].

From a biochemical standpoint, dietary PUFAs impact eicosanoid levels as discussed above with a wide range of consequences. In addition, PUFAs modulate the activity and gene expression of several proteins in either an eicosanoid-dependent or independent manner [54]. For example, PUFAs appear to impact leptin levels in a manner that may be independent of eicosanoid secretion [55-57]. Leptin is a hormone secreted by adipocytes that impacts food intake, energy expenditure, and energy balance while also potentially impacting insulin sensitivity and diabetes. Increased levels of circulating leptin are associated with decreased food intake, increased thermogenesis, and decreased adipose tissue mass [58]. Growing evidence suggests that leptin may be implicated in a variety of pathophysiologies associated with obesity, making it a focus in the battle against this epidemic. Rats fed diets high in n-6 or n-3 fatty acids had significantly higher leptin levels versus animals fed beef tallow, independent of fat mass [55]. In vitro, EPA was shown to significantly increase leptin secretion and mRNA expression in 3T3-L1 adipocytes [56]. Studies in rat and obese human adipose tissue explants showed that exposure to the AA metabolite PGE₂ from exogenous sources increased leptin levels [12, 59]. In contrast, AA was shown to decrease leptin secretion and mRNA expression in isolated rat adipocytes through a prostaglandin-independent mechanism [57]. Given this wide range of contrasting effects on leptin, the exact role and mechanism of PUFA action remains to be clarified.

The most powerful and well-studied role for PUFAs in the battle against excess weight is their impact on gene expression. Peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) have emerged as the

key targets that mediate PUFA effects. Both have affinity for one or more PUFAs, and impact genes involved in lipogenesis or lipolysis.

PPARs are transcription factors belonging to the superfamily of nuclear receptors and exist in three isoforms (α , δ , γ). They recognize and bind DNA response elements as heterodimers with nuclear retinoic acid receptors (RXRs). Activation of PPARs impacts several genes related to adipocyte development and metabolism [60]. PPAR- α is highly expressed in hepatocytes, cardiomyocytes, skeletal muscle, and enterocytes, and is the key player from a weight loss perspective as this isoform primarily promotes lipid oxidation [61]. PUFAs have been shown to act as ligands for all three isoforms, with the affinity for PPAR- α being the greatest [62-64]. In the liver, activation of PPAR- α induces the expression of transport proteins and enzymes involved in lipid oxidation and thermogenesis including carnitine palmitoyltransferase, peroxisomal acyl-CoA oxidase and uncoupling protein-3 [65-67]. The net result of these changes is to increase the use of fatty acids as fuel in mitochondria and peroxisomes and improve glycemic control. This was demonstrated in PPAR- α knockout mice, which developed characteristics of adult-onset diabetes such as fatty livers, elevated blood triglycerides, and hyperglycemia during periods of food deprivation [68]. Less is known at this time regarding PPAR- δ , but some evidence indicates that it may function in a manner similar to PPAR- α [69].

SREBPs are a family of membrane-bound transcription factors that regulate cholesterol and fatty acid homeostasis, with three isoforms (1a, 1c, 2) identified as well. PUFAs have been shown to suppress expression of SREBPs [70-72]. SREBP-2 appears to be involved primarily in cholesterol metabolism with only minor involvement in fatty acid metabolism [73]. The SREBP-1 isoforms (a, c) are derived from the same gene, which undergoes alternative splicing to produce the isoforms [74]. The 1a isoform is a much more potent activator of transcription than the 1c isoform [75], but the 1c form predominates in liver and adipocytes, although both forms are present [76]. Both isoforms have demonstrated a role in increasing lipogenic genes in liver and adipose tissue such as acetyl-CoA-carboxylase (ACC), FAS, and L-pyruvate kinase [71, 75, 77]. They have also been implicated in adipocyte hypertrophy [77]. Increased dietary intake of PUFAs would therefore lead to increasing expression of genes associated with

lipolysis and decreasing expression of genes associated with lipogenesis and a net benefit of decreased adiposity (Fig. 3). It is still uncertain whether SREBPs and PPARs work through the same pathway or distinct pathways, with both possibilities still under investigation.

III. ARACHIDONIC ACID METABOLISM

A. The Arachidonic Acid Cascade

Arachidonic acid obtained from diet or synthesized from LA is incorporated into cell membrane phospholipids. AA can be subsequently cleaved from these phospholipids by the action of various phospholipases, primarily cytosolic phospholipase A₂ (cPLA₂) (fig. 4). This occurs when cPLA₂ binds to the membrane phospholipid and cleaves the ester bond linking AA to the glycerol backbone.

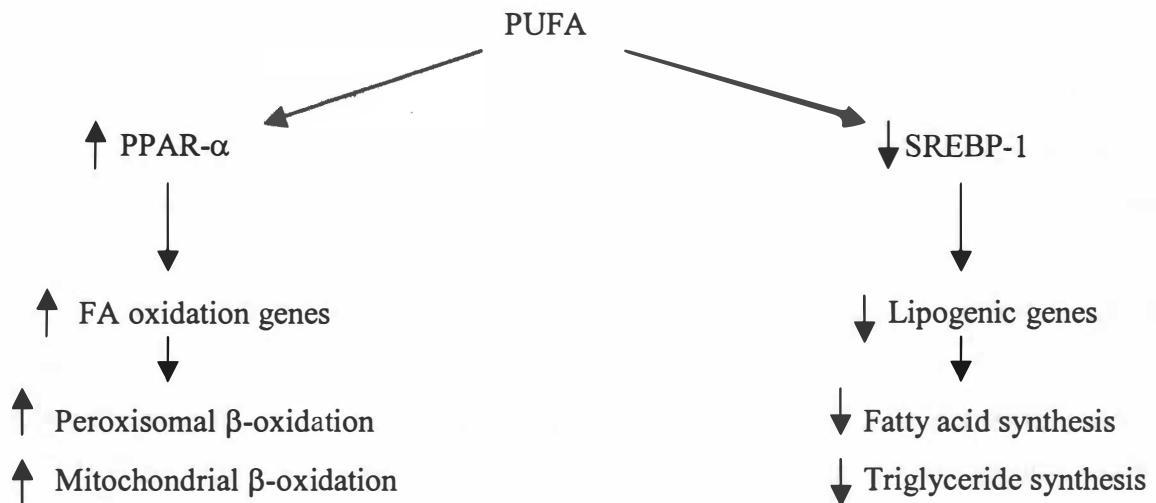


Figure 3. Impact of PUFAs On Gene Expression

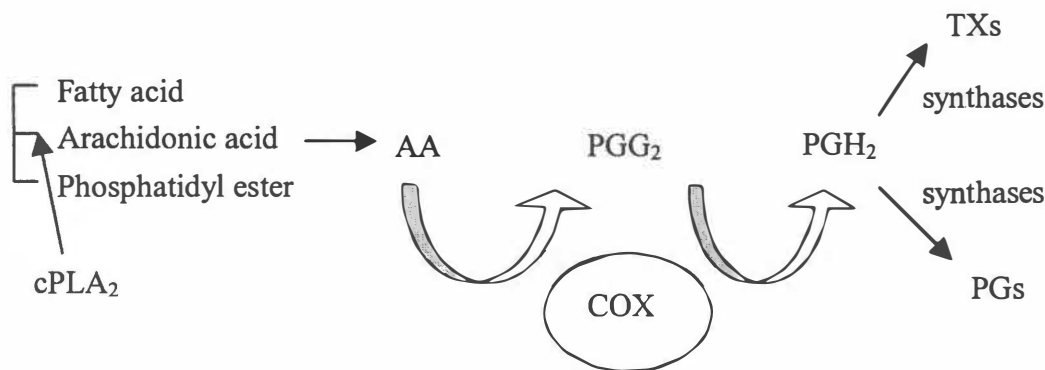


Figure 4. Metabolism of Arachidonic Acid

This initial step is also the rate-limiting step in the metabolism of AA. Liberated AA can then serve as a substrate for cyclooxygenase (COX) resulting in the production of prostaglandins of the 2 series [10].

B. Cyclooxygenase

COX (also called prostaglandin synthase) catalyzes the initial step in the formation of prostaglandins from the AA precursor via two distinct, sequential biochemical reactions [78]. The initial step converts AA via cyclooxygenase activity to the unstable PGG_2 , a peroxide intermediate. PGG_2 then migrates to the peroxidase active site where it is further metabolized to PGH_2 , which is the parent compound for the production of PGs and TXs (Fig. 4). COX exists in two isoforms, designated COX-1 and COX-2. COX-1 is found in most tissues and is constitutively expressed. It is considered a housekeeping enzyme with a role in maintaining tissue integrity. This function is of particular importance in the gastrointestinal (GI) tract, and is largely responsible for the increased incidence of ulcers and other GI complaints associated with the use of non-specific COX inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) [79].

COX-2 is considered an inducible enzyme and is stimulated by growth factors, inflammatory cytokines, and tumor promoters [17], although it is found normally in

tissues such as the kidneys [80]. COX-2 is highly homologous to COX-1, with 61% of the amino acids identical and 84% similar. The relevant difference between the isoforms is a slightly larger active site that forms a pocket on the COX-2 enzyme [79]. This has been the key to the development of the class of specific COX-2 inhibitors such as rofecoxib, celecoxib, and NS 398 [79].

The presence of COX has been demonstrated in preadipocytes and adipocytes [81, 82]. RT-PCR analysis of mRNA in Ob1771 preadipose cells and mature adipocytes demonstrated that both COX isoforms were present in these cells, with COX-1 present in much higher quantities. The levels of both forms decreased markedly during the differentiation process, with COX-1 decreasing approximately 98%, resulting in lower levels in adipocytes when compared to preadipocytes [21, 82]. The development of COX-1 and -2 knockout mice has brought to light a dynamic relationship between these two enzymes. Compared to wild-type mice, the knockout mice expressed the remaining functional COX gene at higher levels and both knockout models expressed 6-8 fold higher levels of basal PGE₂ versus the control mice [83]. In addition, evidence from COX knockout mice indicates that COX-2, when expressed, may be the primary enzyme involved in PGE₂ production in many tissues [22, 83].

C. The Role of EPA

The role of EPA in AA metabolism is one of direct competition. Consumption of preformed EPA bypasses the biosynthetic pathway, allowing increased concentrations to be available for incorporation into cell membrane phospholipids. The similarity in structure of these two fatty acids (Fig. 5) allows EPA to readily substitute for AA in the sn-2 position of triacylglycerols that constitute the cell membrane. Numerous studies have demonstrated that feeding preformed EPA results in higher levels in plasma and tissue phospholipids, with a concomitant decrease in levels of AA [5, 15, 84, 85]. Once incorporated into tissue phospholipids, EPA appears to be mobilized preferentially [86-88], and then competes with AA as a substrate for the COX enzymes. EPA is a poor substrate for both COX enzymes compared to AA [89], but is more efficiently

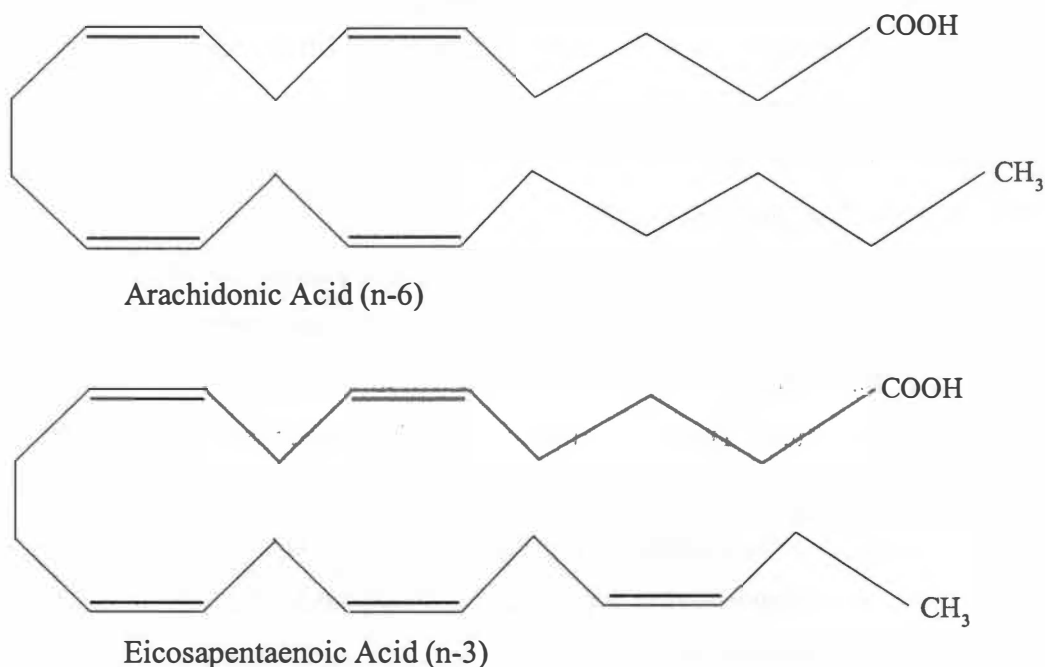


Figure 5. Structural Similarities of AA and EPA

metabolized by COX-2 [90]. Despite being a poor substrate, much evidence exists that the availability of EPA leads to decreased production of metabolites of AA [5, 16, 91, 92] via displacement of AA and also via inhibition of PGE synthase [93]. Due to its 3 double bonds, EPA is converted into 3-series PGs by the action of the COX enzymes. Data are still limited on the impact of these compounds, but they have shown weaker or antithetic effects on measures of inflammation when compared to PGE₂ [16]. One possible impediment to this role of EPA is the consumption of preformed AA in the diet. Consumption of preformed AA, even in the presence of equal amounts of EPA, may lead to increased amounts of AA in tissue phospholipids [14, 94]. This may be problematic in developed countries such as the U.S. where consumption of meat and animal products is high, leading to consumption of relevant quantities of AA [95, 96].

D. PGE₂ Production and Signaling

One of the most thoroughly researched metabolites of AA is PGE₂. It is a potent mediator of inflammation, produces local pain hypersensitivity, plays a role in fever and ovulation, and is associated with tumor promotion/survival and osteoporosis as well as many other normal and abnormal processes [18, 97, 98]. PGE₂ is formed as a result of PGE synthase (PGES) action on PGH₂ (see Fig. 4 on page 12). Two isoforms of PGES exist: cytosolic and membrane-associated. The cytosolic form is expressed in most cells and tissues and is predominantly coupled to COX-1. The membrane-associated form appears to be inducible and is preferentially linked to COX-2 [98]. Once formed, PGE₂ then acts in an autocrine or paracrine fashion via one of four receptor (EP) subtypes designated EP₁ through EP₄ (Table 3). The discovery and cloning of these receptor subtypes provided an explanation for the discordant effects of PGE₂, such as smooth muscle relaxation in some tissues and potent constriction in others [99, 100]. The EP₁ receptor is highly expressed in kidney, gastric mucosa and adrenal tissue, and activation results in signaling via inositol triphosphate (IP₃) generation and increased levels of intracellular calcium [101, 102]. Acting through the same signaling mechanism, activation of EP₂ or EP₄ results in increased levels of cAMP via stimulatory G-protein coupled receptors. Tissues high in EP₂ receptors include the uterus, lung and spleen. Tissues high in EP₄ receptors include the thymus, lung, spleen, adrenal, and kidney [101-105]. Activation of EP₃ results in inhibition of cAMP production via the inhibitory G-protein coupled receptors, with this receptor highly expressed in kidney, uterus, adrenal, and stomach tissue [102, 106]. The complex interplay of the COX enzymes, three subtypes of PGE, and four sub-types of its receptor have made teasing out the function of each individual protein or compound most difficult. The dynamic and compensatory nature of the enzymes, fatty acids, and their metabolites means that isolating one to study its function and mechanisms may cause abnormal alterations of others leading to disruption of normal physiology.

Table 3. Characteristics of EP Receptor Subtypes

Receptor	Tissues with high expression	Signaling mechanism	Selective agonists	Selective antagonists
EP ₁	Kidney Gastric mucosa Adrenal	↑ IP ₃ ↑ Ca ⁺⁺	Sulprostone Iloprost 17-phenyl PGE ₂	SL51089 SC53122P AH6809
EP ₂	Uterus Lung Spleen	G _s Protein ↑ cAMP	11-deoxy PGE ₂ 16,16 Dimethyl PGE ₂ Butaprost AH 13205	
EP ₃	Kidney Uterus Adrenal Stomach	G _i Protein ↓ CAMP ↑ Ca ⁺⁺	Sulprostone MB28767 GR63799 17-phenyl PGE ₂	
EP ₄	Thymus Lung Spleen Adrenal Kidney	G _s Protein ↑ cAMP	1[OH]-PGE ₁ 16,16 Dimethyl PGE ₂ MB 28767 Misprostol Iloprost	

IV. ADIPOCYTES AND PROSTAGLANDINS

A. Functions of Adipose Tissue

Adipose tissue was traditionally considered a passive tissue whose main role was energy storage in the form of triglycerides. It is now well established that contrary to this belief, adipose tissue is in fact a secretory tissue with the potential to impact multiple tissues and pathways. Serving in an endocrine and autocrine/paracrine role, adipose tissue secretes compounds such as leptin, angiotensinogen, adiponectin, PGs such as PGE₂ and PGI₂, cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), and acylation stimulating protein (ASP). These compounds act upon the secreting or neighboring cells and/or distant tissues to impact functions such as appetite and energy balance, immune function, insulin sensitivity and cardiovascular function [107-114]. Clarification and further understanding of the functions of these bioactive compounds, as well as the implications of adipocyte hypertrophy and hyperplasia on their secretion, is vital to understanding the etiology of obesity and its prevention/treatment.

B. 3T3-L1 Cells

The 3T3-L1 cell line is clonally isolated from Swiss 3T3 cells originally derived from 17-19 day old mouse embryos [115], and is one of the most commonly used cell lines to study adipocyte biology and function. These cells are fibroblasts or preadipocytes that are committed to adipocyte lineage. When grown in culture to confluence, they can be differentiated into mature adipocytes by supplementing culture media with dexamethasone (Dex) and methylisobutylxanthine (Mix) with or without insulin [116]. The differentiation process results in the cell morphology changing to a spherical shape, accumulation of lipid droplets, and other genetic and biochemical changes associated with the development of the mature adipocyte [115]. Addition of insulin to the differentiation cocktail serves to accelerate lipid accumulation and results in larger adipocytes. During and after differentiation, activities of enzymes involved in triglyceride synthesis are upregulated and the cells become responsive to insulin and

other hormones, making them excellent models for the study of mammalian adipogenesis [116-118]. In addition, biochemical and genetic conditions in the cell can be compared pre-and post-differentiation to help understand the process itself. This quality makes 3T3-L1 adipocytes an ideal choice for the study of adipocyte development and its role in obesity.

C. PGE₂ and EP Receptors

Numerous studies have demonstrated that adipose tissue and adipocytes in culture have the capacity to secrete PGE₂ under basal conditions and when stimulated by compounds such as catecholamines, theophylline, and the calcium ionophore A23187 [16, 81, 113, 119]. There was some controversy, however, as to whether prostaglandins produced from adipose tissue explants were produced by the adipocytes or were in fact produced by blood vessels and other non-adipose cells found within adipose tissue [120]. Recent work in human adipose tissue explants has demonstrated that human adipocytes do secrete PGE₂ [23, 81]. Adipocytes digested and maintained in culture secreted levels approximately 4% of tissue explants, while cultured adipocytes secreted levels approximately 38% that of tissue explants when both were exposed to endogenous AA [81]. In cultured 3T3-L1 cells, production of PGE₂ was several fold higher in preadipocytes compared to adipocytes, although it was still the main PG secreted during all stages of cell development [113]. In order for PGE₂ to act in an autocrine/paracrine fashion, adipocytes must also express one or more of the EP receptors. Receptors capable of binding PGE₁ were first demonstrated in rat adipocytes [121, 122]. Subsequent studies have demonstrated the expression of EP₁, EP₃, and EP₄ receptors in OB1771 preadipocytes as well as mouse precursor cells and mature adipocytes [123]. Studies conducted on human adipocytes indicated the presence of two or more PGE₂ binding sites with different affinities, but did not identify the receptors [13]. Taken together, these studies indicate that PGE₂ secreted by adipocytes is capable of acting in an autocrine/paracrine manner to regulate adipocyte metabolism.

D. PGE₂ Effects On Adipocytes

The primary and most immediate effect of PGE₂ on adipocyte metabolism is the inhibition of lipolysis through a reduction in cAMP [13, 124, 125], most likely mediated through interaction with the EP₃ receptor [126]. Further evidence indicated that the major impact of PGE₂ was on mature adipocytes versus preadipocytes, and that EP₃ receptor mRNA was expressed only in mature adipocytes [123, 127]. Lipolysis has displayed a biphasic response to PGE₂, with high levels stimulating this process [128], although this study has been refuted on the grounds that the antilipolytic action of adenosine was not controlled for in the earlier study [129]. Concurrent with a decrease in lipolysis, PGE₂ was also found to stimulate leptin release in primary cultures of mouse adipose tissue. Both effects were partially reversed by the administration of NS-398, a selective COX-2 inhibitor [130]. PGE₂ may also play a role in modulating adipocyte gene expression independent from its parent compound AA. Spot 14 is a nuclear protein found primarily in lipogenic tissues, and has been shown to increase in a manner that correlates with expression of lipogenic genes [131]. In 3T3-L1 cultures, AA was shown to suppress S14 and FAS mRNAs as has been demonstrated with other PUFAs, but the activity on S14 expression was reversed by the addition of a COX inhibitor. PGE₂ at a dose of 10 μ M replicated this suppression via a pertussis toxin sensitive alteration in cAMP levels [132]. Additional experiments looking at the effects of PGE₁, PGE₂, and PGE₃ on PPAR isoforms found no effect for any of the PGE variants in activation of these transcription factors [133]. It is clear that PGE₂ can and does impact the metabolic processes of lipolysis and lipogenesis in adipose tissue. What is not clear is the exact mechanism. The presence of multiple isoforms of the EP receptor that are capable of initiating similar, divergent or opposing signaling pathways makes it difficult to elucidate the actions of PGE₂, and may indicate a host of actions depending on the physiological conditions at the time. We therefore set out to clarify whether there is a specific role for PGE₂ in regulating FAS activity and expression.

CHAPTER III

EXPERIMENTAL SECTION

PROSTAGLANDIN E₂ SECRETION AND EFFECT ON MARKERS OF LIPOGENESIS

I. ABSTRACT

A dramatic rise in the incidence of obesity in the U.S. has accelerated the search for interventions that may impact this epidemic. Prostaglandin E₂ (PGE₂) has been shown to decrease lipolysis in adipocytes. Preliminary work conducted in our lab on mice has also demonstrated a potential role for PGE₂ in increasing the activity of a lipogenic enzyme, fatty acid synthase (FAS). Taken together, these two actions would lead to an increased accumulation of triglycerol in the adipocyte and increased adiposity. From a dietary standpoint, one potential means of manipulating levels of PGE₂, and possibly adipose tissue levels, is by increasing the consumption of preformed eicosapentaenoic acid (EPA) in the diet. The purpose of this study was to determine whether the concentration of PGE₂, and lipogenesis as measured by FAS activity, could be decreased by the addition of EPA or a selective COX-2 inhibitor. All studies were conducted using cultures of differentiated 3T3-L1 adipocytes. Dose response studies determined that 150 μ M concentrations of AA and EPA resulted in approximately a 5-fold difference in PGE₂ secretion between the two treatments. Despite effectively mediating the production of PGE₂ ($p < 0.05$), the addition of EPA resulted in no significant decrease in FAS activity. Addition of celecoxib, a selective COX-2 inhibitor, also significantly decreased PGE₂ secretion ($p < 0.05$) and did result in a significant decrease in FAS activity ($p < 0.05$). Unexpectedly, the combination of exogenous PGE₂ and celecoxib further decreased the FAS activity compared to PGE₂ alone or untreated controls. In conclusion, competitive inhibition of AA metabolism by EPA results in significantly

decreased production of PGE₂ when compared to AA treatment, but no significant decrease in FAS activity. Selective inhibition of the COX-2 enzyme also significantly decreases PGE₂ production resulting in a decrease in FAS activity that is not reversed with the addition of exogenous PGE₂, suggesting an additional mechanism that is not mediated by COX-2.

II. INTRODUCTION

The demonstration of endocrine roles for adipocytes has provided new insights helping to explain the etiology of obesity. The secretion of prostaglandins such as PGE₂ has been demonstrated in human and rodent adipocytes [16, 81, 113, 119]. The presence of PGE₂ receptors in these tissues as well [121-123] has led to research into a possible autocrine or paracrine role for this AA metabolite. Several groups have shown that endogenous PGE₂ decreases lipolysis in cultured adipocytes and explants [13, 124, 125], most likely acting through a G-inhibitory protein-coupled EP₃ receptor [126], confirming a paracrine/autocrine role of PGE₂ in adipocytes. Several other roles for PGE₂ in adipocytes have been investigated and include regulation of leptin release and lipogenic gene expression [11, 12].

Growth of adipose tissue is accomplished by increases in adipocyte size (hypertrophy) and number (hyperplasia) [134], and is a hallmark of obesity. Increases in adipocyte size are accompanied by an increase in stored lipids in the form of triglycerides, which can be formed from circulating lipids or *de novo* lipogenesis [135]. To support *de novo* lipogenesis, glucose must be present to form the glycerol backbone of the triglyceride, and enzymes of lipogenesis must be present to generate the fatty acids. AA acid has been shown to stimulate glucose intake in adipocytes by increasing glucose receptor levels in the cell membranes [136]. AA is also the preferential substrate for the COX enzymes and the resultant production of PGs [10, 89]. In mature adipocytes, PGE₂ is the primary PG produced from the COX pathway [113, 119]. Preliminary evidence from our laboratory conducted on adipose tissue samples from *Apc*^{Min/+} mice [18] indicated a possible role for PGE₂ in increasing FAS activity, an indicator of lipogenesis.

Further, adipose tissue from mice given the non-specific COX inhibitor piroxicam or sulindac showed significantly decreased FAS activity versus controls, while the addition of PGE₂ receptor agonists partially and significantly reversed this effect [137]. Coupled with the known effect of PGE₂ in decreasing lipolysis, this effect on FAS activity would lead to increased adipocyte triglyceride accumulation and hypertrophy.

Dietary EPA has demonstrated the capacity to impact adipocyte biological functions via two distinct mechanisms. The first is via transcription factors that impact the expression of genes involved in differentiation and lipogenesis. The second mechanism is via direct competition with AA for incorporation into membrane phospholipids and subsequent conversion to eicosanoids such as PGs via the COX enzymes. The first mechanism has been extensively studied, while few studies have addressed the second mechanism. The PGs (specifically PGE₃) produced by the COX action on EPA appear to have weaker action when compared to those produced by AA (PGE₂), making displacement of AA by EPA a favorable outcome for attempts to control excess adiposity.

Inhibition of AA metabolism to PGs can also be accomplished via pharmacological intervention, and may provide a more direct mechanism to impact PGE₂ actions. Compounds known to inhibit both isoforms of the COX enzymes, as well as selective COX-2 inhibitors, have been shown to reduce PGE₂ production in adipocytes [21-23, 81], and represent a second means to modulate PGE₂ levels and investigate the impact on lipogenesis.

The objective of this work is to investigate the impact of PGE₂ on fatty acid synthesis via regulation of the FAS enzyme. We hypothesized that addition of EPA or selective COX-2 inhibition would lead to a decrease in PGE₂ production and subsequent downregulation of markers of lipogenesis such as FAS enzyme activity (Fig. 6).

III. MATERIALS AND METHODS

A. Animals

Male C57BL/6J *Apc*^{Min/+} mice (Jackson Labs, Bar Harbor, ME) were obtained at 38-45

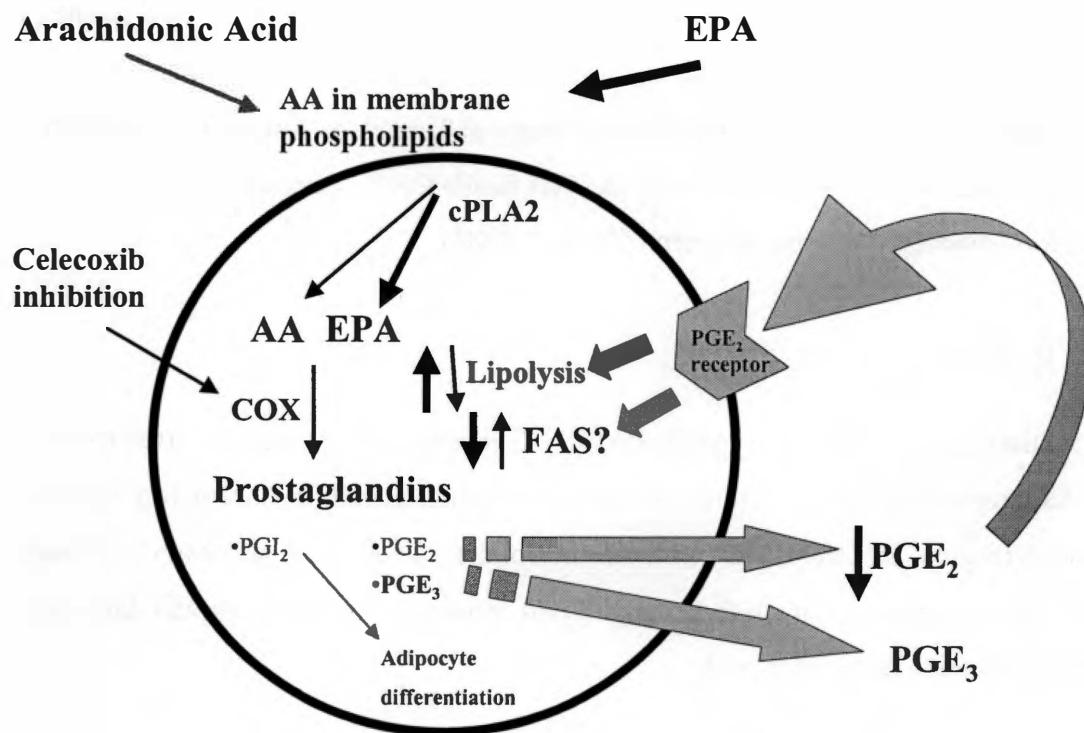


Figure 6. Proposed Mechanism of Action for Decreased PGE₂ Secretion Via EPA Competition

Gray arrows indicate autocrine function of PGE₂/PGE₃. Bold arrows indicate known/proposed EPA mediated changes in PGE₂ metabolism and action in adipocytes

days of age. They were housed in a temperature-controlled room with 14 h periods of light and 10 h periods of darkness and given free access to food and water. The health of the animals was checked daily. Food was withheld overnight prior to sacrifice. All animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985)

B. Diets

Diets were composed of purified AIN93-G powder diet (Dyets, Inc., Bethlehem, PA). Experimental diets containing NSAIDs were prepared daily by thoroughly mixing piroxicam (Sigma, St. Louis, MO) with the control diet. Diets were stored at -20°C and all animals were provided fresh food daily. Food consumption was monitored daily and body weights were recorded weekly.

C. 3T3-L1 Cell Culture

Cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and were grown in 100 mm dishes. Culture medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were plated on day 1 (~200,000 cells/100 mm dish) and grown for 3-4 days to confluence. At confluence, media was changed and supplemented with 250 nM Dex, 0.5 mM Mix, and 10 nM insulin for 48 h to induce adipocyte differentiation, after which cells were cultured with regular media. Differentiation was considered complete at 5-7 days post-confluence (see Fig. 7 for time line). Twenty-four hours prior to treatment, regular media was replaced with starvation media consisting of DMEM, P/S, and 1% fatty acid free bovine serum albumin (BSA). Treatment media consisted of starvation media + 10 nM insulin and individual (FA or inhibitor) treatments. All studies were conducted in differentiated cells (~80-95%).

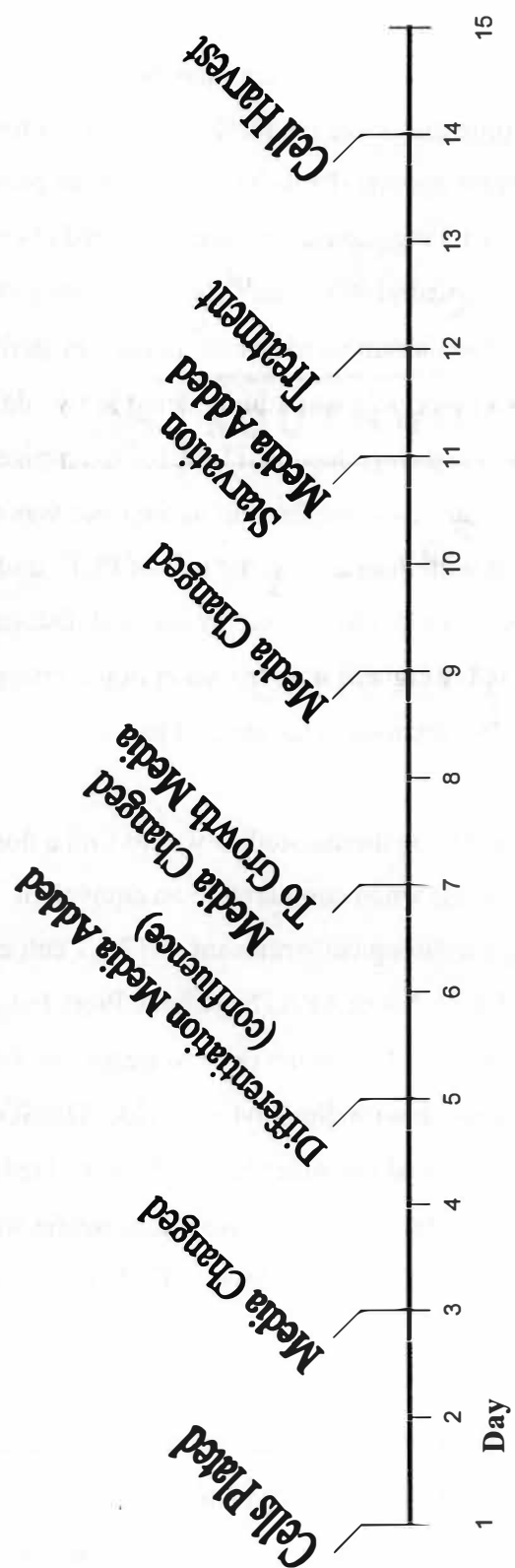


Figure 7. Timeline for Cell Culture

D. Experiment Design

Experiment 1 Male C57BL/6J *Apc*^{Min/+} mice were maintained on the AIN-93G diet until 78-79 days of age at which time they were randomly assigned to one of four groups {control, E-prostaglandin receptor agonist (EPR-A), piroxicam, or piroxicam + EPR-A}. Groups receiving piroxicam (0.5 mg/mouse/day) were pair fed to ensure equivalent dosing. The EPR-A, 16,16-dimethyl-PGE₂ and 17-phenyl-trinor PGE₂ (Cayman Chemical, Ann Arbor, MI), were administered as 10 µg each in sterile phosphate buffered saline. The EPR-A or vehicle was administered in two daily IP injections (0800, 1600) and once daily via gavage feeding (1200) to maximize exposure to the GI tract over a 6 day period. Primary care and feeding of the mice was conducted by Dr. Jay Whelan's lab in conjunction with his study on the role of PGE₂ and colon cancer [18]. The mice were sacrificed at 85-86 days of age by cervical dislocation and epididymal adipose tissue was harvested, weighed, snap frozen in liquid nitrogen and stored at -80°C. Liver weights were also obtained at the time of harvest.

Experiment 2 The goal of our dose response studies was to find a dose of EPA that would significantly reduce PGE₂ levels when compared to an equivalent concentration of AA, while remaining physiologically relevant. 3T3-L1 cell cultures were grown as above, and then treated with AA or EPA (Nu-Check Prep, Inc., Elysian, MN) in 25, 50, 100, 200, and 500 µM doses. Two hours prior to treatment, fatty acids were diluted to the appropriate concentration with dimethyl sulfoxide (DMSO), and then added to treatment media and agitated in a shaking water bath at 37°C to facilitate FA binding to BSA. Treatment time was 48 h for all doses. Two mL of media was removed from each dish prior to cell harvest for analysis of PGE₂ levels. Cell cultures were harvested for cytosolic extracts.

Experiment 3 This control experiment was conducted to verify that changes in PGE from adipocyte cultures treated with EPA were enzymatically driven. 3T3-L1 cell cultures were treated with EPA (50 µM and 150 µM concentrations), the selective

COX-2 inhibitor celecoxib (CI) (5 μ M) (Pharmacia, St. Louis, MO), and EPA (150 μ M) + CI (5 μ M). In addition, culture media with EPA at 150 μ M concentration was exposed to cell culture conditions to facilitate lipid peroxidation. 48 hours following treatments, two mL of media was removed from each dish to measure PGE₂ levels and cells were harvested for cytosolic extracts.

Experiment 4 The role of PGE₂ in regulating the activity of the FAS enzyme was investigated using 3T3-L1 cell cultures. Cell cultures were treated with vehicle (DMSO), CI (1 μ M), OA, EPA, AA, (150 μ M each treatment) or AA + EPA (75 μ M each fatty acid). All fatty acids were added to treatment media and agitated in a shaking water bath for 2 h at 37°C prior to treatment of cells to facilitate binding to BSA. Treatment time was 48 hours. Two mL of media was removed from each dish, and cell cultures were harvested for cytosolic extracts or total RNA.

Experiment 5 To verify that PGE₂ was responsible for the decrease in FAS activity seen with selective COX-2 suppression, PGE₂ was added back to mean levels measured in the control groups of previous experiments. 3T3-L1 cell cultures were treated with vehicle (DMSO), CI (1 μ M), PGE₂ (300 pM) (Cayman Chemical, Ann Arbor, MI), and CI + PGE₂. 48 hours following treatment, two mL of media was removed from each dish, and cells were harvested for cytosolic extracts or total RNA.

E. Methods

FAS assay The activity of FAS was determined in cytosolic extracts from mouse adipose tissue and cell culture by measuring the rate of oxidation of NADPH [138]. Mouse adipose tissue collected from experiment 1 was homogenized on ice in 500 μ L of buffer (pH 7.4) containing 250 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for ~ 30 seconds. Tissue homogenates were centrifuged for 1h (12,000 x g) at 4°C. The supernatant was then removed for analysis of FAS activity and protein concentration. Cell culture plates harvested for cytosolic

extracts were washed twice in Hank's balanced salt solution, and then scraped using 350 μ L of the buffer described above. Cell homogenates were sonicated on ice for 5 seconds and centrifuged for 1h (12,000 x g) at 4°C. The supernatant was then removed for analysis of FAS activity and protein concentration.

Oxidized NADPH absorbs light at a wavelength of 340 nm. The absorption change over a 5-minute time period was measured spectrophotometrically (Spectronic Genesys 5) and was used to calculate the nmol of NADPH oxidized per minute. These values were corrected for protein concentration.

Protein assay Protein concentration was determined in cytosolic extracts by the method of Bradford [139]. This assay utilizes the dye Coomassie blue (Bio-Rad, Melville, NY), which has an affinity for basic and aromatic amino acids. The normally red-brown dye absorbs light at 470 nm, but changes to a blue color when it binds to amino acids and the absorption spectrum changes to 595 nm. Each sample was measured in duplicate using 10 μ L of sample and 200 μ L of dye in a 96-well plate. After addition of the dye, the samples were incubated for 5 minutes and read in a spectrophotometer at 590 nm. A standard curve was plotted using serial dilutions of a BSA standard of known concentration and sample concentrations were extrapolated based on this standard curve.

PGE₂ assay Cell culture PGE₂ concentrations were determined using culture media samples obtained immediately prior to cell harvest. PGE₂ levels were measured by enzyme immunoassay (EIA) using the Correlate-EIA PGE₂ Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. A standard curve was plotted using serial dilutions of a known concentration of PGE₂ and sample concentrations were extrapolated based on this standard curve.

Leptin assay Cell culture media samples were utilized in an attempt to measure leptin levels. Initial attempts at measurement indicated very low leptin levels. Centrifugal concentration was conducted using centricon® YM-3 filters (Millipore, Billerica, MA) centrifuged at 7500 x g for 2 hours. Leptin measurement was attempted

by two different methods. Radioimmunoassay (RIA) is a competitive protein-binding assay, which utilizes a radiolabeled antigen (hormone) that competes with unlabeled hormone in the sample for binding sites on an antibody. Measurement of radioactive hormone bound to the antibody determines the amount of antigen present. This is compared to a standard curve calculated from known concentrations of hormone to determine the concentration of leptin. The RIA kit was purchased from Linco Research (St. Charles, MO). Samples were 100 μ L and were tested in duplicate. The second method attempted, Enzyme Immuno Assay (EIA) utilizing the Mouse Leptin ELISA Kit (Crystal Chem. Inc., Downers Grove, IL), utilized a more sensitive assay to detect the presence of leptin. The assay was conducted using the manufacturer's instruction. Sample volume was 5 μ L with samples tested in duplicate. A standard curve was constructed using samples of known concentration to determine leptin levels in culture media.

Real time RT-PCR Real time RT-PCR utilizes fluorescent dyes to allow quick and accurate quantification of mRNA, and is more sensitive than Northern blot methods [140]. A probe is used that is designed to bind specifically to the target mRNA sequence. This probe is labeled with 2 dyes: a fluorescent reporter dye on the 3' end and a quencher dye on the 5' end. When no target sequence is bound, the quencher dye resides in close proximity to the reporter dye and no fluorescence is detected. When a target sequence is present, the probe will bind it and the distance between the two dyes is extended to the point where fluorescence can now be detected. The intensity of fluorescence thus increases directly with an increase in the number of target mRNA sequences, which will reflect gene expression levels.

FAS mRNA expression was determined using this method. Cells harvested for total RNA were scraped in 350 μ L of Qiazol lysis reagent (Qiagen, Valencia, CA) and sonicated on ice for 5 seconds. RNA was extracted using the RNeasy™ lipid tissue midi kit (Qiagen) following the manufacturer's protocol. RNA was stored at -80°C for use in real time RT-PCR and microarray analysis.

The primers were ordered from Invitrogen (Carlsbad, CA). The sequence of the forward primer is 5'CCCAGAGGCTTGTGCTGACT 3'. The sequence of the reverse primer is 5'CGAATGTGCTTGGCTTGGT 3'. The probe was ordered from Biosearch Technologies, Inc. (Novato, CA). The sequence of the probe is 5' (TET)CCGATCTGGAATCCGCACCGG(TAMRA) 3'. TET is the quencher dye that inhibits the fluorescence of the reporter dye when in close proximity. TAMRA is the reporter dye and is measured at a wavelength of 580 nm. The SmartCycler SC1000-1 and SmartCycler software (Cepheid, Sunnyvale, CA) were programmed as indicated in Table 4.

Microarray analysis Microarray analysis is a relatively new technology used to measure gene expression by determining the amount of mRNA present in a sample [141]. This is usually done for the gene or genes of interest by comparing the expression of the gene(s) in a treatment group versus the expression of the same gene(s) in the control group. The foundation of this technology is a glass slide or “chip” that can contain up to 450,000 spots composed of complimentary DNA (cDNA) sequences for specific genes. mRNA samples from the control and 1 treatment group are converted to cDNA and labeled with a separate dye (normally green for control and red for treatment). The cDNA from each sample is placed on the same slide and incubated for 16-20 hours at 42°C. The slides are then washed and scanned by a laser that measures the intensity of each spot.

Table 4. RT-PCR Programming Menu

Stage	Temperature	Time (seconds)	Number of Cycles
1	48°C	1,800	1
2	95°C	600	1
3	95°C	15	40
	60°C	60	

Genes expressed by both the control and treatment will display approximately equal intensity of both colors, while a gene expressed predominantly by the control or treatment will display an intensity equal to that of the dye it was labeled with. The laser generates separate files with the intensity of the red and green dye for each spot on the chip. This data is then uploaded to software that combines the two files and displays the intensity of each spot. This is then used to calculate gene expression for treatment relative to the control.

Mouse cDNA microarray slides spotted with 15K mouse genes/expressed sequence tags (EST) in duplicate were obtained from University Health Network Microarray Center, Toronto, Canada. Total RNA from the control, CI, AA, EPA, AA + EPA groups in experiment 3 were labeled with Cy3 (control) and Cy 5 (treatments) dUTP dyes (Perkin Elmer, Shelton, CT). Hybridization was performed using the protocol developed by The Institute of Genomic Research [142]. The slides were scanned with a GSI 4000 scanner from GSI Lumonics (Perkin Elmer) and stored as TIFF files. The scanned images were merged using ScanAlyze software (developed at Stanford University) and uploaded into the Stanford Microarray Database (SMD) [143] installed at the University of Tennessee for analysis. Intensity values for each spot were normalized for background intensity and transformed logarithmically (base 2). Expression values are given as a ratio of red/green (treatment/control) intensity, with inter and intra experiment values for the same gene being averaged (control, CI, AA, EPA n = 4; AA + EPA n = 2) to obtain reported gene expression levels. For the purpose of this thesis, microarray analysis was only used to screen expression of a limited number of genes of interest. These genes include FAS, glucose transporters 1 and 4 (GLUT1, GLUT4), SREBP, prostaglandin E synthase (PGES), and two of the prostaglandin E receptor isoforms (EP₂ and EP₄).

Statistics Statistical analysis for all assays was conducted using SPSS (SPSS for windows, version 12.0, SPSS Inc., Chicago, IL). Data were analyzed for homogeneity of variance and for significant F ratios between treatment groups using one-way analysis of variance (ANOVA). Post-hoc analysis was conducted using the Bonferroni (equal

variance) or Dunnett's T3 (unequal variance) test when significant differences were detected. All values are expressed as mean $\pm$ SEM. Values of $p < 0.05$ were considered statistically significant except where otherwise noted.

Statistical analysis for microarray was conducted using SAS (Cary, NC) on log base 2 values. Outliers defined as observations >10 times the median absolute deviation away from the median were discarded. Normalization was done with a loess smoothing parameter of 0.3. Normalized and corrected data were analyzed with SAS mixed models [144], including the random effect of array and fixed effect of dye. Differences in means on the log scale are equivalent to ratios of gene expression, and the latter from SMD are reported in the appendix.

IV. RESULTS

Experiment 1. Effect of COX inhibition and EP receptor agonists in mouse adipose tissue

This experiment was conducted as preliminary work to assist in defining a role for COX specific products in modulating FAS activity. Non-specific inhibition of the COX enzymes with piroxicam resulted in decreased FAS activity ($p < 0.02$), while piroxicam + EP receptor agonists resulted in significant partial restoration of FAS activity ($p < 0.02$) (Fig. 8). Treatment with EP receptor agonists alone also resulted in a significant reduction in FAS activity when compared to control ($p < 0.02$).

Experiment 2. Dose-Response effect of EPA and AA on PGE₂ secretion in 3T3-L1 adipocytes

Consistent with studies that have shown the displacement of AA by EPA in tissue phospholipids [5, 84, 85, 145], the levels of PGE₂ were significantly lower when EPA was added to the cell culture media compared to equivalent concentrations of AA (Fig. 9). As expected, addition of AA led to a powerful dose-dependant increase in PGE₂ production. The two highest doses of EPA (200 μ M and 500 μ M) resulted in PGE₂ levels that were significantly higher than control ($p < 0.03$), although this is most likely

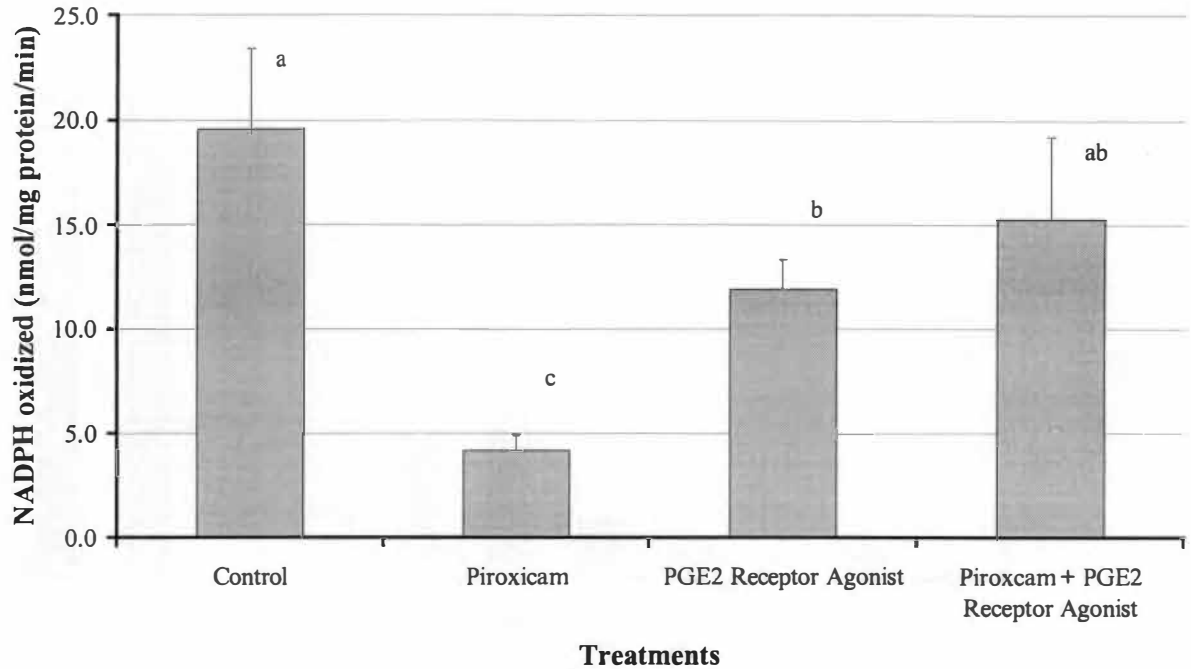


Figure 8. Effects of Piroxicam and PGE₂ Receptor Agonists On FAS Activity In Adipose Tissue From *Apc*^{Min/+} Mice

n = 6 for C and P + EPRA; n = 5 for P and EPRA

Values labeled with different letters are significantly different ($p < 0.02$). Values with the same letters do not differ significantly.

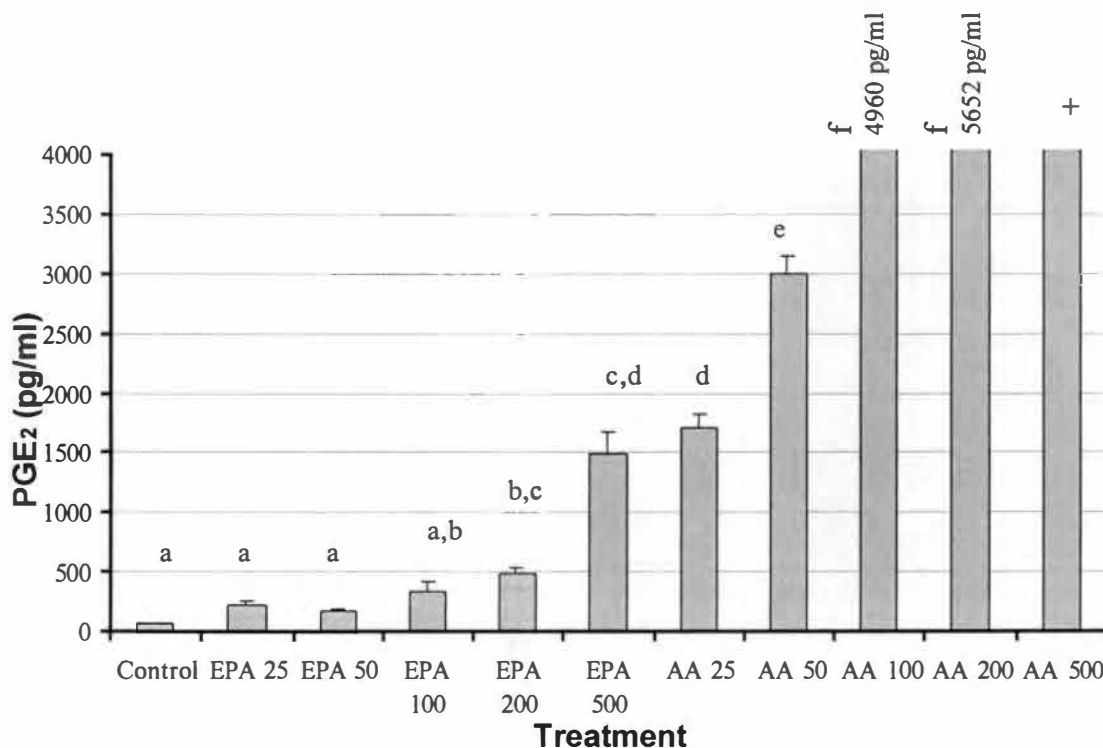


Figure 9. Dose-Response Effects of AA and EPA On PGE₂ Levels In 3T3-L1 Adipocytes

n = 5 for AA/EPA 500; n = 10 for C and AA/EPA 25; n = 15 for AA/EPA 50, 100, 200

Values labeled with different letters are significantly different ($p < 0.03$). Values with the same letters do not differ significantly.

+ Values for AA 500 μ M were beyond the accurate working range of the assay therefore no value is expressed.

attributed to PGE₂ EIA assay cross- reactivity for PGE₃, which is reported by the manufacturer to be 16.3%. FAS enzyme activity was analyzed for the various doses of EPA and AA and displayed no significant changes when compared to control (Fig. 10) except for AA 500 μ M ($p < 0.03$).

Experiment 3. Effect of EPA + CI treatment on measured PGE levels in cultured 3T3-L1 adipocytes

Addition of CI and EPA (150 μ M) led to a significant reduction in measured PGE activity ($p < 0.09$) (Fig. 11). This data recapitulates our previous experiment and demonstrates that the PGE formation is an enzymatic process, supporting the suggestion that the increased levels measured in EPA treated cell cultures are a result of PGE₃ formation. PGE levels for the other treatments used in this experiment were in line with what has been seen in our previous experiments.

Experiment 4. Effect of selective COX-2 inhibition and EPA addition on PGE₂ levels, FAS activity and FAS mRNA levels in 3T3-L1 adipocytes

PGE₂ levels were effectively reduced compared to control by the addition of CI ($p < 0.001$). All fatty acid treatments resulted in measured increases in concentrations of PGE₂ compared to control, with concentrations increasing in the order of OA ($p < 0.05$), < EPA < AA + EPA < AA (all $p < 0.001$) (Fig. 12). Consistent with the decrease in PGE₂ levels, FAS enzyme activity was significantly decreased by treatment with CI ($p < 0.05$). Treatment with EPA and AA resulted in trends similar to those seen from the dose-response study but did not reach statistical significance (Fig. 13). Attempts to measure leptin levels in culture media consistently resulted in concentrations just at or below the minimum sensitivity of both methods attempted. After several trials that included different cultures and culture conditions, we concluded that leptin levels were too low to be used as a marker for our studies and therefore did not include measurements of media leptin levels in this thesis. Expression of FAS mRNA analyzed by real time RT-PCR and microarray showed a significantly decreased expression for all FA treatments versus control ($p < 0.05$) (Fig. 14). The level of reduction of FAS mRNA for the FA treatments

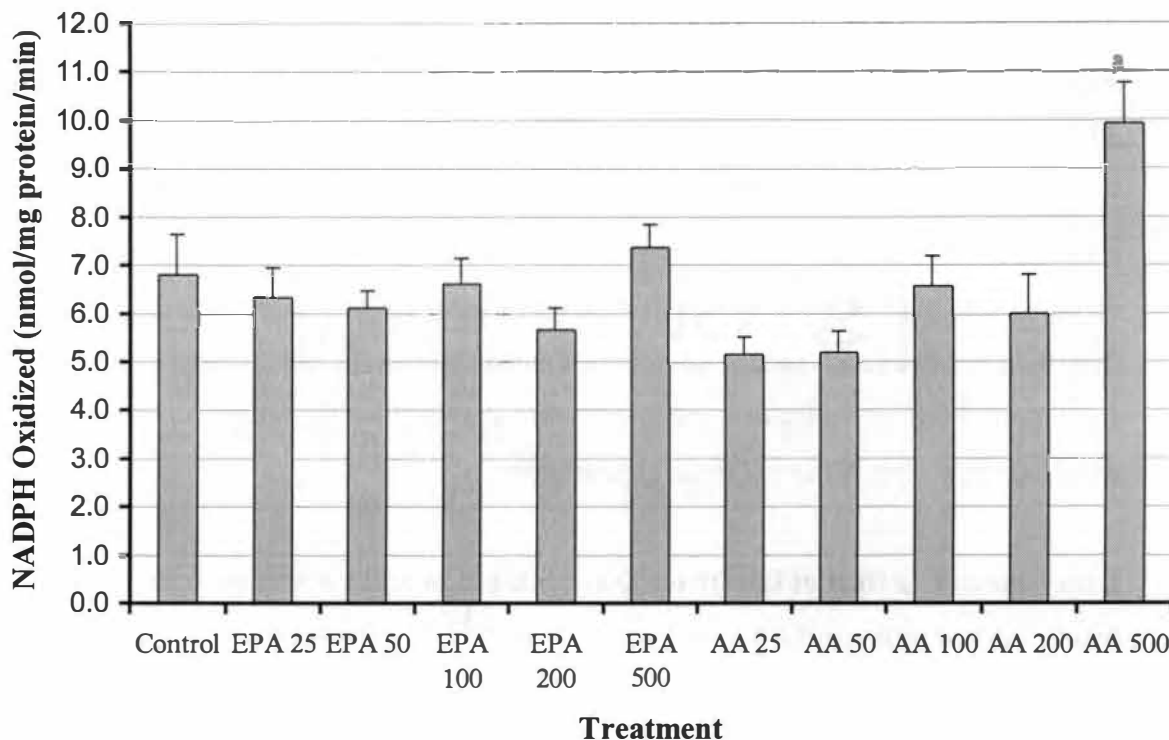


Figure 10. Dose-Response Effects of AA and EPA On FAS Activity In 3T3-L1 Adipocytes

n = 5 for AA/EPA 500; n = 10 for C and AA/EPA 25; n = 15 for AA/EPA 50, 100, 200

Values labeled with different letters are significantly different ($p < 0.03$). Values with the same letters do not differ significantly.

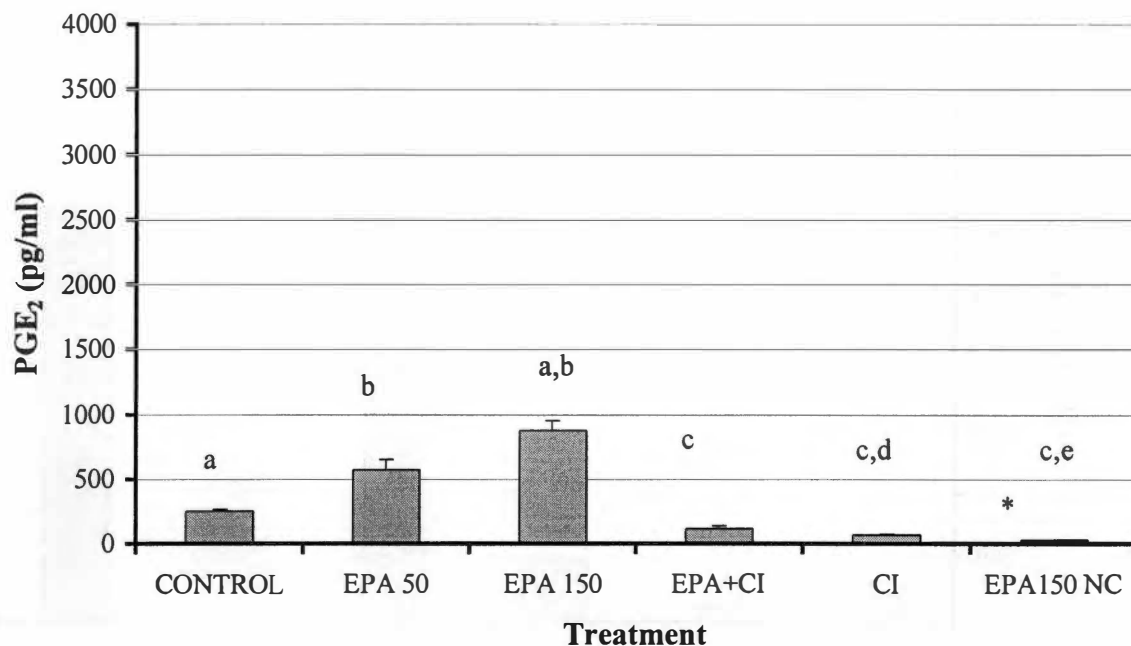


Figure 11. PGE₂ Levels of Cell Culture Media and 3T3-L1 Cell Cultures Treated With EPA and CI

n = 4 for C; n = 3 for all other treatments

* Denotes culture media exposed to cell culture conditions without cells.

Values labeled with different letters are significantly different ($p < 0.09$). Values with the same letters do not differ significantly.

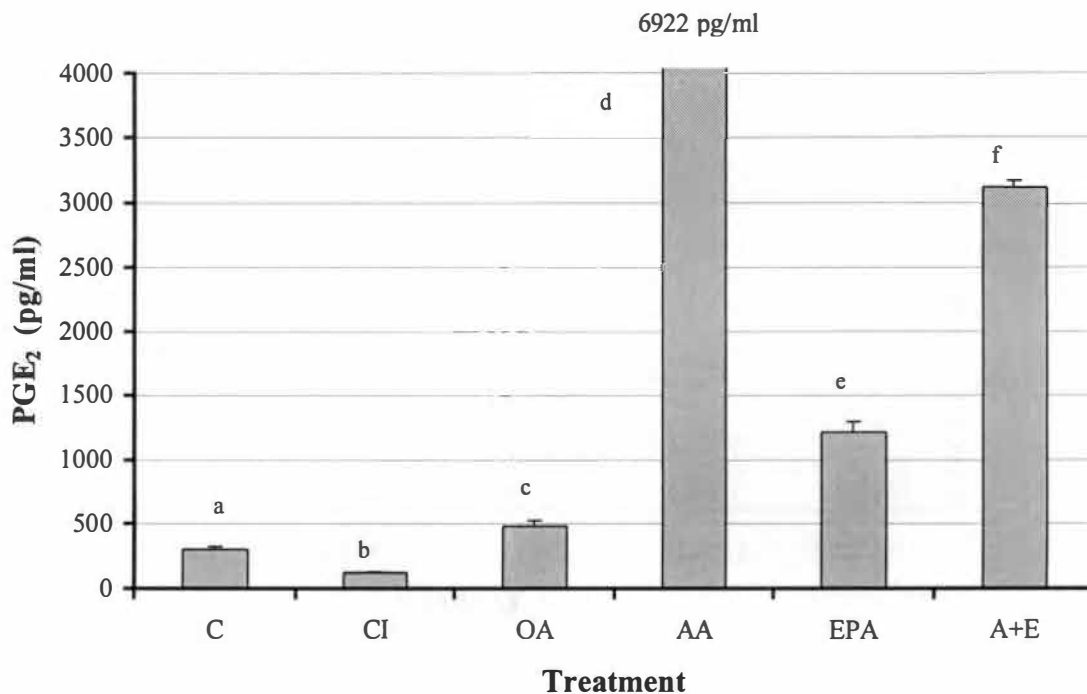


Figure 12. Effects of COX-2 Inhibition and EPA Addition On PGE₂ Levels In 3T3-L1 Adipocytes

n = 10 for all treatment groups

Values labeled with different letters are significantly different ($p < 0.05$). Values with the same letters do not differ significantly.

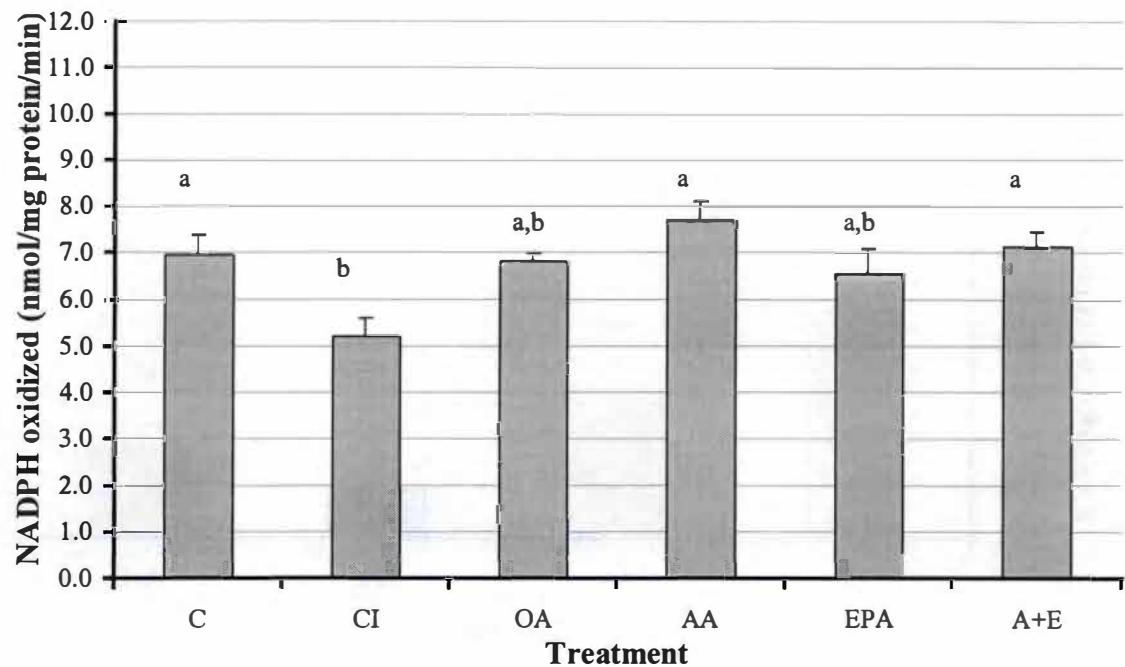


Figure 13. Effects of COX-2 Inhibition and EPA Addition On FAS Activity In 3T3-L1 Adipocytes

n = 10 for all treatment groups

Values labeled with different letters are significantly different ($p < 0.05$). Values with the same letters do not differ significantly.

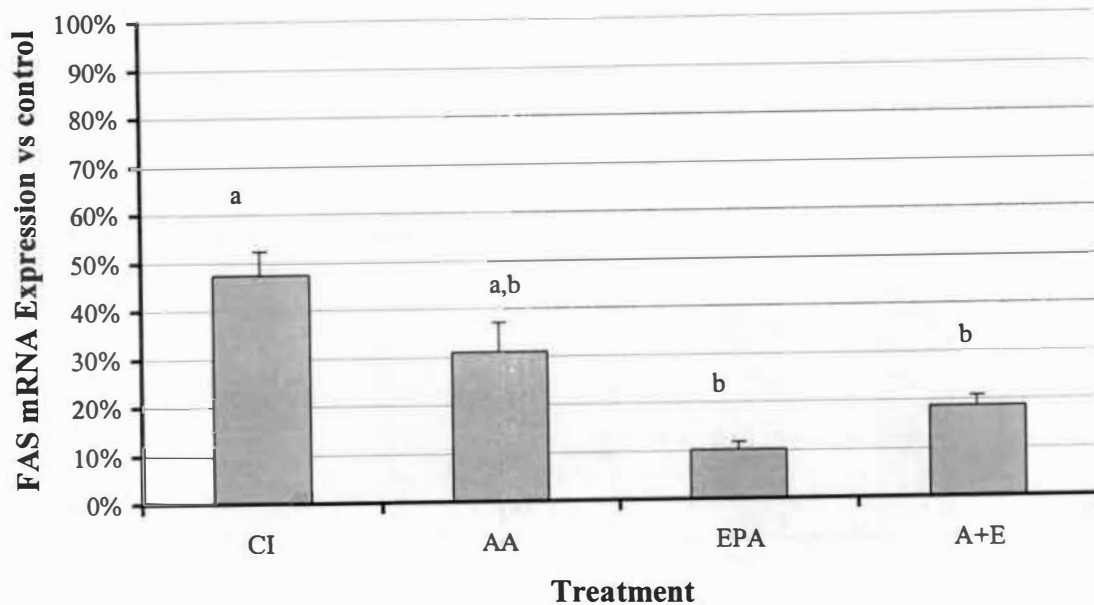


Figure 14. Expression Levels of FAS mRNA In Cultured 3T3-L1 Cells Treated With CI, AA, EPA, or AA + EPA Versus Control

n = 6 for all treatment groups

Values labeled with letters are significantly different from control ($p < 0.05$). Values with the same letters do not differ significantly.

correlates well with the degree of unsaturation and chain length and may therefore reflect a non-specific PUFA effect [70, 72].

Experiment 5. Effect of exogenously added PGE₂ on selective COX-2 inhibition of endogenous PGE₂ secretion, FAS activity, and FAS mRNA levels in 3T3-L1 adipocytes.

As demonstrated in our previous experiments, addition of CI resulted in a significant decrease in PGE₂ production ($p < 0.04$). Addition of PGE₂ to cultures with CI-treated cells restored PGE₂ levels and resulted in significantly higher levels versus CI treatment alone and versus control ($p < 0.02$) (Fig. 15). Addition of PGE₂ to cultures without CI resulted in the expected significant increase over control levels ($p < 0.02$). The level of PGE₂ added was based on the mean of previously measured levels of PGE₂ secreted by 3T3-L1 control groups (300 pM).

Analysis of FAS enzyme activity and mRNA expression, however, produced unexpected results. Treatment with CI resulted in a consistent decrease in enzyme activity that did not reach statistical significance as in the previous experiments. Treatment with CI plus exogenous PGE₂ actually resulted in a significant decrease in FAS activity ($p < 0.03$) instead of reversing it (Fig. 16). Addition of PGE₂ alone, in the absence of CI, resulted in no change in FAS activity. Real time RT-PCR analysis of FAS mRNA expression indicated that CI treatment had no significant effect in this experiment. In contrast, and in agreement with the combined effects of CI and PGE₂ on FAS activity, FAS mRNA expression was also decreased by the combined treatment vs. all other individual treatments (Fig. 17).

V. Discussion

Despite the vast amount of research that has been conducted on PGE₂, much still remains to be learned about its impact on adipocyte metabolism. It is well documented that PGE₂ can and does decrease lipolysis in adipocytes [13, 124-126], but we questioned

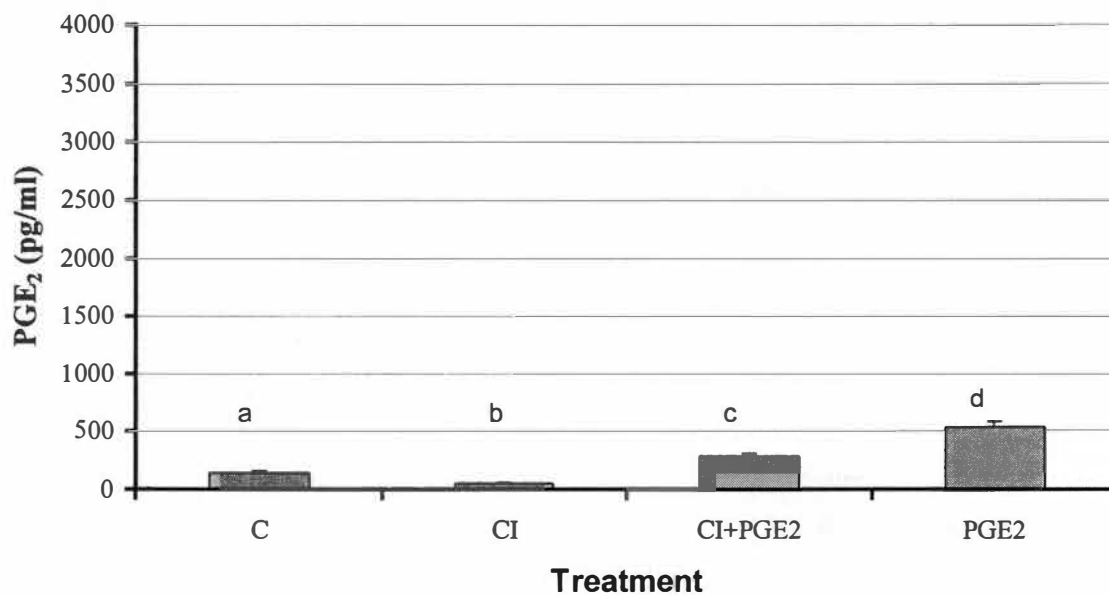


Figure 15. Effect of Exogenous PGE₂ Addition On PGE₂ Concentration In Culture Media of 3T3-L1 Cells Treated With and Without CI

n = 8 for all treatments

Values labeled with different letters are significantly different ($p < 0.05$). Values with the same letters do not differ significantly.

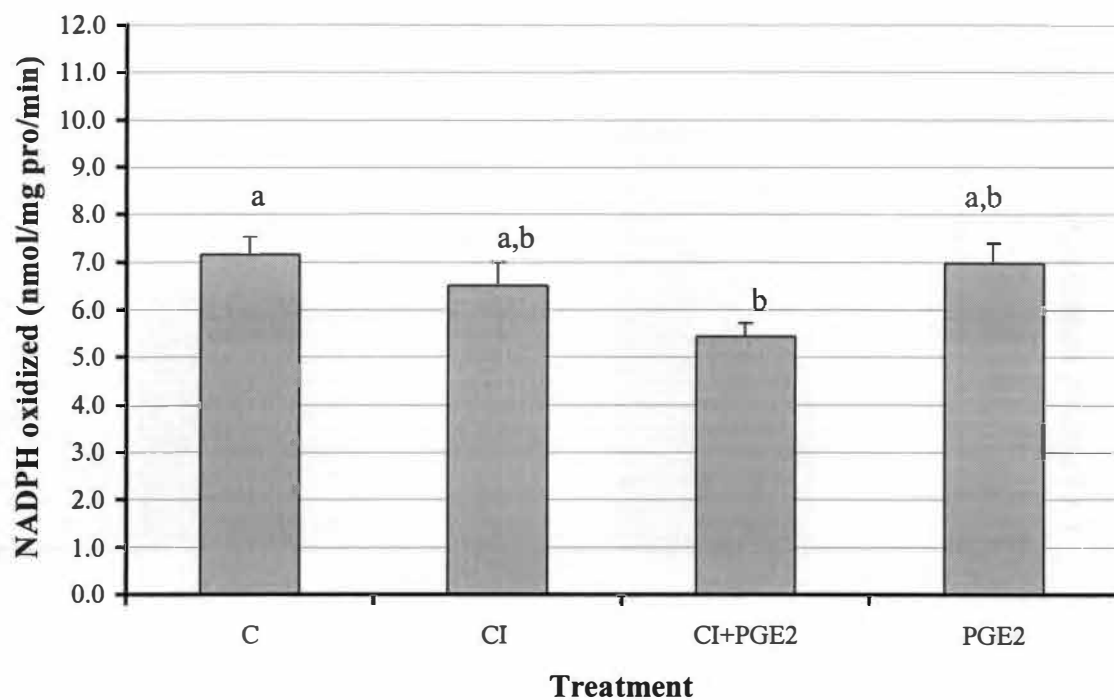


Figure 16. Effect of Exogenous PGE₂ Addition On FAS Activity In Cultured 3T3-L1 Cells Treated With and Without CI

n = 8 for all treatments

Values labeled with different letters are significantly different ($p < 0.04$). Values with the same letters do not differ significantly.

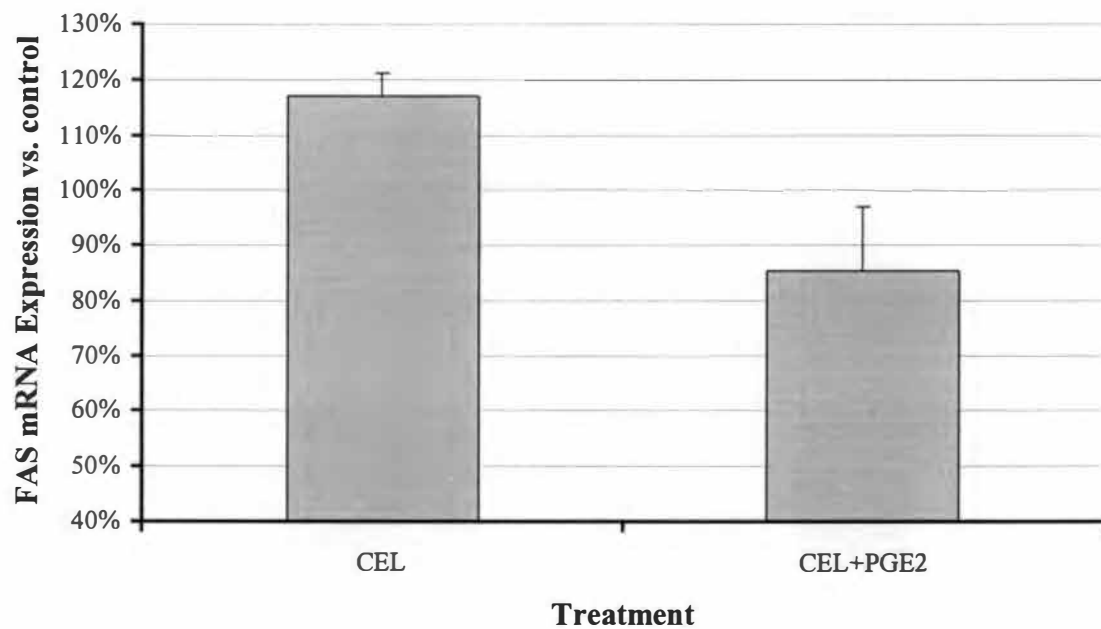


Figure 17. Effect of Exogenous PGE₂ Addition On 3T3-L1 Cultures With and Without CI On FAS mRNA Expression

n = 4 for all treatments

whether this action was associated with other actions that could be considered anti-lipolytic or lipogenic. Data from in vivo studies performed in tissues harvested from the *Apc*^{Min/+} mice clearly indicated that inhibition of the COX enzymes by piroxicam, sulindac, or aspirin (unpublished data) led to decreased FAS activity. Reversal of this inhibition by EP receptor agonists pointed to a lipogenic role that might be coupled with the decrease in lipolysis already known to occur. Several factors inherent in this study required us to continue this investigation in adipocyte models to further delineate possible direct actions of PGE₂ in adipocytes. The most prominent shortcoming of these earlier studies was an inherent defect in the *Apc* gene of this CRC mouse model [146], potentially contributing to several metabolic inconsistencies that might impact adipocyte metabolism. For example, due to their condition, fat mass accretion is typically below that of age-matched controls. These mice also exhibit elevated PGE₂ levels associated with high numbers of intestinal tumors [18]. The results found in these studies would therefore require verification using other wild type mice models more suited for the study of adipocyte metabolism in vivo; or use of adipocyte cell models in culture to determine direct cellular and molecular actions of PGE₂ in these cells.

3T3-L1 adipocytes were chosen as a model to further investigate the direct actions and role for PGE₂ in modulating FAS activity. Two separate mechanisms were selected to attempt modulation of PGE₂ levels secreted by the adipocytes. Pharmacological intervention utilized celecoxib, a selective COX-2 inhibitor, to inhibit metabolism of AA to PGH₂, which we proposed would lead to a decrease in PGE₂. EPA was also utilized to competitively inhibit AA metabolism, representing a dietary intervention that we also proposed would decrease adipocyte PGE₂ production.

Dose response studies demonstrated an approximately linear increase in measured PGE₂ levels for AA and EPA, with a highly significant difference ($p < 0.001$) between the two treatments at the 200 μ M concentration. Very little information exists in the literature regarding physiological levels of EPA. Liebich et al measured free EPA levels in plasma using gas chromatography and found them to be approximately 6.6 μ M [147]. Studies conducted on postmenopausal women fed fish oil found the EPA levels of all plasma lipids was 750 μ M, and that LDL phospholipids and cholesterol esters contained

57.5 nmol/mg protein [84, 145]. Given the approximate ten-fold reduction in measured PGE₂ levels between EPA and AA at the 100 to 200 μ M concentrations, 150 μ M was chosen for our dosage. Consistent with the findings of other studies [5, 16], we demonstrated the ability to manipulate secreted PGE₂ by using EPA to compete with AA for incorporation into membrane phospholipids and subsequent PG production.

PGE₂ levels in the CI treatment group were significantly lower than controls, providing a clear-cut reduction in PGE₂ production. Although the CI is a selective COX-2 inhibitor, it is uncertain as to the specific mechanism of PGE₂ reduction. Most published evidence indicates that COX-2 is not expressed at physiologically relevant levels in mature adipocytes under normal conditions [21, 82]. One possible explanation for the activity of the CI may be that the IC₅₀ of this compound for COX-1 is reported as 1.2 μ M [148], thus putting our 1 μ M concentration high enough to inhibit some COX-1 function.

To determine the impact of manipulating PGE₂ levels, we used FAS enzyme activity as a marker of adipocyte lipogenesis. We report a significant decrease in FAS activity with the CI treatment. Lu et al, using aspirin for non-specific COX inhibition, have also reported similar effects by measuring accumulation of triacylglycerols in adipocytes [21], although the duration of their treatment was 6 days making direct comparison impossible.

The overall comparison of PGE₂ levels for the AA, EPA, and AA + EPA treatment groups correlated well with previously published data measuring tissue concentrations of these fatty acids in mice fed diets supplemented with them [5], although the end points measured and experimental models were different [5, 85, 149]. This served to help verify that our method of incubating the free fatty acid treatments with the fatty acid free BSA did in fact result in the albumin binding the fatty acids, and that the fatty acids were being incorporated into the cell membrane phospholipids.

Although the measured PGE₂ levels were significantly lower in EPA treatment versus AA treatment, they were still significantly higher than control levels. This finding may be partially accounted for by considering PGE₃ cross-reactivity, but because the actual media content of PGE₃ was not measured it is impossible to know the

ratio of the PGE isoforms. Complicating this issue further is the fact that very little is known concerning the actions of PGE₃. Studies in NIH 3T3 fibroblasts found that PGE₃ activated the same signaling pathways as PGE₂, but with much less efficiency [16]. This would imply that high levels of PGE₃ may duplicate the actions of PGE₂, suggesting there would be a point of diminishing returns with EPA supplementation. It is also possible that PGE₃ may bind EP receptors with affinities that differ significantly from PGE₂, whereby the type and number of receptors in adipocytes would also influence the impact of EPA supplementation. An additional study was undertaken to verify that the measured changes in PGE formation in EPA treated cells was mediated via an enzymatic process. This data recapitulates our previous experiment and demonstrates that the PGE formation is an enzymatic process, and since no exogenous AA was added to the cells these data collectively support the suggestion that production of PGE₃ is responsible for the increases seen in PGE production.

It is important to note that measuring enzyme activity only gives a picture of the quantity of enzyme present at the time of harvest and does not reflect regulation at other levels such as transcription, translation, and protein half-life that impact the quantity of enzyme present and therefore the product formed (in this case FAs and subsequently triacylglycerols). Since FAS is well documented to be primarily regulated -whether nutritionally or hormonally- at the level of gene transcription [150-152], we examined the impact of our treatments on the genetic regulation of the FAS enzyme using real time RT-PCR and microarray analysis. The use of these small- and large-scale gene analysis methods, respectively, relays information regarding the expression of FAS mRNA or other genes of interest for a given treatment relative to the control. Both RT-PCR and microarray analysis indicated a clear reduction in FAS mRNA for all treatments versus the control. The effect of fatty acid treatment was substantial, with the reduction correlating well with degree of unsaturation and chain length as previously reported for hepatic FAS and other lipogenic gene regulation [153, 154]. This effect also very closely resembles results from Deng et al showing that degree of unsaturation correlated highly with suppression of the SREBP-1c promoter [72].

Treatment with EPA and AA led to significantly increased PGE levels versus control, yet both treatments suppressed FAS mRNA expression compared to control values. Treatment with CI led to reductions in PGE production, yet treatment with CI in experiment 4 also led to reduced FAS mRNA when compared to control. These data demonstrating opposing changes in PGE levels with similar reductions in FAS mRNA seem to indicate nuclear regulation as playing a key role in these changes for AA and EPA treatment independent of PGE levels. Since mRNA stability was not measured in these experiments, it is possible that increased stability was responsible for the discrepancy created by decreased FAS mRNA expression and unchanged FAS activity for PUFA treatments. It is also possible this discrepancy is due to a longer half-life of the FAS protein such that we were unable to detect changes in enzyme activity within our treatment times (24-48 hours). Indeed, previous studies have shown changes in FAS mRNA half life depend on cell culture treatment and state of differentiation [152, 155].

When exogenous PGE₂ was added to cells treated with and without CI, we obtained very surprising results. Although PGE₂ levels in media were significantly higher than controls, FAS activity with PGE₂ added to CI was significantly lower than controls. This was in direct opposition to what was expected, and what has been shown by other experiments [21]. In addition to the duration of treatment, another possible explanation for this result may be linked to leptin secretion. PGE₂ was shown to stimulate leptin secretion in mouse adipose tissue in primary culture [130], and elevated leptin levels decrease lipogenesis [156] and specifically impact FAS by decreasing gene expression [157, 158]. It is unfortunate we were not able to measure cell culture leptin levels, as this would have enabled us to determine whether the exogenous PGE₂ did result in increased leptin levels. A second possible mechanism involves the antithetic actions of the EP receptors. Long et al investigated the role of COX mediated products of AA metabolism on regulation of glucose transporter 4 (GLUT 4) regulation. They found that a 50-fold increase in endogenous PGE₂ or exposure to 10 μ M exogenous PGE₂ resulted in an increase in cAMP concentrations, consistent with activation of the EP₂/EP₄ receptor [159]. Additionally, studies using the specific COX-2 inhibitor NS-398 on cortical collecting duct cells found that NS-398 treatment increased EP₃ and EP₄ receptor

expression 3-fold [160]. Although the concentration of exogenous PGE₂ added in our treatments was much lower, our preliminary microarray data indicated that celecoxib also influenced the expression of EP receptors (specifically EP₄) and may have resulted in findings different from other experiments utilizing aspirin or other non-specific COX inhibitors. An increase in EP₄ receptors and the resulting increase in cAMP would activate a pathway that would oppose the decrease in cAMP responsible for the PGE₂ mediated decrease in lipolysis. If our hypothesis was correct that this pathway was also responsible for an increase in FAS activity, then activating the opposing pathway should lead to a decrease in FAS activity. Further work with EP receptor concentration and mechanism of action is necessary to delineate the impact of each receptor and how they function in a mixed company.

Measurement of FAS mRNA expression by RT-PCR showed a reduced expression with the CI + PGE₂ treatment (as seen with the enzyme activity), and increased expression with treatment by CI alone when compared to control. This result also seems to indicate an action of the CI that is further impacted by the addition of exogenous PGE₂. This may be tied to EP receptor expression and signaling or may be a separate and additional action of the CI acting through regulation of transcription.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Our studies demonstrated the ability to pharmacologically decrease production of PGE₂ using the selective COX-2 inhibitor celecoxib, which resulted in a significant reduction in measured FAS activity. The use of EPA was also shown to decrease the production of PGE when compared to AA treatment, but not when compared to control. The PG products resulting from COX action on EPA did not result in a significant decrease in FAS enzyme activity, most likely due to higher levels of the two PGE isoforms compared to control. Attempts to restore CI reduced FAS activity with exogenous PGE₂ resulted in further suppression of activity, contrary to what was expected. This suggests a possible alternate pathway in the action of the CI treatment independent of COX suppression.

FAS mRNA expression was decreased by FA treatment in a manner similar to the PUFA effects seen in liver tissue. Given that SREBP1c, an insulin responsive transcription factor, mediates PUFA regulation of hepatic lipogenic genes it is possible that PUFA regulation of adipocyte metabolism modulates insulin sensitivity. Indeed, in the absence of insulin, our cells expressed significantly higher PGE₂ levels than in the presence of insulin.

Further experimentation is required to determine the mechanism of action of celecoxib and PGE₂ in adipose tissue, and to resolve the seeming inconsistency in the role of PGE₂ in modulating FAS enzyme activity and mRNA content. While we were able to demonstrate regulation of PGE₂ levels in adipocytes by n-3 PUFA, we were limited by the small responsiveness of the FAS model, especially at the enzyme level. Lack of simultaneous analysis of several markers of adipocyte metabolism is a weakness in our experiments and should be included in future studies. Finally, additional data on the receptor affinities and actions of PGE₃, although difficult to approach at this time given the lack of data in this area, is necessary in order to understand the mechanism mediating EPA and PGE₃ effects on lipid metabolism.

We have demonstrated in vitro using a well-accepted model of adipocyte metabolism that EPA does reduce tissue AA levels in adipocytes and results in a significant reduction in the PGE₂ produced. In addition, pharmacological intervention via inhibition of the COX pathway also resulted in significant inhibition of PGE₂ production. Although a direct impact on the activity of FAS was not consistently demonstrated, a reduction in PGE₂ levels would still result in less inhibition of lipolysis. Less inhibition of lipolysis, coupled with the other known gene-regulated actions of EPA and other polyunsaturated fatty acids, would still favorably impact adipose tissue levels and result in decreased adiposity. Given the widespread use of celecoxib as an arthritis medication, and the problem of lack of mobility for many who suffer from arthritis, the implication of increased lipolysis and a potential for decreased lipogenesis may prove a benefit for those who take it.

This study also brings to light the need for studies on the function and impact of PGE₃ in adipocytes to determine if it does in fact elicit the same responses as PGE₂. If this is the case, then it may indicate a point of diminishing benefit and the need to specify a consumption range instead of recommending minimum consumption levels. Understanding the mechanisms involved with EPA metabolism takes on further importance with the recent FDA move to allow qualified health claims for omega-3 fatty acids [161], as this will most certainly bring more attention to these fatty acids and increase their consumption even further.

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APPENDIX

The recent development of microarray analysis has given researchers a tool with almost unlimited potential to identify and understand the complex nutrient gene interactions that impact obesity. Although microarray analysis was not part of the original proposal for this thesis, the opportunity to use this incredible tool was too good to pass up. Microarray analysis was conducted following the procedures for experiment #3 on two separate occasions. Since each slide had duplicate spots, this gave a total of 4 values for each gene tested, with the exception of the AA + EPA treatment group which only had two values due to insufficient RNA from the second trial. Statistics were run on all genes for each treatment, but due to the low number of values ($n = 4$), no statistical significance was found on any of our genes of interest. Despite these results, the expression of specific genes in the treatment group relative to the controls provided an interesting snapshot of the impact of a given treatment. The following chart gives the relative expression of treatment versus control for several genes of interest to this work.

Table A1. Microarray Results for Selected Genes.

Gene	Treatment			
	CI	AA	EPA	AA + EPA
GLUT 1	1.11	1.51*	1.58	1.14*
GLUT 4	0.84	1.32	0.66	2.21*
SREBP	1.20	1.80*	2.80	1.20*
FAS	0.41*	0.47*	0.14*	0.24*
PGES	0.92	1.54	2.03	0.19*
EP ₂	0.75	1.83	2.10	1.41*
EP ₄	1.20	3.30	1.98	3.30*

Values reported are means for 4 spots except for * which indicates mean for 2 spots.

Values indicate relative expression of each gene versus control, where 1.00 would indicate expression identical to control.

GLUT = glucose transporter

SREBP = Sterol regulatory element binding protein

FAS = Fatty acid synthase

PGES = Prostaglandin E synthase

EP = Prostaglandin E receptor

VITA

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