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Arachidonic Acid Regulation of Lipogenic Gene Expression in Adipocytes and Hepatocytes

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ARACHIDONIC ACID REGULATION OF LIPOGENIC GENE EXPRESSION IN ADIPOCYTES AND HEPATOCYTES

By

Michelle Kay Mater

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ABSTRACT

ARACHIDONIC ACID REGULATION OF LIPOGENIC GENE EXPRESSION IN ADIPOCYTES AND HEPATOCYTES

By

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Previous observations have shown that dietary polyunsaturated fatty acids (PUFA) can inhibit the expression of lipogenic genes in both liver and epididymal fat. Transcriptional regulation of genes by PUFA have given rise to three hypothetical mechanisms of action: a prostanoid pathway, a PPAR pathway or a prostanoid/PPAR independent pathway. Using the S14 gene as a model for lipogenic gene expression, preliminary studies showed that in cultured adipocytes, arachidonic acid (20:4n-6; AA) inhibited both mRNA expression and transcriptional activity in reporter studies more potently than eicosapentaenoic acid (EPA). This result prompted the analysis of AA inhibition in both adipocytes and hepatocytes. In adipocytes, AA inhibition of S14 gene expression was reversed by the cyclooxygenase inhibitor, flurbiprofen, implicating a prostanoid pathway. Results also indicated PGE_2 inhibited S14 expression and S14CAT activity. The PGE_2 effect was blocked by pertussis toxin, a G_i-protein inhibitor.

Prostaglandin (PG) agonist results, coupled with inhibitors of specific signal transduction pathways, suggested PGE_2 action was mediated through a Ca^{2+} linked pathway.

In hepatocytes, AA and PG also inhibited S14 expression. However, the AA effect was not blocked by flurbiprofen, indicating the PUFA control of gene expression did not require cyclooxygenase. In liver, PG are produced by Kupffer cells and act on hepatocytes. In adipocytes PG are synthesized by both preadipocytes and adipocytes. PG-agonist studies implicate involvement of EP3 receptors, but failed to reveal the mediator of PGE_2 action in hepatocytes.

Promoter deletion analysis indicated that both AA and PGE_2 targeted similar regions within the S14 promoter, showing that AA can act directly on parenchymal cells or through a paracrine mechanism to alter parenchymal cell gene expression. Presumably, AA enters Kupffer cells, is converted to PG and secreted to interact with EP3 receptors on parenchymal cells. Interestingly, both PGE_2 and AA have similar effects on gene in parenchymal cells.

In conclusion, these studies have shown that dietary n-6 PUFA conversion to PG can provide another route for the control of hepatic and adipogenic lipogenic gene expression. I speculate this route plays a significant role in hepatic gene expression under conditions stimulating Kupffer cell PG synthesis, *i.e.* inflammation.

To my family:

Dad
Mom
Thinh
Pamela
Michael
Janet
Mindi
Terry
Morgan
Cassandra
Whitney
Marc
Michelle
Kayleigh
Ryan
Blade

and

my PHD (Pretty Happy Dog)

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AA	Arachidonic acid (20:4, n-6)
ACS	Acyl CoA synthetase
ADD1	Adipocyte determination and differentiation dependent factor 1 (also known as SREBP)
AOX	Acyl CoA oxidase
CamK	Calcium calmodulin kinase
C/EBPα	CCAAT enhancer binding protein alpha
CYP	Cytochrome P450 (many subtypes:2E1, 4A2, 2C23, etc.)
DEX	Dexamethasone
EGF	Epidermal growth factor
EPA	Eicosapentaenoic acid (20:5, n-3)
FAS	Fatty acid synthase
FGF	Fibroblast growth factor
GH	Growth Hormone
GLUT4	Glucose transporter 4 (in adipose and muscle tissue)
HSL	Hormone sensitive lipase
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor 1
IP3	inositol triphosphate
LAP	Liver activating protein
LIP	Liver inhibitory protein
LPL	Lipoprotein lipase
OA	Oleic acid
Ob17	Adipogenic cell line from ob/ob mice
Ob1771	Subclone of Ob17 cells
PEPCK	Phosphoenolpyruvate carboxykinase
PDGF	Platelet derived growth factor
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator activated receptor response element
PRR	Pluripotent response region
PUFA	Polyunsaturated fatty acids
RXR	Retinoid X receptor
SCD	Stearoyl CoA desaturase (1 or 2)
SRE	Sterol response element
SREBP	SRE binding protein
TA1	Adipogenic cell line from mice embryo 10T1/2 mesenchymal cells treated w/ 5'azacytidine
TGF	Transforming growth factor
TH	Thyroid hormone
TRE	Thyroid hormone response element
TRR	Thyroid hormone response region

VLDL	Very low density lipoproteins
WAT	White adipose tissue
3T3-L1	Adipogenic cell line from mice
3T3-F442A	Adipogenic cell line from mice

INTRODUCTION

Nearly 40% of the calories in a typical American diet is derived from fat. Approximately half of this is from animal fat (meat and dairy) while the other half is from plants, in the form of oils. Research has shown that high dietary fat intake is correlated with heart disease, diabetes, several forms of cancer and obesity. These health risks led to the recommendation of decreasing dietary intake to a maximum of 30% of calories from fat. However, recommendations have not changed the eating habits of the majority of Americans. Nearly one third of American adults are considered obese and perhaps more frightening, a quarter of American children are overweight (Food Fats and Health, 1991).

Dietary fat and fat synthesized *de novo* contribute to total body fat. The liver and adipose tissues are the main organs involved in fat synthesis and storage. Fats, endogenous or dietary, can follow different pathways in these cells. In the liver, triglycerides can be converted into phospholipids for cell membranes, packaged as VLDL and

secreted or oxidized. Adipose tissue is the principal site for triglyceride storage but also releases fatty acids from triglycerides when the body requires energy. Fatty acids derived from the diet or adipose tissue can regulate gene expression of many genes. For example, polyunsaturated fatty acids can lower the expression of lipogenic genes, those involved in synthesizing fatty acids (Jump et al., 1996).

The focus of my research has been to study the regulation of genes involved in lipid metabolism, particularly how PUFA regulate lipogenic gene expression. It is not well understood how PUFA regulate gene expression. Using the S14 gene as a model of lipogenic gene expression, my studies have shown that PUFA can regulate the same gene in two different tissues, i.e. liver and adipose, through different mechanisms. However, at the promoter level, there appears to be a common target of action (Jump et al., 1993; 1996; 1997a).

This dissertation will describe my findings of how dietary PUFA regulates lipogenic gene expression in cultured 3T3-L1 adipocytes and primary hepatocytes. These studies, coupled with previous work from our lab (Ren et al., 1996; 1997) reveal the presence of multiple mechanisms in the liver for PUFA regulation of lipogenic gene expression.

CHAPTER 1

CHAPTER 1

LITERATURE REVIEW

Our laboratory focuses on the study of lipid metabolism and its regulation by hormones and dietary factors. This literature review will describe lipid metabolism in liver and white adipose tissue. I will also describe the effect of dietary fat on these tissues. Finally I will discuss the regulation of S14, a model for hormonal and dietary regulation of lipogenic gene expression, by the polyunsaturated fatty acid, arachidonate, in both adipocytes and hepatocytes.

Lipid Metabolism

Lipids play an important role in energy storage, cell membrane structures and the synthesis of important bioactive compounds. Dietary lipids have also been shown to regulate gene expression. This section will discuss lipid metabolism, synthesis, oxidation and the role of dietary fat.

Fat in the body is derived either from the diet or synthesized *de novo*. In humans, dietary fats or

triglycerides, are broken down into fatty acids as a result of bile salts, pancreatic lipase and intestinal lipase in the small intestine. The fatty acids are absorbed into the intestinal villi and packaged into chylomicrons. The chylomicrons are transported to the lymph system from the villi and enter the blood stream (Stryer, 1988; Alberts et al., 1994; Vance and Vance, 1985). Fatty acids released from chylomicrons by lipoprotein lipase (LPL) are taken up by the adipose, muscle or other tissues. The fatty acids enter adipocytes and are re-esterified into triglycerides for storage. The chylomicron remnants, containing cholesterol and apo-proteins, are transported to the liver and taken up by receptor mediated endocytosis (Cooper, 1997).

The liver packages triglycerides into very low density lipoproteins (VLDL). Triglycerides in VLDL are synthesized endogenously or are derived from the diet. Once released, lipases act on VLDLs, releasing fatty acids. The triglyceride level decreases as fatty acids are removed, increasing the density of the particle. These remaining components are either converted to low density lipoproteins (LDL) or taken up by the liver. LDL contain cholesterol and are considered the main transporters of this molecule. Another transporter of triglycerides are high density lipoproteins (HDL). These molecules contain a small amount

of triglycerides and mainly function to pick up cholesterol in the plasma for esterification (Stryer, 1988; Alberts et al., 1994).

Lipid Synthesis

VLDL from the liver contain triglycerides and phospholipids which are derived from endogenous or dietary fatty acids. Fatty acids can be synthesized in the liver and the adipose tissue of humans. However, fatty acid synthesis occurs predominantly in the liver and only when a high carbohydrate diet is consumed (Jungermann and Kietzmann, 1996; Hellerstein et al., 1996). The first step in fatty acid synthesis requires acetyl CoA and acetyl CoA carboxylase (ACC) to form malonyl CoA. Fatty acid synthase (FAS), using acetyl CoA as a primary molecule, sequentially adds malonyl CoA to form palmitate (16:0) (Stryer, 1988; Volpe and Vagelos, 1976). Palmitate can be elongated and/or desaturated to either stearate (18:0) or oleate (18:1). Oleate can also be elongated but desaturation beyond n-9 is not possible in mammals due to the lack of appropriate desaturases. Linoleic and linolenic acid (18:2n-6 and 18:3n-3, respectively) are therefore required in our diets (see Figure 1.1) (Vance and Vance, 1985). These fatty acids are used in the production of triglycerides which are used

for the synthesis VLDLs, phosphoglycerides or sphingolipids (Stryer, 1988; Alberts et al., 1994).

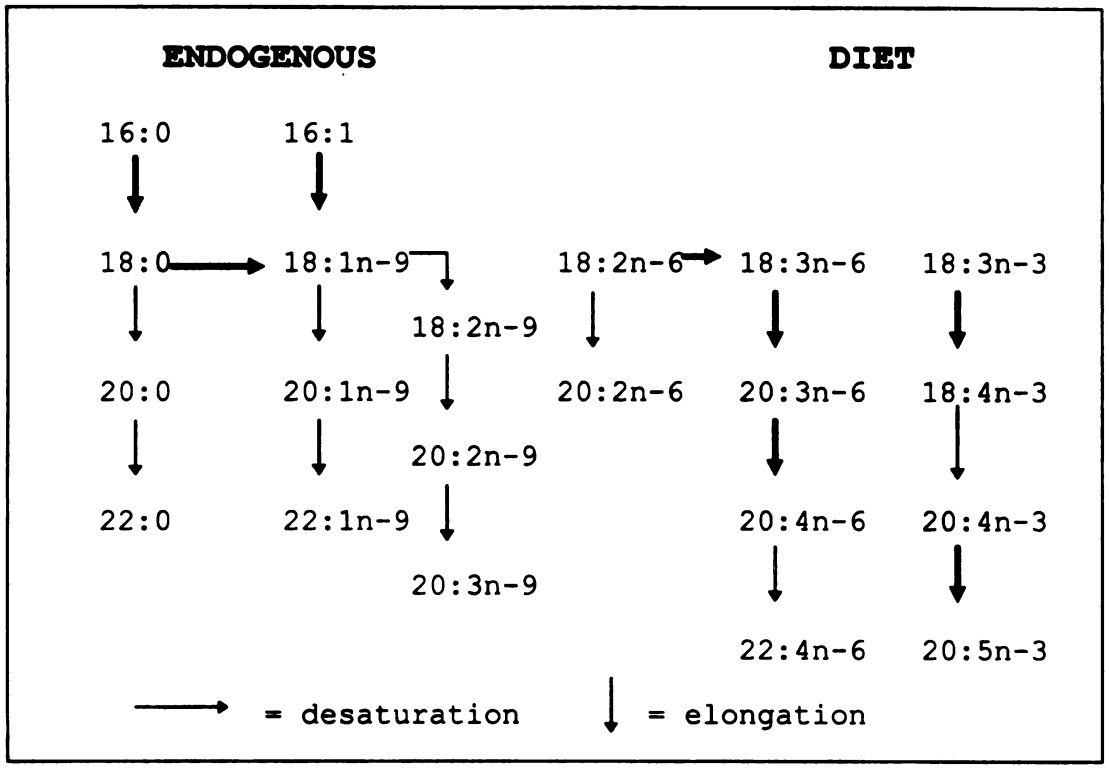


Figure 1.1. Fatty Acid Elongation and Desaturation. Fatty acids can be elongated and desaturated *in vivo*. This figure represents the pathways present in mammals. Thicker arrows indicate more prominent pathways, horizontal arrows indicate desaturation steps and vertical arrows indicate elongation steps. This figure was adapted from a figure in Vance and Vance, 1985.

Enzymes involved in fatty acid synthesis are regulated at both transcriptional, translational and post-translational levels. For example, ACC activity is induced by citrate and suppressed by palmitoyl CoA. Phosphorylation of ACC by protein kinase A (PKA) following treatment of

cells with epinephrine or glucagon will suppress ACC, while insulin will induce ACC activity (Stryer, 1988). Transcription of ACC is also regulated by epinephrine, glucagon and insulin. Diet also affects these enzymes. For example, high carbohydrate diets increase ACC and FAS activity while polyunsaturated fatty acids (PUFA) decrease activity of these enzymes (Kim and Freake, 1996; Jump et al., 1996; Hellerstein et al., 1996). Most of the activity level of FAS is due to protein and RNA levels and is therefore controlled at the pretranslational level rather than the post-translational level. The same is true for the lipogenic gene, S14 (Clarke and Jump, 1993). A more extensive discussion of the regulation of FAS and S14 will be presented in a later section.

Lipid Oxidation

Most humans store enough energy in the form of fat to last a month. When energy is required, these stored triglycerides are degraded by lipases, releasing fatty acids and one glycerol molecule. The glycerol can be used for glucose synthesis but the fatty acids can not (hence sugar can not be synthesized from fats). Fatty acid oxidation occurs in the mitochondria of many tissues, including liver, heart, muscle and brain. The major form of oxidation is β-oxidation where the chain length is reduced by two carbons

each cycle. The result is the release of acetyl CoA which is further oxidized in the mitochondria to produce ATP and CO_2 (Stryer, 1988; Alberts et al., 1994; Vance and Vance, 1985).

Fatty acids are released by lipases on the cell surface and must be taken into the mitochondria for oxidation. Once inside the cell, the fatty acid is converted to a CoA thioester by acyl CoA synthetases at the outer mitochondrial membrane. Once through the outer mitochondrial membrane, the fatty acid thioesters are transported across inner the mitochondrial membrane to the matrix by carnitine. This translocation requires carnitine palmitoyltransferase I and II (CPTI and CPTII), present on the inner and outer side of the inner mitochondrial membrane, respectively. CPTII transfers the thioester to carnitine and CPTI removes the carnitine and the fatty acid thioester is released into the intermembrane space where oxidation can occur. Thus the fatty acid thioester is converted to an acylcarnitine and back to a thioester before oxidation begins (Vance and Vance, 1985).

Fatty acid oxidation is induced when energy is needed by the body. Thus during times of hunger, fatty acid oxidation is increased. The enzymes involved in fatty acid oxidation, 3-hydroxyacyl CoA dehydrogenase and thiolase, are inhibited by NADH and acetyl CoA which indicate that energy

is high. Glucagon and epinephrine increase their activities and transcription while insulin will inhibit this (Stryer, 1988). Fatty acids are synthesized or oxidized upon changes in body energy status. Thus, the enzymes involved in these processes are regulated by the energy status in the cells. A summary figure for lipid metabolism is given in Figure 1.2.

Fatty acids are also oxidized in peroxisomes. Peroxisomes are organelles which have hydrogen peroxide-producing flavin oxidases and catalases. These organelles function to metabolize hydrogen peroxide, but also contain several lipid oxidative enzymes. Peroxisomal β-oxidation is increased by peroxisome proliferators such as clofibrate, gemfibrozil, hypolipidemic agents and high fat diets. Peroxisomes contain oxidases which oxidize fatty acids and release heat in contrast to the energy released from mitochondrial β-oxidation (Reddy and Mannaerts, 1994). One peroxisomal β-oxidative enzyme, acyl CoA oxidase (AOX), is regulated at the transcriptional level by peroxisome proliferators. AOX mRNA levels increase in rats fed a diet containing fish oil (contains long chain n-3 fatty acid) or gemfibrozil, a hypolipidemic drug (Ren et al., 1997). Very long chain fatty acids are almost exclusively oxidized by peroxisomes. Prostaglandins and bile acids are also oxidized by the peroxisome (Reddy and Mannaerts, 1994).

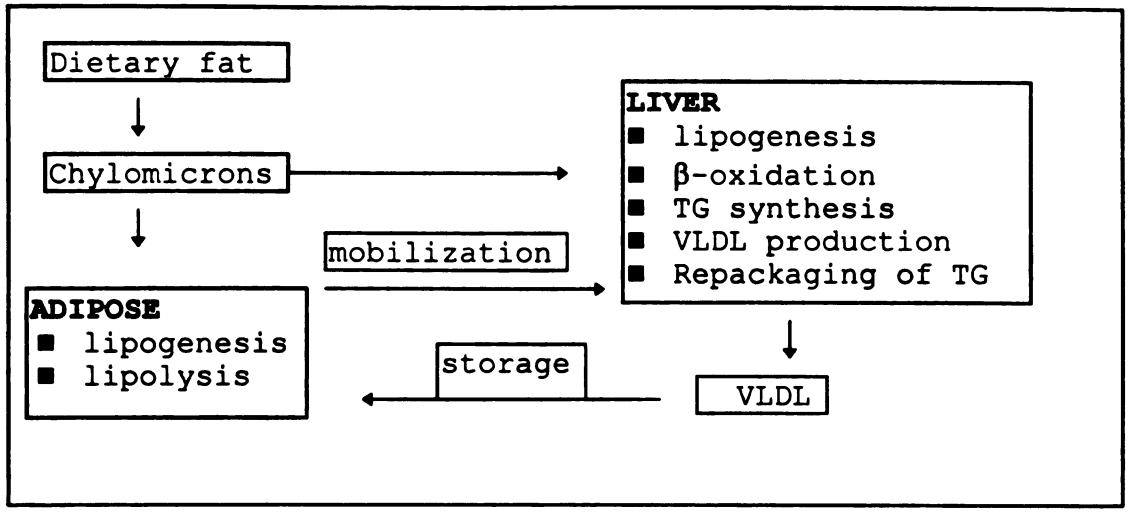


Figure 1.2. Lipid Metabolism. Dietary fat is taken in, absorbed as chylomicrons and dumped into circulation. The adipose depot can store TG from chylomicrons while the liver can pick the remnants or the chylomicrons for repackaging into VLDL. The liver can also synthesize fatty acids (lipogenesis) for TG formation and release.

Dietary Fat

Dietary fat is required in the diets of humans and animals. Fats are necessary for energy, steroid and prostanoid production and cell membrane components. Dietary fat also improves taste and palatability of food. However, too much dietary fat can have negative side effects (Food Fats and Health, 1991).

Both amount and type of dietary fat are important when considering health factors. Too much fat is linked to obesity, diabetes, heart disease and cancer. Perhaps even more importantly, the type of fat consumed is linked to these diseases (Food Fats and Health, 1991). Dietary fat is

either saturated or unsaturated. Saturated fat, fatty acids with no double bonds, has been linked to these diseases as well as insulin resistance and hypertension. In contrast, fats with more than one double bond, polyunsaturated n-3 fatty acids, are correlated with lower heart disease (Food Fats and Health, 1991). Gene expression is also affected by degree of saturation. Polyunsaturated n-3 fats lower VLDL production from the liver and both n-6 and n-3 PUFA decrease enzymes involved in fatty acid synthesis while saturated fats do not (Jump et al., 1997a). This dissertation will focus on the regulation of gene expression by n-6 polyunsaturated fats. A more extensive review of PUFA follows.

Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFA) are fatty acids containing two or more double bonds. Two long chain PUFA discussed here are eicosapentaenoic acid 20:5n-3 (EPA) and arachidonic acid 20:4n-6 (AA). AA and EPA are synthesized by animals from linoleic 18:2n-6 and α-linolenic acid 18:3n-3, respectively. Animals can not synthesize these precursors because they can not add double bonds past the ninth carbon on a fatty acid. Linoleic and α-linolenic acids are therefore essential in animal diets. Most human

diets also include some AA and EPA. For example, fish (or fish oil) contains n-3 PUFA (Food, Fats and Health, 1991).

Arachidonic acid contains twenty carbons with four double bonds, an n-6 fatty acid. EPA also has twenty carbons but has five double bonds and is an n-3 fatty acid (see Figure 1.3). The difference being one more double bond in EPA.

PUFAs suppress lipogenic enzyme activity by inhibiting gene transcription. N-3 PUFA also inhibit triglyceride synthesis but the mechanism is not known (Clarke and Jump, 1993). Early work with rats fed linoleic and linolenic acid determined that diets containing these fatty acids could lower G6PD and FAS activity (Clarke et al., 1976; Flick et al., 1977; Hodge and Salati, 1997). Since that time, transcription, RNA stability and enzyme activity of lipogenic enzymes have been studied to determine how PUFAs affect these processes. Most lipogenic genes have been shown to be suppressed at the transcriptional level, rather than RNA stability or direct inhibition of the enzyme. FAS, SCD1, pyruvate dehydrogenase, ACC and S14 all have lower expression in the liver as a result of dietary PUFA (Clarke and Jump, 1994; 1993; Clarke and Abraham, 1992; Clarke et al., 1990a). In contrast G6PD is suppressed at the post-transcriptional level as a result of changes in RNA stability (Hodge and Salati, 1997).

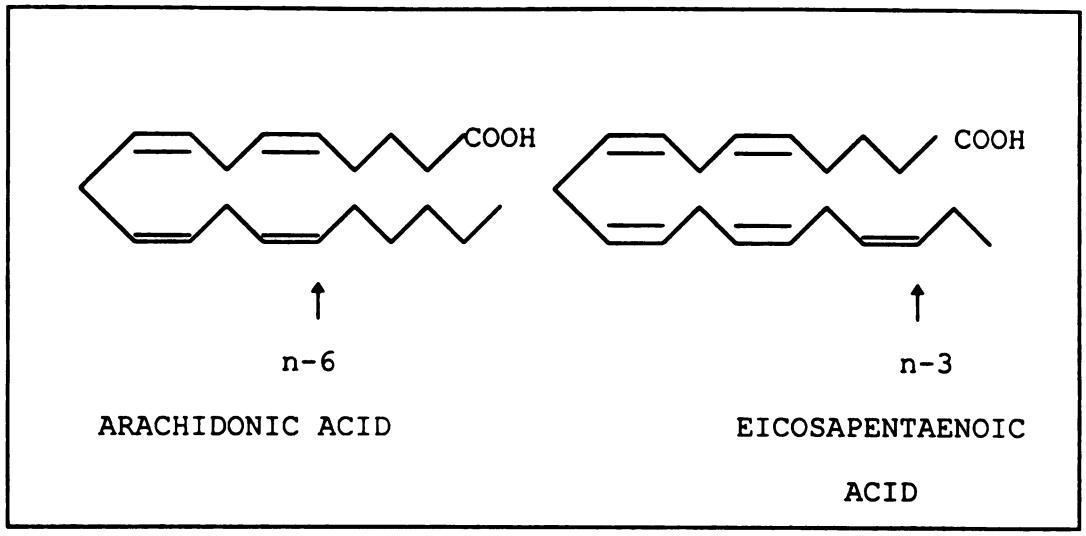


Figure 1.3. Structure of AA and EPA. Each fatty acid is shown above. The arrows indicate the first carbon double-bonded from the opposite end of where the numbering system starts. Hence, AA contains a double bond on the 5, 8, 11 and 14^{th} carbon.

Fish oil has been extensively studied because it has been shown to lower serum triglycerides, VLDL secretion and increase fatty acid oxidation in humans. A diet high in fish, such as that of Eskimos, is highly correlated to lower heart disease as well (Food, Fat and Health, 1991; Jump et al., 1997a). In our lab, fish oil has been used in several rat feeding studies. In every instance, rats fed diets containing fish (menhaden) oil, have lower expression of S14, FAS and PK mRNAs. Serum triglyceride levels are also lower compared to olive oil fed animals. In mice, mRNAs encoding proteins involved in peroxisomal and microsomal fatty acid oxidation, such as AOX and CYP4A (a P-450 gene),

are increased with this dietary regime as compared to the olive oil fed animals (Ren et al., 1997). These effects on hepatic gene expression are due to direct effects on hepatocytes. There is no evidence to indicate that PUFA regulation of hepatic gene expression requires extraheptic metabolites or hormones. Adipose tissue also responds to fish oil feeding leading to a suppression of S14 and FAS mRNA. However, the fish oil effect on adipose tissue is slower than in liver. Moreover, the mechanism for this control is unknown (Jump et al., 1993).

Like EPA, AA suppresses lipogenic gene expression in both hepatocytes and adipocytes. AA has previously been shown to lower FAS expression in hepatocytes (Armstrong et al., 1991; Jump et al., 1994), but not increase the peroxisomal β-oxidative enzyme AOX (Jump et al., 1997a). Interestingly, EPA increases in AOX and CYP4 mRNA *in vivo* and primary hepatocytes. One proposed mechanism of how EPA regulates gene expression is through peroxisome proliferator activated receptors (PPAR), which bind DNA and are transcription factors (these will be discussed later). Using the PPARα knockout mouse, Ren et al. (1997) reported that EPA required PPARα to induce AOX and CYP4 mRNA levels. In this same report, Ren also showed that while S14 and FAS were suppressed by EPA, their suppression did not require PPARα (Ren et al., 1997). These results indicate that PPAR

are required for PUFA regulation of some genes (AOX, CYP4A) but not others (S14, FAS). Because AA failed to activate PPARα (Ren et al., 1997), the AA regulation of hepatic gene expression does not require PPARα. This thesis will examine how AA suppresses lipogenic gene expression in cultured adipocytes and primary hepatocytes. The following section describes the possible mechanisms for PUFA control of S14.

Possible Mechanisms for PUFA Control of Lipogenic Gene Expression

Efforts to determine how PUFA are suppressing S14 (and FAS) have led to several possibilities. One possibility is that peroxisome proliferator activated receptor (PPAR) is the PUFA regulatory factor (Jump et al., 1996; Clarke and Jump, 1996). PPAR are a family of transcription factors including PPARα (predominant in liver), PPARγ (γ2 predominant in adipose tissue, γ1 ubiquitous) and PPARβ (many tissues) which bind to DNA as heterodimers with retinoid X receptor (RXR) at peroxisome proliferator response elements (PPRE) and affect transcription (Braissant et al., 1996). Activators include fatty acids, leading to the hypothesis that PUFA are ligands for PPAR, and therefore can regulate transcription via PPAR. However, PPARα, the

most abundant PPAR in liver, does not bind the S14 promoter although PPARα can interfere with the T3 response by sequestering RXR (Ren et al., 1996). AOX and CYP4A have been shown to require PPARα to be regulated by PUFA but this was not the case with S14 and FAS (Ren et al., 1997; Ren et al., 1996). The lipogenic gene, stearoyl CoA desaturase (SCD1), is also suppressed by PUFA but is induced by the peroxisome proliferator clofibrate (Miller and Ntambi, 1996). Opposing this theory that PUFA use PPAR to regulate gene expression is the fact that saturated fats bind and activate PPAR as well (Gottlicher, et al., 1992). It is known that PPAR bind several arachidonic acid metabolites including 15-deoxy-$\Delta^{12,14}$ PGJ_2, 8S-HETE, 8S-HEPE, PGI (Forman et al., 1997). In primary hepatocytes arachidonic acid failed to activate PPARα. These studies suggest that PPAR may not be involved in the AA control of lipogenic gene expression (Ren et al., 1997).

A second possibility is that another PPAR such as γ or β/δ is involved in the PUFA response (Jump et al., 1996; Clarke and Jump, 1996). The peroxisome proliferator WY-14,643 inhibits S14 mRNA expression and S14CAT activity in primary hepatocytes (Jump et al., 1995; Ren et al., 1996). Perhaps this suppression is through a PPAR other than PPARα.

A third possibility is that PUFA suppression is through the production of prostanoids from PUFA (Jump et al., 1996; Clarke and Jump, 1996). Both AA and EPA can be metabolized to prostaglandins although much less EPA than AA is metabolized in this way (Smith, 1989). However, prostanoid inhibitors did not reverse the inhibition of FAS activity seen with corn oil treatment (Flick et al., 1977). However, it is not known if arachidonic acid is acting through a prostanoid dependent pathway, and this question will be addressed in this thesis. Arachidonic acid metabolites, eicosanoids, are abundant and will be discussed in more detail later in the literature review.

Other possible mechanisms also present potential modifiers of transcription by PUFA. For example, alteration of the redox state of the cell could change transcription. PUFA may change the phosphorylation state of proteins such as PKA or protein kinase C (PKC), which phosphorylate transcription factors. PKC is known to be activated by unsaturated fats, including monounsaturated fats. However, monounsaturated fatty acids do not suppress lipogenic enzymes (Clarke and Jump, 1994). PUFA decrease lipogenesis and SCD1, leading to a decline in 16:0 and 18:1. Perhaps this reduces the fatty acid availability for cell membrane components, changing membrane fluidity (Clarke and Jump, 1996; Clarke and Jump, 1993). However, studies in primary

hepatocytes do not support a generalized effect on plasma membrane signaling (Liimatta et al., 1994).

The regulation of S14 by PUFA has been a focus of our lab. Several hypotheses exist but definitive answers do not. This dissertation will focus on how arachidonic acid is suppressing S14 in adipocytes and hepatocytes.

Adipocytes

Because a large portion of this thesis deals with fatty acid regulation of adipocyte gene suppression, I will now present a brief overview differentiation and fatty acid metabolism in adipocytes. Formation of adipocytes begins with "determination" of stem cells destined to become either myoblasts, chondroblasts or preadipocytes. Once determination is complete, preadipocytes are destined to become adipocytes. After preadipocytes become confluent, or contact inhibited, and the proper hormonal signals are present, differentiation begins. During the differentiation process there are changes in the levels of 300 proteins, nearly a third of which change in the first five hours (Sadowski et al., 1992). The cells go on to accumulate lipids until the adipocyte has multilocular or a unilocular lipid droplet. In adult animals adipocytes can also be formed by induction of replication and differentiation of preadipocytes. Regardless of when the preadipocyte becomes

an adipocyte, once differentiated, it is an extremely efficient lipogenic and lipolytic machine (Smas and Sul, 1995; Shillabeer and Lau, 1994; Cornelius et al., 1994; Ailhaud et al., 1991).

Adipocytes function to store energy in the form of triglycerides in humans and animals. When energy is needed, fatty acids are released into the circulation where they are taken up by tissues and oxidized for energy. During times when energy is plentiful, the adipocytes takes up triglycerides for storage (Alberts et al., 1994). Adipose tissue and cultured adipocytes also produce the hormone leptin. Adipose production of leptin is important in regulation of satiety and body weight maintenance. In mice, leptin production is reduced by fasting and then increased by refeeding. Mice with defective leptin (ob/ob) are hyperphagic and extremely obese, even when pair fed to their lean littermates (MacDougald et al., 1995; Coleman, 1978).

Differentiation involves a multitude of factors including various inducers and inhibitors as well as expression of specific transcription factors. Though most of the research presented here is done using only fully differentiated adipocytes, the many signaling compounds that trigger adipocyte formation and the transcription factors expressed in adipocytes also induce lipogenic gene expression. Each of the following sections, *Adipogenic*

Regulators and *Adipogenic Transcription Factors*, will describe the multitude of factors involved in the complexity of adipocyte differentiation.

Adipogenic Regulators

Regulators of adipocyte differentiation include hormones, fatty acids, peroxisome proliferators, prostaglandins, growth factors and other assorted compounds. Each of these are used in adipogenic cell culture to increase the percentage of cells which become lipid-filled adipocytes. Most adipogenic cell lines are cultured and differentiated in the presence of serum but recently the use of serum-free medium has necessitated the determination of which regulators are required for adipocyte differentiation.

The importance of growth hormone (GH) in adipocyte differentiation was discovered when attempting to remove it from serum. Without GH, differentiation does not occur in serum-free cultured preadipocytes (Ailhaud et al., 1992a; Doglio et al., 1986). GH works through a cell surface receptor linked to a Janus Kinase (JAK), a tyrosine kinase which signals to a number of transduction pathways (Alberts et al., 1994). Research showed that some adipogenic cell lines require growth hormone for differentiation while others allow IGF-1 substitution. PKC activators can also substitute for growth hormone to induce differentiation.

Another mediator of growth hormone action may be c-Fos activation. GH may act by activation of c-Fos, PKC or IGF-1, but all adipogenic cell lines require growth hormone or IGF-1 to develop (Smas and Sul, 1995; Ailhaud et al., 1991).

Thyroid hormone also induces adipocyte differentiation, although it does not play a main role. For example, in Ob17 cells thyroid hormone is necessary for differentiation when these cells are cultured in serum-free conditions. However, cAMP can fulfill the thyroid hormone requirement (Cornelius et al., 1994; Smas and Sul, 1995).

Glucocorticoids induce differentiation of preadipocytes to adipocytes. Dexamethasone, a synthetic glucocorticoid, is routinely used to differentiate the adipogenic cell lines 3T3-L1 and 3T3-F442A, as well as other cell lines (Smas and Sul, 1995; Chapman et al., 1985). One possible mechanism of action by glucocorticoids is the ability to block synthesis of prostaglandins. Indomethacin, a potent inhibitor of cyclooxygenase, is used to differentiate another adipocyte cell line, TA1 (Smas and Sul, 1995). Treatment with cortisone has proven to increase the breakdown of AA into PGI_2. The PGI_2 can increase cAMP levels which will increase differentiation of preadipocytes to adipocytes. This is supported by the fact that prostaglandins or arachidonic acid can substitute for glucocorticoids in some cell differentiation media (Ailhaud

et al., 1992). Glucocorticoids can also act by directly increasing gene expression. Glucocorticoids bind DNA via the glucocorticoid receptor and may increase expression of genes involved in adipocyte differentiation. Glucocorticoids also increase the expression of the lipogenic genes FAS and S14 in adipocytes post differentiation (Lepar and Jump, 1989; Jump and MacDougald, 1993).

Insulin is one of the most common hormones used to induce differentiation of fat cells. Insulin is used at high concentrations and is probably acting through the IGF-1 receptor (Cornelius et al., 1994). However, insulin may also play a role by increasing the glucose transporters GLUT-1 and GLUT-4, which increase expression of other lipogenic genes (Ailhaud et al., 1992). In adipocytes, insulin binds to its receptor, causing phosphorylation of the receptor and other proteins including insulin receptor substrate (IRS). IRS subsequently activates Ras which phosphorylates Raf which phosphorylates MAP kinase. Through this pathway, insulin increases glucose storage and lipogenesis in adipocytes. If the activation of Ras by IRS-1 is inhibited, 3T3-L1 cells will not differentiate, indicating the requirement of the insulin signaling pathway for differentiation (Porras and Santos, 1996; Lamphere et al., 1994; Yenush and White, 1997).

Fatty acids can also increase adipocyte differentiation. In Ob17 and Ob1771 cells, arachidonic acid (AA) is an inducer of differentiation, acting through an increase in cAMP and diacylglycerol (Smas and Sul, 1995; Gaillard et al., 1989). However, in other cell lines, AA is an inhibitor of differentiation (Smas and Sul, 1995). Long term treatment of Ob1771 cells (an adipogenic cell line) with palmitate led to differentiation. Furthermore, a nonmetabolizeable form of palmitate could repeat this finding (Ailhaud et al., 1995). Shillabeer and Lau (1994) reported that the saturated fats were better inducers of adipocyte formation than polyunsaturated fatty acids in rats fed different diets. Grimaldi et al. (1992) used palmitate to induce adipogenesis as well as lipogenic genes. These data indicate that fatty acids, especially saturated fats, in the diet can increase fat cell numbers and differentiation.

Some peroxisome proliferators have recently been shown to induce differentiation of adipocytes. Peroxisome proliferators include a variety of chemical compounds including plasticizers, thiazolidinediones, fatty acids, steroids and xenobiotics. Thiazolidinediones are particularly active on preadipocytes. Treatment of preadipocytes with these compounds can quicken the transition into lipid-filled adipocytes (Chawla and Lazar,

1994; Chawla et al., 1994; Tontonoz et al., 1994b; 1994c). The actions of these compounds will be discussed further in the next section.

Prostaglandins both inhibit and induce adipocyte differentiation depending on the cell line and the prostaglandin tested. The three prostaglandins produced in adipose tissue and adipocytes (*in vitro*) are PGE_2, PGI_2 and small amounts of $PGF_2\alpha$ (Aubert et al., 1996). These PGs are synthesized from arachidonic acid by cyclooxygenase. PGI_2 (or prostacyclin) is reported to act via an increase in cAMP and is thought to be an inducer of differentiation in Ob17 and Ob1771 cells. PGE_2 and $PGF_2\alpha$ do not act via an increase in cAMP but $PGF_2\alpha$ is known to induce differentiation through the stimulation of PKC in Ob17 cells (Ailhaud et al.,1991; Smas and Sul, 1995). However, in 3T3-L1 cells, $PGF_2\alpha$ is an inhibitor of differentiation, as is PGE_2. As mentioned above, the cyclooxygenase inhibitor indomethacin, induces differentiation in the adipogenic cell line TA1. It is unknown if blocking prostaglandin synthesis is the reason that indomethacin induces differentiation in these cells (Smas and Sul, 1995; Shillabeer et al., 1996). Recently Lehmann et al., (1997) found that indomethacin itself binds PPARγ. Perhaps it is this pathway that indomethacin is using to induce differentiation of adipocytes.

Prostaglandins may bind PPARγ, which is known to enhance differentiation. One prostaglandin, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, has been identified as a ligand for PPARγ2, although this ligand has never been detected *in vivo* (Forman et al., 1995; Kliewer et al., 1995). While prostaglandins can play a role in differentiation, it is unknown exactly how they are involved in the process.

Growth factors generally inhibit differentiation of adipocytes, with the exception of IGF-1. Epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factor (TGF) and fibroblast growth factor (FGF) all inhibit fat development either in rats or in adipogenic cell lines (Smas and Sul, 1995; Cornelius et al., 1994). Growth factors activate mitosis (cell division), not differentiation. These mitogenic factors will not cause differentiation even once the cells are confluent. Overexpression of c-myc, a mitogenic signaling molecule, also prevents differentiation (Cornelius et al., 1994).

IGF-1 is the only growth factor required for differentiation. It can also substitute for GH in some cell lines requiring GH in serum-free differentiation media. IGF-1 acts at the cell surface through its receptor which is linked to a tyrosine kinase signaling cascade including the GTP-activated *ras*. This is further substantiated by the

fact that cells transfected with H-ras can be differentiated without GH or IGF-1 (Smas and Sul, 1995; Cornelius et al., 1994).

Other compounds are important in blocking adipocyte differentiation. Tumor necrosis factor α (TNFα) and other cytokines are inhibitors of differentiation. Even in combination with normal differentiation inducers, TNFα will not allow differentiation and actually depresses the expression of several lipogenic genes in adipocytes (Smas and Sul, 1995; Hauner et al., 1995; Ninomiya-Tsuji et al., 1993). TNFα also interferes with the insulin receptor substrate IRS-1 and may be a cause for insulin resistance. By blocking phosphorylation of IRS-1, TNFα blocks lipogenesis and glucose utilization initiated by insulin (Hotamisgil et al., 1996b; Yenush and White, 1997). TNFα has also been shown to reduce C/EBP and PPARγ expression in treated adipocytes. C/EBP and PPARγ, which will be discussed in the next section, are inducers of adipocyte differentiation. Each is involved in maintaining the adipogenic state of fat cells (Zhang et al., 1996; Stephens and Pekala, 1992). Retinoic acid also inhibits differentiation, causing de-differentiation or cell death. Retinoic acud is thought to suppress the expression of the adipogenic transcription factor C/EBP (Schwarz et al., 1997;

Cornelius et al., 1994). Other differentiation inhibitors include phorbol esters, dimethylsulfoxide (DMSO) and endothelin-1 (Cornelius et al., 1994). [TNF will be further discussed later.]

In summary, adipocyte differentiation has many regulators. Over all, there appears to be a requirement for an increased cAMP levels, a glucocorticoid stimulation and activation of the insulin/IGF-1 tyrosine kinase pathway in order to induce differentiation (Cornelius et al., 1994; Smas and Sul, 1995; Ailhaud et al., 1991). As will be discussed below, most of these differentiation regulators also affect the expression of S14.

Adipogenic Transcription Factors

The adipocyte transcription factors involved in adipocyte differentiation have been described. They include PPARγ, the C/EBP family and SREBP/ADD1. These function to induce transcription of other genes involved in differentiation and lipogenesis. Each of these have putative binding sites in the S14 promoter as well.

Peroxisome proliferator activated receptors (PPARγ) are a part of the nuclear hormone receptor family which includes PPARγ1 PPARγ2, PPARα and PPARβ (NUC1, FAAR, PPARδ are all homologs to PPARβ). PPARγ1 and 2 differ only in the 5′

untranslated region. PPARγ2 mRNA is slightly longer at 2.1 kb vs. 1.8 kb for PPARγ1 in rodents. The most important PPAR in adipocyte differentiation is PPARγ2. PPARγ2 is specifically expressed in high levels in adipocytes and at much lower levels in other tissues (Tontonoz et al., 1994b; Braissant et al., 1996; Schoonjans et al., 1996b; 1996c). PPARγ2 can be also be phosphorylated as a result of insulin or MAP kinase activation, perhaps indicating some crosstalk between insulin and PPARγ in adipocyte differentiation (Zhang et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997; Hu et al., 1996a; Reginato et al., 1998).

Endogenous PPARγ2 is expressed in 3T3-L1 and 3T3-F442A cells within 1-2 days post confluence of preadipocytes (Tontonoz et al., 1994b) but PPARγ expression alone is often enough to induce adipocyte formation. Overexpression of PPARγ2 can cause differentiation in fibroblasts (Tontonoz et al., 1994c). Overexpression of FAAR in conjunction with fatty acids will convert muscle cells into lipid containing cells (Teboul et al., 1995; Hu et al., 1995). The PPARγ2 ligand, pioglitazone, used with PPARγ2 expression is an even stronger inducer of differentiation in non-adipogenic cells (Brun et al., 1996). Clearly, PPARγ plays a leading role in adipocyte differentiation.

Activators of PPARs include long chain fatty acids and their metabolites, plasticizers and hypolipidemic drugs. Although considered orphan receptors only a few years ago, several ligands have now been determined for each of the PPARs. PPARγ2 ligands include 15-deoxy-Δ12,14 prostaglandin J2 (15-PGJ_2) and the thiazolidinediones (Kliewer et al., 1995; Forman et al., 1995; Lehmann et al., 1995). Leukotriene B4, 8(S) HETE and carbaprostacyclin bind PPARα and carbaprostacyclin also binds PPARβ. It is interesting to note that all of the naturally occurring ligands are derivatives of arachidonic acid (Mandrup and Lane, 1997; Brun et al., 1996; Spiegelman and Flier, 1996; Yu et al., 1995).

Indomethacin and ibuprofen have also been shown to be PPARγ ligands. This further substantiates the finding that indomethacin activates differentiation in TA1 adipocytes, perhaps by acting through PPARγ rather than by blocking prostaglandin production. Ibuprofen was shown to increase activity of PPARα as well. The authors concluded that adipocyte differentiation and peroxisome proliferation may be initiated by common pathways (Lehmann et al., 1997).

Because PPARs are activated by fatty acids, it has been suggested that they are the mediators of PUFA regulation of genes. Fatty acids and peroxisome proliferators increase β-

oxidation of fatty acids. For example, both the peroxisome proliferator, WY-14,643 and eicosatetraynoic acid (20:4,n-6 triple bonds), activate PPARα and the peroxisomal enzyme, acyl CoA oxidase (AOX) (Keller et al., 1993). AOX contains an PPRE and binds PPARα (Varanasi et al., 1996). Another gene regulated by fatty acids and peroxisome proliferators is apolipoprotein A-II. The authors here suggest that fatty acids and peroxisome proliferators are both acting to modulate gene transcription at through the PPRE (Vu-Dac et al., 1995). These findings suggest that the two regulators, fatty acids and peroxisome proliferators, may use the same pathway to influence gene transcription (Keller et al., 1993; Kliewer et al., 1997).

Some of the thiazolidinediones are now being used to increase insulin sensitivity in non-insulin dependent diabetes (NIDDM) patients (Brun et al., 1996). Pioglitazone and other thiazolidinediones can lower plasma insulin, triglycerides and glucose in NIDDM humans and animals. In the obese Zucker (*fa/fa*) rat, pioglitazone increased the use of glucose and insulin sensitivity. However, the treatment also increased adipocyte differentiation and expression of specific lipogenic genes: fatty acid synthase (FAS), phosphoenolpyruvate carboxykinase (PEPCK), C/EBPα, GLUT4 and decreases leptin expression. The increase in adipocytes is not a desirable side effect in the treatment of NIDDM

patients, who already tend to be obese. However, humans in clinical trials with the thiazolidinedione troglitazone have not yet shown weight gain (Hallakou et al., 1997).

PPARγ2 is known to activate several lipogenic genes including the adipocyte gene (aP2), malic enzyme (ME), lipoprotein lipase (LPL) and PEPCK as well as some β-oxidative genes (AOX) and P450 cytochrome genes (P450 4A6) (Hunter et al., 1996; Castelein et al., 1994). Like the other PPARs, PPARγ2 binds to DNA at its peroxisome proliferator response element (PPRE), a DR-1, as a heterodimer with retinoid X receptor (RXR). Usually the PPRE is located far upstream of the transcription start site and acts as an enhancer of transcription (Bernlohr et al., 1985; Amri et al., 1991; Tontonoz et al., 1994a; Graves et al., 1992; Spiegelman and Flier, 1996).

Several genes contain PPRE sites. The aP2 gene is often used as an early marker of adipocyte differentiation. This gene has two binding sites for PPARγ2, its key regulator and inducer (Tontonoz et al., 1994a). LPL was known to be activated in adipocytes and liver by treatment with peroxisome proliferators. Later a PPRE was found in the LPL promoter by gel shift assays (Schoonjans et al., 1996a). Similarly, the acyl-coenzyme A synthetase gene was found to contain a PPRE and be activated by peroxisome proliferators (Schoonjans et al., 1995). Kallen and Lazar

(1996) and De Vos et al. (1996) reported that leptin was down regulated by thiazolidinediones. These findings indicate that PPAR can both increase and decrease transcription of target genes.

Another adipogenic transcription factor is the CCAAT enhancer binding protein (C/EBP) family. C/EBPα, β and δ are leucine zipper transcription factors each involved in differentiation. This transcription factor tends to bind near the proximal promoter in target genes. The members of this family have basic helix-loop-helix binding domains and homo- or heterodimerize with other family members through a leucine zipper region. Both β and δ are expressed early, then α is produced during the last part of differentiation. Inhibition of C/EBP expression in preadipocytes but not in adipocytes may play a role in regulation when the cells are allowed to differentiate (Brun et al., 1996; Cornelius et al., 1994; Spiegelman and Flier, 1996; Mandrup and Lane, 1997; Jiang et al., 1998).

Like PPARγ, overexpression of C/EBP can induce differentiation of nonadipogenic cells (Wu et al., 1996; Freytag et al., 1994) or preadipocytes (Lin and Lane, 1994; Christy et al., 1991). Furthermore, antisense C/EBP expression in 3T3-L1 cells prevented expression of aP2, GLUT4 and stearoyl CoA desaturase 1 (SCD1) as well as inhibited triglyceride accumulation (Lin and Lane, 1992).

In C/EBPα knockout mice, the adipocytes do not contain as much lipid and have lower lipogenic gene expression than wildtype mice (Cornelius et al., 1994; Mandrup and Lane, 1997; Darlington et al., 1995). These findings indicate that the C/EBP family plays a key role in adipocyte differentiation.

Many variants of the C/EBP family exist and have been described. C/EBPβ is also known as liver activator protein (LAP) and its alternative splice product is liver inhibitory protein (LIP). LIP, which does not have a trans-activation domain, can block activation caused by LAP. C/EBPα also has two isoforms, a 30kDa protein (p30C/EBPα) and a 42kDA protein (p42C/EBPα). These are different products of a single gene. The p42 but not p30 inhibits mitosis in 3T3-L1 adipocytes but both activate gene transcription of aP2 (Cornelius et al., 1994). C/EBPβ and δ appear to be expressed first and transiently in preadipocytes treated with the hormonal inducers; then C/EBPα expression occurs and growth is arrested, initiating differentiation. C/EBPβ expression requires cAMP while δ requires glucocorticoids, two of the requirements for differentiation (Mandrup and Lane, 1997).

C/EBPα interacts with other gene promoters as well. Miller et al. (1996b) and Hwang et al. (1996) determined

that the leptin gene contains a C/EBPα binding site, and mutation of this site removed activation by this transcription factor. GLUT4 also contains a C/EBP binding site, which allows for transactivation of this gene (Kaestner et al., 1990). Both aP2 and SCD have promoter binding sites for C/EBP as well (Christy et al., 1989; Herrera et al., 1989).

Even more confusing is the interaction between the two transcription factors, C/EBP and PPARγ2. C/EBPβ and δ induce expression of C/EBPα, which can also up-regulate itself. C/EBPβ induces PPARγ which, in turn, can induce C/EPBα. Both C/EBPα and PPARγ induce the expression of two lipogenic genes already mentioned: aP2 and PEPCK (Tontonoz et al., 1994b; 1995; Cheneval et al., 1991). The leptin gene promoter also contains sites for each of these transcription factors but the factors themselves seem to antagonize one another, perhaps explaining why thiazolidinediones depress leptin expression (Hollenberg et al., 1997; Kallen and Lazar, 1996). The interactions between these transcription factors make the process of adipocyte differentiation and gene expression extremely complex.

A third transcription factor involved in adipocyte differentiation is adipocyte differentiation and

determination factor 1 (ADD1), also know as sterol response element binding protein 1 (SREBP-1). This protein also contains a basic helix-loop-helix domain like the C/EBP family but binds to "E-boxes" or CANNTG sequences as a dimer. This transcription factor was originally found to play a role in cholesterol regulation. However, ADD1 has been shown to play a role in adipocyte differentiation (Kim and Spiegelman, 1996). Specifically, overexpression of ADD1 under nonadipogenic conditions will induce the expression of the lipogenic genes LPL and FAS. These authors also showed that ADD1 expression will increase the activity of PPARγ. These results indicate that ADD1 may play a role in differentiation itself and/or through another of the transcription factors, PPARγ. Another indication of the role SREBP plays in adipocytes is its binding to the lipogenic gene, FAS. Both Bennet et al. (1995) and Tontonoz et al. (1993) showed that the FAS promoter binds SREBP, and with another transcription factor Sp1, could increase activity in a FAS promoter-luciferase reporter system. S14 also contains an E box in its carbohydrate response region (-1457 to -1428 bp). This E box binds ADD1 and transcription is activated in NIH 3T3 cells transfected with a S14 reporter vector (Kim et al., 1995).

The three major transcriptional regulators of adipocyte differentiation are PPARγ, C/EBP and ADD1. All can increase

differentiation of preadipocytes into adipocytes directly or indirectly as well as influence the expression or activity of each other and adipogenic genes. Spiegelman and Flier (1996) describe these interactions as shown in Figure 1.4.

Lipogenic Gene Expression in Adipocytes

Once differentiation has begun in adipocytes, several lipogenic and lipolytic genes are expressed. An early marker of differentiation is lipoprotein lipase (LPL). Other markers include aP2, adipsin, glycerol-3-phosphate dehydrogenase (GPD) and PEPCK. Some of the genes with increased expression include acetyl CoA carboxylase (ACC), malic enzyme (ME), fatty acid synthase (FAS), ATP-citrate lyase, S14, GLUT4, hormone sensitive lipase (HSL), stearoyl-CoA desaturase, angiotensinogen, insulin and β adrenergic receptors and glucocorticoid receptors (Butterwith, 1994; Mackall et al., 1976; Cornelius et al., 1994). All of these genes appear after differentiation of adipocytes (Lepar and Jump, 1989; Cornelius et al., 1994).

In general, lipogenic gene expression increases with insulin, glucocorticoid or carbohydrate treatment and decreases with polyunsaturated fatty acid treatment and agents which cause an increase in cAMP in both liver and adipose tissue. For example, PUFA, but not saturated fat diets, lower expression of liver stearoyl CoA desaturase

(SCD) in both lean and obese rats (Jones et al., 1996) and in mice and hepatocytes (Landschulz et al., 1994). A discussion of the control fatty acid synthase and S14 in liver and adipose tissue is included at the end of this chapter.

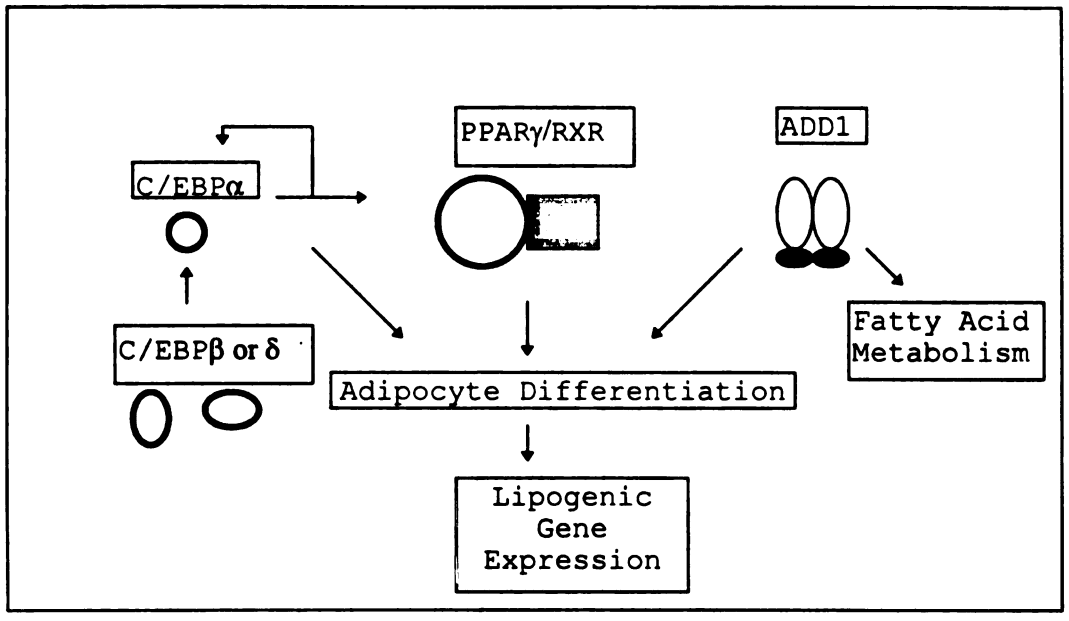


Figure 1.4. Adipocyte Transcription Factors. Initially C/EBPβ and/or δ is induced by hormonal changes which give rise to expression of PPARγ, ADD1 and C/EBPα. C/EBPα may allow for the continued presence of PPARγ, both of which are thought to be important for adipocyte differentiation (Spiegelman and Flier, 1996).

Arachidonic Acid

While both EPA and AA are polyunsaturated fats and suppress lipogenic gene expression, AA also plays a significant role as a precursor for eicosanoids (all AA

metabolites are termed eicosanoids). Very little AA is free within the cytosol. Most AA in the body is present in cell membrane phospholipids in the sn-2 position. With the proper signals, phospholipase A_2 cleaves the ester linkage, and AA is released into the cell. It can then be metabolized by one of the three major enzyme groups: epoxygenases, lipoxygenases and cylcooxygenases (Irvine, 1982; Makita et al., 1996; Clark et al.,1995; Camandola et al., 1996). Figure 1.5 describes the metabolism of AA.

The metabolites produced by epoxygenases include hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs). These compounds are produced from a cytochrome P-450 pathway which functions to monooxygenate arachidonic acid. Some of the epoxy products are involved in vasodilation/contraction and regulation of ion flux. For example, 14,15-EET has been shown to cause an increase of calcium output from liver cells while 5,6-EET increases intracellular calcium in liver. Inhibitors of epoxygenases have also been reported to block the calcium release induced by arachidonic acid. However, the incredible lack of stability of these compounds makes studying their effects difficult (Makita et al., 1996; Fitzpatrick and Murphy, 1989; Graber et al., 1997; Oliw, 1994).

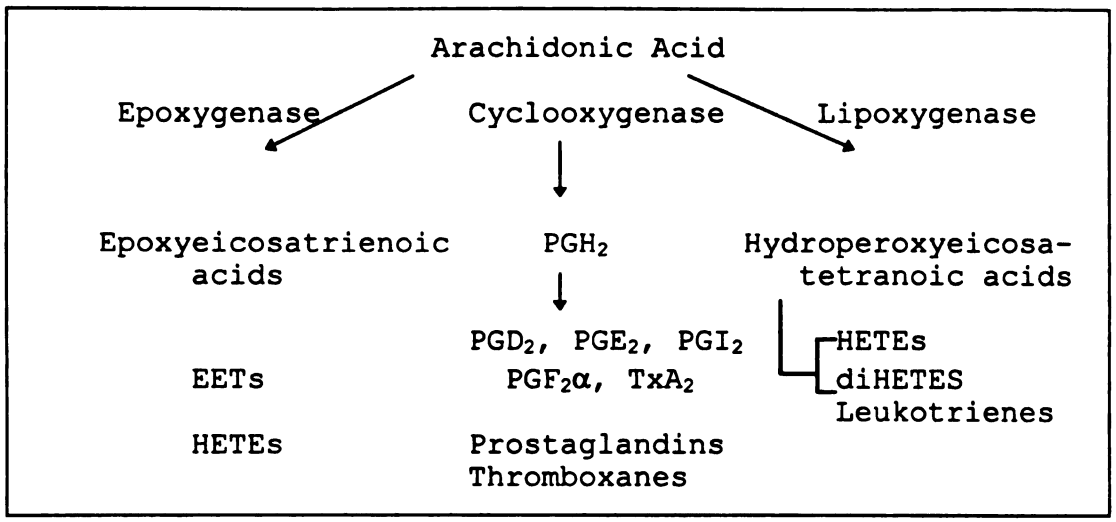


Figure 1.5. Arachidonic Acid Metabolism. Arachidonic acid can be metabolized in three separate pathways: epoxygenase, cyclooxygenase and lipoxygenase to result in several metabolites listed here.

The cytochrome P450s (CYP) are microsome associated enzymes that oxidize hydrophobic compounds. There are several families of CYP and some, e.g. 2C, 2E and 4A, monooxygenate fatty acids. For example, CYP4A oxidizes ω-1 mono and polyunsaturated fatty acids in liver microsomes (Fitzpatrick and Murphy, 1989; Oliw, 1994). Peroxisome proliferators increase the activity of some of these enzymes in the liver and some are regulated by PPAR (Demoz et al., 1994). Some metabolites produced in the liver include 20-HETE, 19-HETE, 18-HETE, 17-HETE and 16-HETE by CYP4A. CYP2E1 produces 19R-HETE, 19S-HETE and 18R-HETE. Of these, 20-HETE has been found in urine and 19-HETE has been proven to have biological activity. Some epoxides can be used in

phospholipid synthesis while others are metabolized into epoxy prostaglandins by PGH synthase in the liver (Oliw, 1994). Unlike liver, I found no evidence for epoxygenase products in the adipocyte or in adipose tissue.

The lipoxygenase pathway produces leukotrienes, HETEs and HPETEs. Lipoxygenase is stimulated by calcium in activated neutrophils, monocytes and macrophages. The leukotrienes produced include LTA_4, LTB_4, LTC_4 and LTD_4 depending on the cell type involved. Leukotrienes act mostly to induce chemotaxis, chemokinesis and aggregation of cells. LTB_4 in particular, is proinflammatory. The involvement of leukotrienes and their connection to asthma is a focus of much research (Christie et al., 1993). In another study, dietary fish oil depressed cyclooxygenase product formation but increased lipoxygenase product levels. The significance of more lipoxygenase products produced in these rats is not known (Hwang et al, 1988).

In the rat liver, liver fatty acid binding protein (L-FABP) binds lipoxygenase metabolites. Although L-FABP binds fatty acids like AA and OA, PGE_1, PGE_2 and leukotriene B4; 15-HPETE, 5-HETE and 15-HETE all bind with greater affinity (Raza et al., 1989). L-FABP binding of these metabolites may reduce their rate of further oxidation. It may also function to carry a specific fatty acid, such as arachidonic acid, to the nucleus to regulate transcription (Ek et al.,

1997). In liver, FABP has also been shown to bind PGA and PGJ_2 with greater affinity than arachidonic acid (Khan and Sorof, 1990). In the adipocyte, the fatty acid binding protein is similar to L-FABP: adipocyte lipid binding protein (ALBP or aP2). ALBP has a higher affinity for oleoyl CoA than L-FABP in the liver. However, both bind long chain fatty acids very well and play a role in fatty acid metabolism (Gossett et al., 1996).

Prostaglandins and thromboxanes are produced from the cyclooxygenase metabolism of arachidonic acid. EPA can also be converted to prostaglandins (3-series), however, AA is a much preferred substrate of the cyclooxygenase enzyme even with equal release of the two fatty acids. EPA can also act to suppress AA metabolism in platelets (Belury et al., 1989; Hwang et al., 1988). One reason for this suppression is due to the fact that EPA is a poor substrate for the constitutively produced cyclooxygenase in most tissues, which much prefers arachidonic acid (Laneuville et al., 1995). AA is also more mitogenic than EPA. In studies with Swiss 3T3 cells, AA was shown to activate the expression of c-fos and Egr-1, both early growth gene products, whereas EPA did not. This study also determined that the mitogenic response of AA was due to PGE_2 production followed by PKC activation (Sellmayer et al., 1996). Further discussion of the cyclooxygenase products of AA will follow this section.

Arachidonic acid can induce other signaling pathways without being metabolized. Wolf et al. (1986) showed that in permeabolized islet cells, AA caused an immediate release of calcium similar to levels induced by IP_3. In a similar experiment with neutrophils, calcium was also released upon treatment with arachidonic acid (Beaumier et al., 1987). Blobe et al. (1995) hypothesized that arachidonic acid activates PKC if calcium was present. Using rat liver microsomes, Chan and Turk (1987) showed that arachidonic acid could cause an intracellular increase in calcium which could not be blocked by lipoxygenase or cyclooxygenase inhibitors. These data indicate that arachidonic acid itself may be involved in cell signaling, possibly via a calcium mediated process.

Other examples of direct action of AA on gene expression include GLUT4 and SCD1. AA suppresses GLUT4 in adipocytes treated with 50μM AA for forty-eight hours (Tebbey et al., 1994). In adipocytes treated with cyclooxygenase, epoxygenase or lipoxygenase inhibitors, AA still caused the RNA stability of the SCD1 gene to be depressed while oleate and stearate had no effect on expression (Sessler et al., 1996).

Arachidonic acid is also involved in the inflammatory response. AA can block transcription of inflammatory genes such as IL-8, E-selectin and ICAM-1, however, some AA

metabolites are pro-inflammatory increasing cytokine and coagulant levels (Stuhlmeier et al., 1997). In humans who voluntarily consumed a lower n-6:n-3 fatty acid diet, the cytokines involved in inflammation, IL-1 and TNFα, are decreased (Camandola et al., 1996).

Arachidonic acid, cytokines and peroxisome proliferators have been shown to act by increasing the activity of NF-κB. This transcription factor binds to and activates transcription of several genes involved in inflammation. Gel shift binding of NF-κB increased with AA, was reversed by AA + indomethacin and could not be induced by EPA. For example, when the promonocytic cell line U937 is treated with arachidonic acid, the transcription factor NF-κB is stimulated. Further experiments indicated that PGE_2 (a metabolite of AA, the synthesis of which can be blocked by indomethacin) could also increase NF-κB binding (Camandola et al., 1996). Leung et al. (1996) reported NF-κB is activated in rat liver upon treatment with the peroxisome proliferator ciprofibrate.

NF-κB must move from the cytoplasm to the nucleus to induce transcription of its target genes. In the cytoplasm, NF-κB is bound by IκB. Phosphorylation of IκB cause the release of NF-κB, which in turn increases transcription of

the target genes after nuclear translocation (see Figure 1.5). TNFα causes phosphorylation of IκB, for example (Alberts et al., 1994). AA can inhibit the phosphorylation of IκB and hence, AA inhibits transcription of some inflammatory genes, suppressing the immune system (Stuhlmeier et al., 1997). Glucocorticoids also inhibit NF-κB activation by increasing IκB, acting as an immune suppressant (McKay and Cidlowski, 1998). At the same time, most AA metabolites, such as PG, are proinflammatory, possibly by increasing NF-κB action (Stuhlmeier et al., 1997). These results imply that AA action can depend on its metabolism and on which metabolic pathway is most active in the cell.

The production of eicosanoids from arachidonic acid (n-6) is suppressed by EPA, an n-3 fatty acid (Kinsella et al., 1990). Overproduction of eicosanoids can lead to immune suppression and high levels of inflammation, as discussed above. Hence a diet containing the correct ratio of n-6:n-3 is very important for body homeostasis. Although some n-6 fatty acid is required for eicosanoid synthesis, n-3 long chain PUFAs can substitute for membrane phospholipid needs. N-3 fatty acids have also been shown to decrease eicosanoid production and inhibit conversion of linoleate to arachidonate (Hwang et al., 1988). Releasing too much arachidonic acid from cell membranes may lead to

overproduction of eicosanoids. This suggests that consumption of more n-3 fatty acids may help off set this possibility (Kinsella et al., 1990). However, it is not well understood how dietary fat levels relate to membrane phospholipid. Currently, an equal balance of n-6:n-3 fatty acids in the diet is recommended (Kinsella et al., 1990; Food Fats and Health, 1991).

Prostaglandins

As indicated above, one possible pathway of PUFA suppression of S14 is through a prostanoid regulatory mechanism. Prostanoids are produced in both the liver and adipose tissue from fatty acids. The most common precursor is arachidonic acid, and therefore, the 2-series prostanoids most prevalent (Coleman et al., 1994). These prostaglandins have diverse effects in their target tissues. The actions of prostaglandins in adipose tissue and liver are focused on after a general description of prostaglandin receptors.

Prostaglandin Receptors

Before discussing prostaglandin receptors, below is a list of definitions which are helpful for the remainder of this discussion.

G-Proteins = are trimeric membrane proteins activated by a ligand binding to its receptor. Upon activation the alpha

subunit can further activate adenylate cyclase (G_s) or phospholipase C ($G_{q/o}$) or activation of phosphodiesterase (G_i) or other pathways. Prostaglandin receptors are linked to G-proteins.

G_s - activates adenylate cyclase, increasing cAMP and subsequent activation of protein kinase A.

G_i - activates a phosphodiesterase, decreasing cAMP and inhibition of protein kinase A.

$G_{q/o}$ - activates phospholipase C, causing IP_3 and diacylglycerol (DAG) formation. IP3 increases intracellular calcium while DAG with the calcium activates protein kinase C.

Pertussis toxin = acts by ADP-ribosylating the alpha subunit of G proteins. This disconnects the G protein from its receptor. It has been used to study prostaglandin signaling pathways. Reversal of PG action by PT indicates a G_i or G_o pathway (inhibition of cAMP or activation of phospholipase C).

Prostaglandins act on cell surface G-protein linked receptors. PGE_2 has four receptors:EP1, EP2, EP3 and EP4 while $PGF_2\alpha$ binds to the FP receptor. EP2 and EP4 receptors activate a G_s protein and increase cAMP. EP1 is linked to a pertussis toxin-insensitive G protein which activates phospholipase C, PKC and intracellular calcium release. The EP3 receptor is both sensitive or insensitive to inhibition by pertussis toxin and depending on the subtype, linked to at least three separate pathways. There are four splice variants of the EP3 receptor in the cow: a, b, c and d; each of which have different actions. The A subtype of EP3 inhibits adenylate cyclase, B and C induce cAMP and D can activate G_q, G_i, G_s to increase turnover phosphoinositides

and calcium levels. EP3-d is similar to the EP1 receptor (Coleman et al., 1994; Asboth et al., 1996).

EP receptors have a variety of actions in many different cell types depending on which subtype is present. EP3 subtypes are also present in mice, humans and rabbits and like the cow, each subtype has different actions. In the kidney, EP3 receptors can be inhibited by pertussis toxin to block the decrease of cAMP formation. The EP3 receptor linked to G_q is insensitive to pertussis toxin in bovine adrenal glands. EP3 receptors are also present in adipocytes where they function to inhibit lipolysis by blocking the epinephrine increase of cAMP (Coleman et al., 1994; Negishi et al., 1995). Bone cells use EP1 and EP4 receptors to increase calcium and cAMP by PGE_2, respectively (Suda et al., 1996). Myometrial cells contain all four EP receptors but EP1 is sensitive to pertussis toxin and EP3 is not, though both increase intracellular calcium (Asboth et al., 1996). In summary, PGE_2 increases cAMP, decreases cAMP or activates phospholipase C to increase intracellular calcium depending on the receptor present on the target cell.

The other prostaglandins, PGI_2 and $PGF_2\alpha$, also have cell surface receptors. PGI_2 binds the IP receptor, which is found in vascular smooth muscle tissue, nerve tissue, thymus, platelets and lung. IP receptors are linked to both

adenylate cyclase activation via G_s and intracellular calcium increase (Coleman et al., 1994). In WAT, PGI_2 was shown to activate G_s and increase cAMP coupled to release of calcium but also may act through a PPAR system (Aubert et al., 1996). PGI_2 has also been shown to act through PPARα (Hertz et al., 1996). $PGF_2\alpha$ binds FP receptors which act by increasing intracellular calcium. The G_q protein is thought to be involved (insensitive to pertussis toxin here) and causes a concomitant increase in IP_3 with calcium (Coleman et al., 1994). In preadipocytes, $PGF_2\alpha$ inhibits differentiation. This inhibition was reversible when cells were also treated with a calcium calmodulin-dependent protein kinase blocker, KN-62 (Miller et al., 1996a). Lepak and Serrero (1995) reported that $PGF_2\alpha$ could induce transcription of TGFα which prevented differentiation. These examples demonstrate the diversity and complexity of prostaglandin action.

Liver is known to have PG receptors, although how many types are present is not known. Hashimoto et al. (1997) reported that EP3 receptors were present in hepatocytes although in mice liver EP3 receptors were not detected by Sugimoto et al. (1992). It is also likely that because PGE_2 has been shown to increase or decrease cAMP levels in both adipocytes and hepatocytes that these cells contain EP2 or

EP4 receptors (Watanabe et al., 1986). Down regulation of the PGE_2 receptor involved in activation of cAMP has also been shown in rat liver (Robertson et al., 1980).

Actions of Prostaglandins

Primarily PGE_2 and PGI_2 are produced in adipose tissue of humans and rats. PGE_2 and PGI_2 act to control blood flow and lipolysis in adipose tissue (Vassaux et al., 1992). In cell culture, adipocytes also produce prostaglandins, although at a much lower level than preadipocytes (Hyman et al., 1982; Vassaux et al., 1992; Richelsen et al., 1992).

The PGE series is usually referred to as "antilipolytic" in adipose tissue. PGE_2 acts primarily to decrease cAMP, countering the glucagon effect (Christ and Nugteren, 1970; Castan et al., 1994; Watanabe et al., 1986; Richelsen, 1987). Antilipolytic effects by PGE_2 in adipose tissue is thought to be through EP3 receptors (Negishi et al., 1995; Strong et al., 1992).

Acting opposite of PGE_2, PGI_2 increases adenylate cyclase activity and is considered lipolytic (Aubert et al., 1996). Insulin can inhibit both PGE_2 and PGI_2 release in adipose tissue, perhaps relating blood pressure to insulin levels (Chatzipanteli et al., 1996). PGE_2 and PGI_2 also appear to counteract the action of one another in rat adipose tissue when both adipocytes and endothelial cells

are present (Chatzipanteli et al., 1992; Parker et al., 1989). PGI_2, while considered lipolytic in adipose tissue, can be adipogenic and will substitute for arachidonic acid or cAMP in the differentiation media of Ob1771 cells (Negrel et al., 1989).

In the adipocyte, prostaglandins can act through multiple pathways. Some studies suggest PGE_2 action on adipocytes is anti-lipolytic and acts via blocking an increase in cAMP (Vassaux et al., 1992; Christ and Nugteren, 1970; Chatzipanteli et al., 1992). Others suggest that PGE_2 acts through protein kinase C via a receptor coupled to phospholipase C in Swiss 3T3 fibroblasts (Danesch et al., 1996). Long and Pekala (1996a and 1996b) showed that GLUT4 expression in adipocytes was depressed by 20:4,n-6 and PGE_2 and further showed that cAMP was produced in adipocytes upon treatment with PGE_2. These studies indicate that adipocytes have the capacity to respond to prostaglandins by all three mechanisms discussed above: increasing cAMP, decreasing cAMP or altering intracellular calcium levels.

Prostaglandins are also produced in the liver by Kupffer cells upon injury, sepsis or other stimulus. Released PG act in a paracrine fashion on the surrounding hepatocytes. After liver injury, PGE_2 can increase hepatocyte proliferation through the EP3 receptor (Hashimoto et al., 1997). PGE_2 and $PGF_2\alpha$ can also induce DNA synthesis

in cultured hepatocytes in conjunction with hepatocyte growth factor (Adachi et al., 1995; Refnes et al., 1995). Skouteris and Kaser (1991) measured DNA synthesis in hepatocytes treated with PGE_2 and $PGF_2\alpha$ (which are induced by EGF). Not only did indomethacin (cyclooxygenase inhibitor) block the EGF effect, but PG treatment increased DNA synthesis, and this increase could be reversed with the calcium ion channel blocker, verapamil. This work implies that arachidonic acid was metabolized as a result of the growth factor treatment to produce the PG to cause the increase in DNA synthesis in hepatocytes. Later, this indomethacin inhibition was repeated in TGF treated hepatocytes. Again, prostaglandins seem to be actively involved in liver regeneration by increasing DNA synthesis (Skouteris and McMenamin, 1992).

Both PGE_1 and $PGF_2\alpha$ have been shown to block the effect of glucagon treatment of hepatocytes and be reversed by pertussis toxin (Melien et al., 1988). Glycogen breakdown is induced by PGE_2 in the liver. In Kupffer cell culture, glucagon induced PGE_2, PGD_2 and $PGF_2\alpha$ synthesis. These PG then inhibited the glucagon induced breakdown of glycogen. Furthermore, pertussis toxin could block this PGE_2 effect in hepatocytes. These results indicate that PGE_2 is acting through a pertussis toxin-sensitive G_i-linked receptor which acts by decreasing cAMP levels (Garrity et al., 1983; 1989;

Hespeling et al., 1995a; 1995b; Okumura et al., 1988). In contrast, others have found that pertussis toxin could not reverse the PGE_2 effects on glycogen breakdown but was likely acting through a calcium mediated process (Kanemaki et al., 1993; Mine et al., 1990). In other experiments, EGF stimulation of hepatocytes increased a G_i protein to activate phospholipase C resulting in an increase in calcium. The calcium release was blocked when the rats were pretreated with pertussis toxin before hepatocyte isolation (Yang et al., 1991; 1993). Clearly G proteins which activate calcium release exist on hepatocyte membranes, but which EP receptor PGE_2 is acting through is not clear (see Figure 1.6).

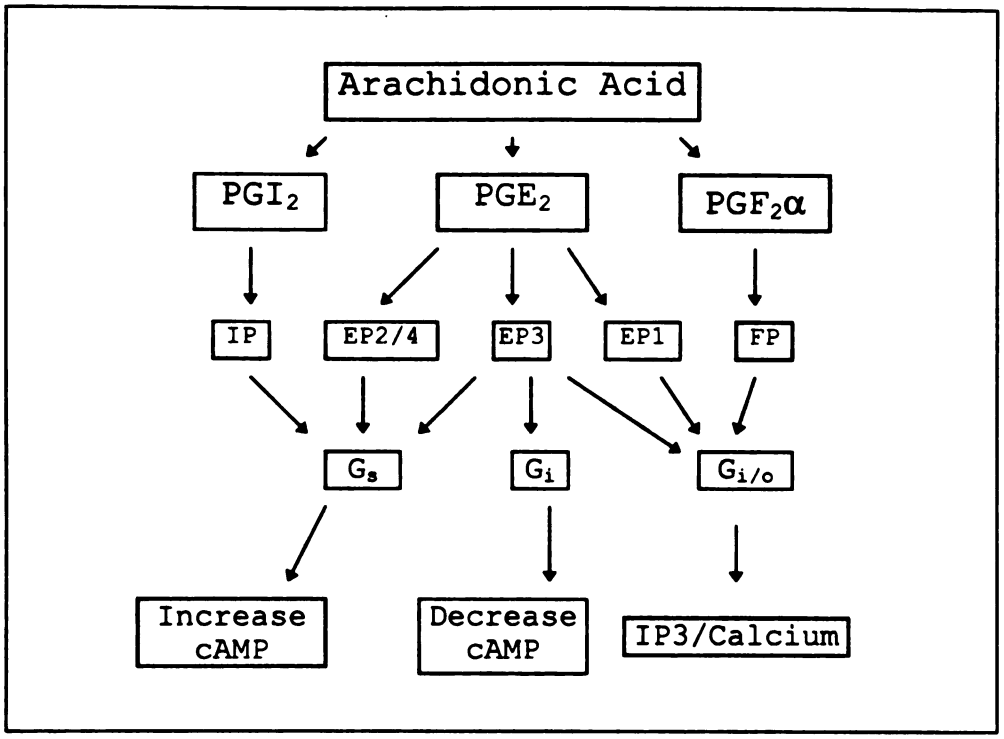


Figure 1.6. Overall Prostaglandin Synthesis and Signaling. Prostaglandins are synthesized from arachidonic acid and act on cell surface receptors to activate G proteins. This diagram is a general overview of the synthesis and signal pathways PG can act through. Notice that PGE_2 has 4 receptors and that EP3 has subtypes that work through all three pathways.

Hepatocyte and Kupffer Cell Interactions

The nonparenchymal liver cells include Kupffer cells, pit cells, fat cells and endothelial cells. Kupffer cells were discovered by von Kupffer in 1876. He called them "sternzellen" or star cells due to their shape. Although the star shaped cells and their associating cells later proved to be the above four separate types of cells, all are located in the sinusoidal walls of the liver. Of these cell

types, Kupffer cells are the most important in their interactions with hepatocytes (Billiar and Curran, 1992).

Kupffer cells are macrophages and vary in location, shape and size. Internally, Kupffer cells contain many lysosomes, vacuoles and phagosomes, typical of the average macrophage. They can be mobile and associate with pit cells and lymphocytes following inflammation. Kupffer cells function to fight infections and in detoxification. They are also in close contact with hepatocytes. Kupffer cell-hepatocyte interactions include receptor mediated, gap junction mediated, factor mediated as well as cell-to-cell interactions. The most important of these is the receptor mediated communication (Billiar and Curran, 1992).

The most common interaction between hepatocytes and Kupffer cells is receptor mediated communication. For example, stimulation of Kupffer cells causes a release of cytokines which interact with nearby hepatocytes via a receptor mediated process. Only the Kupffer cells secrete these compounds, not the hepatocytes. For example, one effect of Kupffer cells cytokines on hepatocytes is the inhibition of cytochrome P-450 enzymes. TNFα, prostaglandins, IL-6 and IL-1 are released from Kupffer cells during acute phase response to bacteria, viruses, immune complexes, colloidal compounds and other substances. These molecules go on to cause a variety of actions on all

the liver cell types. Growth factors are also secreted by Kupffer cells and induce hepatocytes to proliferate via a receptor mediated system in response to liver injury (Billiar and Curran, 1992; Mion et al., 1995). Some of these cytokines will be discussed further in the following sections.

Tumor Necrosis Factor (TNF)

Tumor necrosis factor (TNF) is produced by Kupffer cells in the liver. A portion of this thesis project involved using TNF to determine its effect on lipogenesis in hepatocytes. A brief review of TNF and its action follows. Most of this information was taken from an excellent review by Tracey, 1997.

TNF was simultaneously discovered in 1975 by two separate laboratories as an agent causing cancer cell death and a cause of wasting in chronic infectious diseases. After isolation and cloning, TNF was found to be a 17kDa protein having two forms: α and β. Although originally thought to be an endogenous anti-cancer protein, TNF is not specific to cancer cells but can target nearly all types of cells. TNF can cause all the symptoms of septic shock. Furthermore, antibodies to TNF can prevent these side effects of septic shock in baboons, even when bacteria is

present in their blood. This cytokine has a variety actions in many different physiological states (Tracey, 1997).

TNF is produced mainly by macrophages, which upon stimulus by endotoxin or lipopolysaccharide (LPS), will release the TNF. Other stimuli include enterotoxins, nitrites, calcium ionophores, irradiation, oxygen radicals, IL-1, phorbol esters, viruses and TNF itself, just to name a few. After release, the macrophages will not secrete further TNF until a 3-7 day recovery period has passed. Expression of TNF is increased through transcriptional regulation at a Y-box and an NF-κB enhancer sequence. Removal of the κB sequence in the TNF promoter will eliminate the LPS stimulated TNF release. In immunocytes, NF-κB is translocated to the nucleus to allow for transcription of proinflammatory genes (as well as TNF itself). Other transcriptional regulators of TNF include AP-2, CREB and AP-1. RNA stability and translational modifications also play a role in controlling TNF levels. Synthesis of TNF can depend on leukotrienes, cAMP, prostaglandins, tyrosine kinases and calcium levels (Tracey, 1997).

TNF binds cell surface receptors and activates several secondary pathways and mediators (Figure 1.7). Some of these include leukotrienes, prostaglandins, ceramides, interleukins, nitric oxide, cortisol, insulin, glucagon,

epinephrine, tyrosine kinases and calcium depending on the target cell. These responses to TNF depend on the cell types but include the following physiological effects: shock, lactic acidosis, hypoxia, fever, anorexia, release of hormones to regulate glucose, diarrhea, sodium uptake in muscle cells, depressed motility of the gastrointestinal tract and system inflammation and many others. For example in the vascular system, TNF causes production of nitric oxide (NO) causing vasodilation of blood vessels and leakage. NO levels are generated at toxic levels, causing myocardial and endothelial cell death, although the TNF levels can be toxic itself to these cells (Tracey, 1997; Fournier et al., 1997). In contrast, PGE_2 has been shown to inhibit NO in cultured hepatocytes (Harbrecht et al., 1996).

TNF is also involved in diabetes and obesity. TNF is lethal to islet cells and is often produced at higher levels in diabetic patients. In a septic shock, TNF depresses appetite and induces thermogenesis hypothalamically as well as causes insulin resistance. In obese animals, appetite is not suppressed but TNF levels are increased from adipocytes. Although TNF levels are high in these obese animals, they do not show the typical wasting seen in subjects with sufficient appetite control systems when treated with TNF (Tracey, 1997).

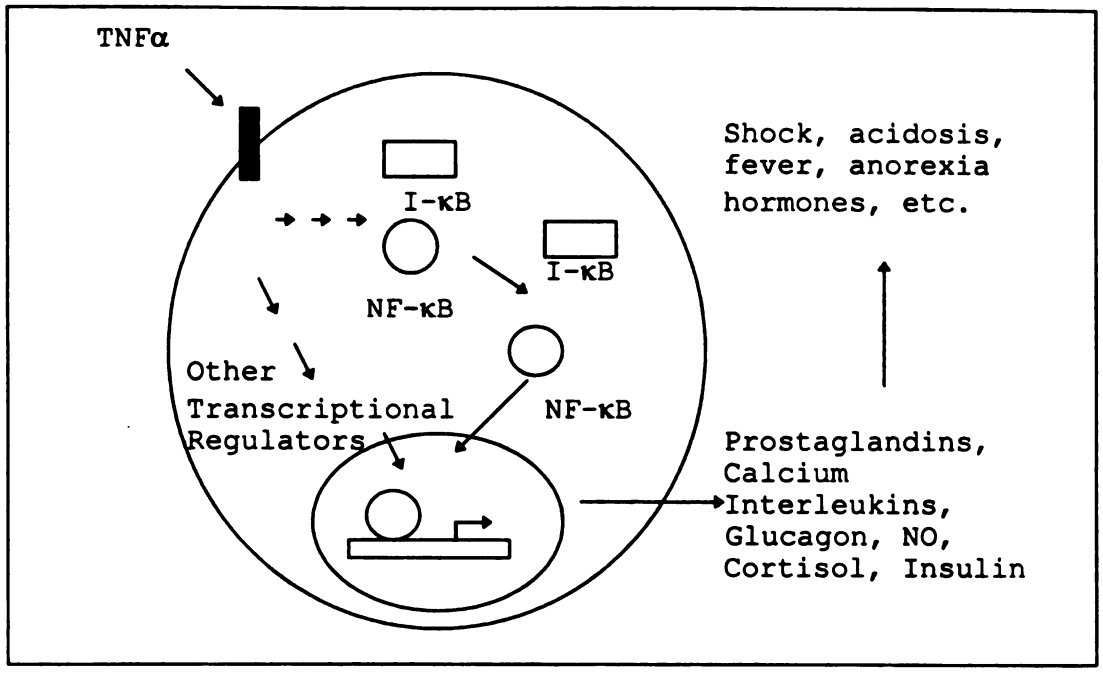


Figure 1.7. Summary of TNF Action. This diagram is a simplified summary of how TNF may act to produce in vivo responses. NF-κB is bound by I-κB in the cytosol. Upon TNF binding to its receptor, I-κB is phosphorylated and this releases NF-κB which can then translocate to the nucleus and activate transcription of several genes. Other transcriptional regulators are also likely playing a role in TNF stimulation of gene transcription.

Adipose tissue significantly contributes to TNFα levels. In obese mice models and in obese humans, TNFα levels are greater than in lean or normal controls. High levels of TNFα correlate extremely well with hyperinsulinemia, an indicator of insulin resistance in euglycemic states. It is thought that TNFα interferes with the normal insulin signaling pathway by blocking the

tyrosine kinase activity of the insulin receptor which in turn causes insulin resistance (Hotamisligil et al., 1995; 1996b). In contrast to adipocytes, in preadipocytes TNFα can increase glucose transport and upregulate the GLUT1 transporter (Cornelius et al., 1990). In adipocytes, TNFα downregulates GLUT4 and aP2 (Stephens and Pekala, 1992). TNFα is both depressing differentiation and causing insulin resistance in adipocytes.

The actions of TNFα released from Kupffer cells and acting on the hepatocyte appear somewhat contradictory to the in vivo and adipocyte data. In rats treated with TNFα, liver lipogenesis and serum triglycerides increase and LPL activity decreases (Grunfeld et al., 1990; Feingold et al., 1990). Using mice resistant to LPS (they could not produce TNF when treated with LPS), Adi et al. (1992) demonstrated that TNF was required for the lipogenic effects of LPS. The wild type mice had increased lipogenesis as measured by fatty acid synthesis in the liver while the mutant strain had lower fatty acid synthesis and much less TNF and IL-1 message. However, in hepatocyte cell culture, TNFα did not increase lipogenesis in experiments reported by Brass and Vetter (1994). TNFα also increases lipolysis in adipocytes, which *in vivo*, causes a release of fatty acids into circulation. The liver then re-esterifies and releases

these in the form of VLDLs which may account for the higher triglyceride levels seen with TNFα treatment (Feingold et al., 1992; Grunfeld et al., 1990).

The search for the TNFα mediator involved in the induction of lipogenesis has resulted in several possibilities: IL-1, IL-6, sphingomyelin, interferon α and prostaglandins. Grunfeld et al. (1990) ruled out prostaglandins as a TNFα mediator but suggested that IL-6 could be involved. Brass and Vetter (1994) reported that IL-6 and a prostaglandin E agonist both increased hepatocyte lipogenesis but TNFα did not. Wang et al. (1995) reported that TNF does not increase cAMP in hepatocytes but does increase cAMP levels in Kupffer cells, possibly as a result of higher PGE_2 levels. These findings make it difficult to determine what compounds are released from Kupffer cells and their cumulative result on lipogenesis as they collectively target hepatocytes.

One TNF signaling pathway is through the sphingomyelin (SM) cycle. TNFα causes hydrolysis of sphingomyelin, generating ceramide. The ceramide, a signaling molecule, can mimic TNF action in some cell lines. Both lead to apoptosis in leukemia cells, for example. This action is blocked by the use of PKC activators, suggesting that the action of ceramide (or TNF via ceramide) may involve the

inhibition of PKC. The PKC inhibition hypothesis is further substantiated by the activation of protein phosphatase by ceramide although ceramide can activate certain PKC isoforms and MAPK. Ceramide can also increase NF-κB activity and PGE_2 secretion (Hannun, 1997; Hannun and Obeid, 1995). Also in leukemia cells, Jayadev et al. (1994) reported that TNFα increased AA release which in turn increased sphingomyelin metabolism and generation of ceramide. Both TNF and ceramide have been shown to lower GLUT4 expression in adipocytes. This report indicates this signaling pathway exists in adipocytes in addition to the usual inflammatory cells (Long and Pekala, 1996a; 1996b).

As mentioned above, another factor indicated in TNF action is IL-1. IL-1 is a proinflammatory cytokine which binds a separate and distinct cell surface receptor from TNF. Both can cause similar effects, however. Several inducers of IL-1 such as LPS, phorbol esters, radiation and viruses also induce TNF. Kupffer cells also produce both of these cytokines, although monocytes produce much more IL-1 than Kupffer cells. IL-1 binds a receptor which causes activation of a MAP kinase also activated by LPS. Blocking this MAP kinase phosphorylation can stop production of TNF and IL-1. There is some evidence that this MAP kinase phosphorylation involves a Janus kinase (JNK) or Stat pathway. IL-1 can also increase prostaglandin synthesis

(Tocci and Schmidt, 1997). IL-1α can also activate nuclear translocation of NF-κB, like TNF does (Brasier et al., 1990). Others have reported that IL-1, acting through the sphingomyelin/ceramide pathway, regulates the oxidative gene CYP2CII in hepatocytes (Nikolova-Karakashian et al., 1997). It is unknown how IL-1, TNF and PG interact to influence S14 expression in the hepatocyte. One part of this thesis will be to look at the effect of these three players in the suppression of S14.

TNF and PGs in Primary Hepatocyte Culture

Isolation of hepatocytes from adult rats using the collagenase perfusion technique is selective for hepatocytes only. This implies that the primary culture contains only hepatocytes. However, it is possible that small amounts of Kupffer or other nonparenchymal cells are carried through into the culture. Moreover, it is Kupffer cells, not hepatocytes, that produce most, if not all, the prostaglandins. PGD_2 is the most predominant prostaglandin produced by Kupffer cells but PGE_2 is thought to be important as well. Hepatocytes, although they are not synthesizing the prostaglandins, have a great capacity for metabolizing prostanoids and leukotrienes (Billiar and Curran, 1992; Billiar et al., 1990).

During sepsis, Kupffer-hepatocyte interaction becomes very important in glucose and fatty acid metabolism. Sepsis increases output of interleukins (IL), prostanoids and TNFα from the Kupffer cells. IL-1 is linked to an increase in gluconeogenesis while TNFα increases glucose metabolism and insulin levels. PGD_2 increases glycogenlysis in hepatocytes, an effect that can be blocked with indomethacin (an inhibitor of cyclooxygenase). Lipolysis and gluconeogenesis are also stimulated in both the liver and adipose tissue upon sepsis via the nervous system and subsequent elevation of adrenaline, cortisol and glucagon. Sepsis patients then become hyperglycemic, even with adequate levels of insulin present followed by hypoglycemia. Patients also become hypertriglyceridemic as triglycerides are released from the adipose tissue from TNFα action. In the liver, fatty acid oxidation and release of free fatty acids is increased. In some experiments, TNFα and IL-1 have lowered LPL activity, which may contribute to the higher levels of triglycerides in the blood. If no epinephrine or cortisol is present, TNFα has also been shown to lower triglyceride production in hepatocytes (Billiar and Curran, 1992; Rodriguez de Turco and Spitzer, 1990).

Although both TNFα and prostaglandins are released from Kupffer cells, they can have different effects. In contrast to TNFα, PGD_2 and PGE_2 were shown to decrease VLDL production in hepatocytes by a calcium or cAMP mediated pathway (Bjornsson et al., 1992). PGE_2 has also been reported to increase glycogenolysis (i.e. increase glucose output) by a calcium mediated pathway (Mine et al., 1990), but inhibit glucagon induced PEPCK expression by the same reported mechanisms (Valera et al., 1993; Puschel and Christ, 1994).

There has been some research with PGE_2 and its effect in hepatocytes. It is thought that PGE_2 inhibits PEPCK expression by decreasing cAMP levels through an EP3 receptor in hepatocytes. This receptor, linked to a G_i protein, is sensitive to pertussis toxin inhibition. Pertussis toxin has been used to reverse the PGE_2 effect on PEPCK activity (Puschel and Christ, 1994). These authors also reported that PGE_2 increased the decay of PEPCK mRNA. However, if the PGE_2 was added two hours after the addition of glucagon, there was still some inhibition of PEPCK activity. This inhibition was not reversible by pertussis toxin, suggesting another pathway than inhibition of cAMP by PGE_2. Others have reported that an increase in calcium causes inhibition of PEPCK in hepatocytes. By decreasing extracellular calcium with EGTA, Valera et al. (1993) reversed the

decrease in PEPCK message. However, these authors did not indicate reversal of PGE_2 action with any agent. Another example in cultured hepatocytes, is the report by Mine et al. (1990) in which glucose output was increased by PGE_2. These authors could find absolutely no increase in cAMP but did measure an increase in intracellular calcium and IP_3 production. These findings suggest that PGE_2 is most likely involved in activating more than one signaling pathway in the hepatocyte when regulating glucose output.

Calcium Mediators

As discussed above, many of the mentioned compounds use calcium as a signaling pathway. Arachidonic acid and prostaglandins both can increase intracellular calcium levels in many different cell types. Vasopressin is often used to activate IP_3 to increase calcium. This section will briefly discuss calcium cell signaling and some examples of cell systems in which calcium levels were measured in response to various treatments.

The change in intracellular calcium levels is commonly used as a signaling pathway in cells. Calcium release is one pathway used by G_q-proteins, for example. After binding of a ligand to a cell surface receptor, the G-protein is activated, causing phospholipase C (PLC) to be activated. PLC cleaves phosphatidylinositol bisphosphate to give rise

to diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 is coupled to a calcium channels on the endoplasmic reticulum which releases calcium into the cytoplasm. DAG can activate PKC, which is calcium dependent. PKC has a variety of actions including activation of MAP kinases and subsequently NF-κB. Calcium ionophores like A23187 can mimic the IP_3 effect in many cell types while phorbol esters like TPA mimic the DAG effects. Another protein activated by calcium is calmodulin which can regulate calcium/calmodulin-dependent protein kinases (CaM Kinases) or calcium ion pumps (Alberts et al., 1994).

Calcium levels are also affected by activation of ion channels on the cell surface. The hetero-trimeric G_i protein can directly act upon a calcium ion channel in the plasma membrane to allow calcium influx when the receptor is activated. (The G_i protein also acts to decrease cAMP, as discussed in the prostaglandin receptor section.) Calcium ion channels can also be "voltage gated", which respond to membrane polarity. In muscle cells for example, calcium ion channels on the cell surface are voltage gated but ion channels on the sarcoplasmic reticulum are not (Alberts et al., 1994). Figure 1.8 summarizes calcium signaling.

Calcium is a major player in many of the different signaling pathways. Several examples of calcium release in hepatocytes or adipocytes in response to the above mentioned

compounds can be found. Vasopressin, acting through IP_3, has been used in hepatocytes to determine the effects of intracellular calcium release on gene expression (Duddy et al., 1989). Several have reported (Hughes et al., 1987; Butta et al.,1993; and Fernando and Barritt, 1994; Fernando et al., 1997; Mellgren et al., 1997) that vasopressin stimulates calcium release through a pertussis toxin sensitive GTP protein in hepatocytes. In another example, Kass et al. (1990) reported that vasopressin caused a calcium influx through ion channels in hepatocytes as a secondary
response after the initial intracellular calcium release. Sphingosine has also been shown to activate calcium release from intracellular stores to activate glycogen phosphorylase in hepatocytes (Im et al., 1997). Berven and Barrit (1994; Berven et al., 1995; 1994) localized a G-protein sensitive to pertussis toxin that upon activation, increased calcium release into hepatocytes. In WAT, Izawa et al. (1994) used adrenal corticotropin hormone to show that this hormone increased arachidonic acid release which increased calcium in the cells. Furthermore, they could block this calcium release by pertussis toxin. Calcium is also released in human platelet cells treated with arachidonic acid, even when metabolism of AA is blocked by lipoxygenase or cyclooxygenase inhibitors (Tohmatsu et al., 1989). These

examples demonstrate just a few of the wide variety of compounds using calcium as a second messenger in their signal transduction pathways.

Fatty acids can also change calcium levels. In hepatocytes, palmitoyl CoA has been shown to cause a release of intracellular calcium (Fulceri et al., 1993). Gomez-Munoz et al. (1991) reported that palmitate and linolenate increased glycogen phosphorylase activity through a calcium mediated pathway. In rat adipocytes, palmitate increases glucose uptake. In experiments using a intracellular calcium chelator (quin2-AM), the palmitate was unable to stimulate the glucose uptake indicating the importance of calcium in the signaling pathway (Thode et al., 1989).

S14 is suppressed by an increase in intracellular calcium. Sudo and Mariash (1996) showed that hepatocytes treated with the calcium ionophore A23187, had suppressed S14 mRNA. Vasopressin treatment did not result in any change in S14. Blocking the calcium release from the endoplasmic reticulum with thapsigargin prevented the increase in S14 expression normally seen with glucose treatment. These results suggest that the endoplasmic reticulum calcium pool is necessary for the glucose induction of S14 but the IP_3 induced calcium release (via vasopressin) is not.

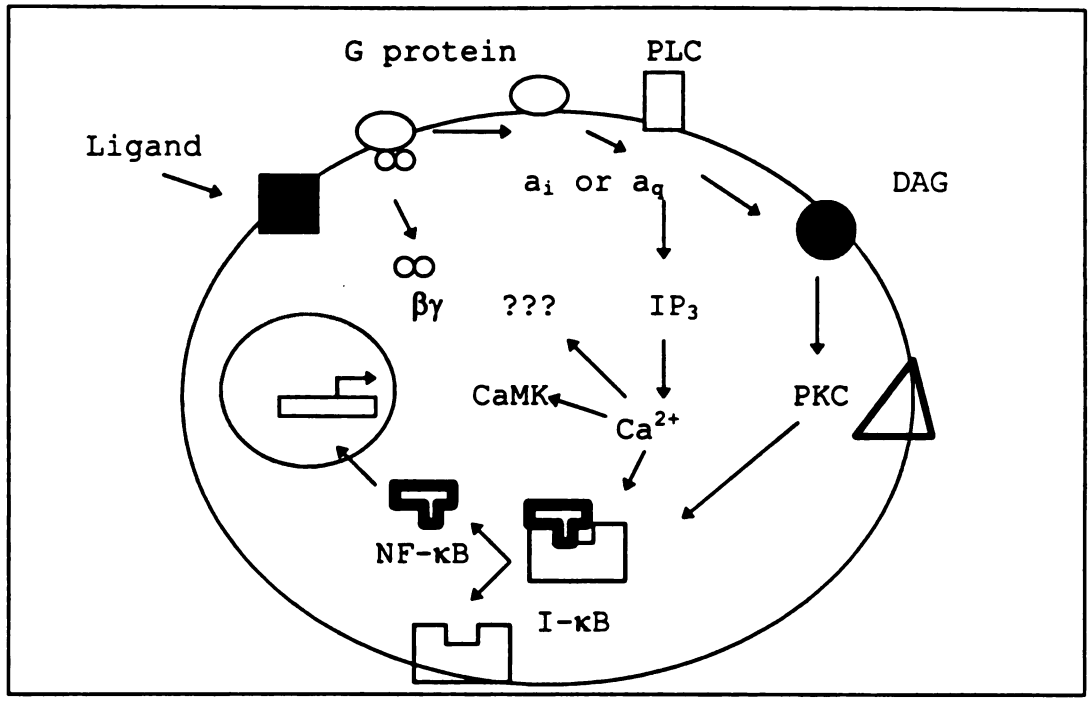


Figure 1.8. Calcium Signaling Pathways. Binding of a ligand to the receptor on the target cell activates a G-protein. G-proteins can activate PLC which generates DAG and IP3. IP3 can activate calcium release from intracellular stores. PKC is activated by DAG and calcium. One target of PKC activation is a MAP kinase which phosphorylates I-kB to cause release of NF-kB. NF-kB, once released, can translocated to the nucleus and activate gene transcription of targeted genes. Calcium can also activate CaMK through calmodulin. Other possible effects may also result as calcium levels change in the cell.

Calcium seems to be involved in insulin-induced lipogenesis in adipocytes as well as regulate some lipolytic and lipogenic enzymes via other agents such as parathyroid hormone and vitamin D (Ritz et al., 1980; Avasthy et al., 1988). Very few examples exist for direct measurement of lipogenic gene expression regulation by calcium per se. As discussed previously, $PGF_2\alpha$ acts through a calcium mediated

pathway to inhibit adipocyte differentiation, and therefore, lipogenic genes requiring the differentiated state (like FAS) would not increase (Sessler and Ntambi, 1998).

These examples represent only a few of the wide variety of mediators that can cause an increase in calcium in the cells. Control of each of these and how they interact is not always clear. While the focus of this thesis is not on calcium signaling, the regulation of lipogenic gene expression by AA and PGE_2 might involve calcium mediated mechanisms and will be addressed in Chapters 2 and 3.

Regulation of FAS and S14

Both FAS and S14 are expressed in the adipocyte and hepatocyte and have been used as models to study lipogenic gene expression. Neither is detectable in the preadipocyte but mRNA levels for FAS dramatically increase in 3T3-L1 cells upon differentiation while S14 expression increases only after adipocytes are treated with glucocorticoids (Lepar and Jump, 1989). Glucagon or cAMP lowers and insulin raises FAS and S14 mRNA levels in these cells (Moustaid et al, 1993; Paulauskis and Sul, 1988; Jump and MacDougald, 1993; Sul et al., 1993; Jump et al., 1990a). Triiodothyronine (T3) has proven to increase transcription of these genes, leading to increases in mRNA. T3 also increases mRNA stability of FAS in 3T3-L1 adipocytes

(Moustaid and Sul, 1991). S14 mRNA expression in 3T3-L1 adipocytes is not induced by T3 treatment, but interestingly, S14 mRNA is increased in epididymal fat after rats are treated with T3. Fat treatment also changes S14 expression in adipose tissue. WAT S14 message levels in rats fed fish oil are approximately 50% of the levels in rats on high carbohydrate diets even after only a few hours (Jump and Oppenheimer, 1985; Lepar and Jump, 1989; Jump et al., 1993).

In the rat liver or hepatocyte, S14 and FAS regulation is again nearly identical. Thyroid hormone increases mRNA expression of both genes, as does insulin or carbohydrates. Starvation and polyunsaturated fatty acids (PUFA) inhibit transcription. Rats fed a diet of menhaden oil (contains n-3 PUFA), have depressed S14 and FAS levels within a few hours as well as lower serum triglycerides (Jump et al., 1996; 1993; Blake and Clarke, 1990). After starvation, refeeding induces S14 and FAS expression in the liver about 25-fold but in adipose tissue only FAS is induced at these levels after refeeding (Kim and Freake, 1996).

The greatest differences between FAS and S14 are the sizes of their genes and promoters. FAS is a large gene and protein. The FAS gene includes over 18,000 base pairs containing forty-two introns. The 250kDa protein functions as a homodimer with each monomer comprising seven separate

enzyme functions in three domains. Sequence analysis revealed that its promoter contains transcriptional regulatory sequences similar to the estrogen response element, glucocorticoid response element, thyroid response element and the progesterone response element. Eight sequences of the cAMP transcriptional regulatory element (CRE) are also present (Amy et al., 1990; 1992).

The S14 protein has been extensively studied in our laboratory as a model for lipogenic gene regulation. S14 is a small protein (M=17,000) with mRNA lengths of 1.3 and 1.47kb (the 2 sizes differ only in the 3′ untranslated region). This gene is expressed in lipogenic tissues, and like FAS, is extremely responsive to nutritional status of the cell or animal. Carbohydrates (or eating a normal chow diet in rats) induce transcription while starvation and polyunsaturated fatty acids suppress transcription. Thyroid hormone greatly induces expression in rat liver or hepatocytes (Jump, 1989; Jump et al., 1990; 1984; 1993).

High expression of S14 only occurs in the liver, lactating mammary tissue and adipose tissue. The S14 promoter and gene is also nearly homologous between humans and rats (Ota et al., 1997). The protein has been localized to the nucleus by immunohistochemical methods (Kinlaw et al., 1993). Recently experiments using antisense S14 expression in hepatocytes showed that S14 is involved in the

induction of ATP citrate lyase, FAS, ME activity, PEPCK and PK (Brown et al., 1997; Kinlaw et al., 1995). Cunningham et al. (1997) reported that S14 is a homodimer which co-precipitates with an unknown hepatic protein. S14 has also been shown to be expressed in mammary gland (Jump and Oppenheimer, 1985). In breast cancer cells lipogenesis is increased and overexpression of FAS implies a poor prognosis. S14 has also been found to be increased in human breast cancer (Moncur et al., 1998). These results suggest that S14 may be a transcriptional regulator of lipogenic genes in all three of the tissues where it is expressed.

In summary, S14 and FAS are both lipogenic genes with similar regulation. Each has hormonal controls: T3, insulin, glucagon and glucocorticoids. Both are controlled by diet: fasting, feeding, carbohydrates and dietary fats. Both also have tissue specific regulation, being expressed in the liver and adipocyte. The regulation of these genes has led to great interest in the promoters of each gene. Because S14 has been very amenable for research purposes and the promoter is well characterized, S14 has been used as a model to study lipogenic gene expression in our laboratory. The following section will focus on several aspects of the S14 promoter.

FAS and S14 Promoter

As discussed in the above section, FAS is regulated much like S14. Soncini et al. (1995) used a transgenic mouse expressing the reporter gene chloramphenicol acetyl transferase (CAT) under the control of the FAS promoter to -2.1kb to study regulation of FAS by various compounds. CAT was expressed in the correct tissues (liver, WAT and mammary gland) and was induced by glucocorticoids and suppressed by cAMP as expected. However, diets containing fish oil greatly suppressed the endogenous FAS but only diminished CAT activity about 50%. This observation was repeated in hepatocytes upon transfection of the reporter vector (-2195 to -60. Several sites were located upon promoter analysis including putative Sp1, T3 and two GRE binding domains. CAT activity increased when hepatocytes were treated with insulin or T3 treatment and dexamethasone compared to control treated cells but not with insulin or T3 treatment alone. It was unexpected that T3 and insulin would not increase CAT activity since both of these agents increase in vivo levels of FAS mRNA (Clarke and Jump, 1993). Others have mapped the insulin sensitive region in the FAS promoter to -67 to -52bp (Moustaid et al., 1993; 1994). These results indicate that the FAS promoter is not limited to -2500 base pairs. Work with the FAS promoter in CAT reporter systems often gives disparate responses. This lab and

others have also attempted to determine the PUFA response region in FAS but efforts to date have not yet been successful. These responses with FAS have prompted our use of S14 as a model to study lipogenic gene regulation.

Unlike FAS, the promoter region of S14 is well defined and has been more amenable to experimentation. Several regions in the S14 promoter which bind specific factors have been mapped. The thyroid hormone response region (TRR) at -2.7 kb was first detected by DNase I treatment of rat liver nuclei (Jump, 1989). Two other important regions, the pluripotent response region (PRR) from -1.6 to -1.4 kb and the proximal promoter region (+19 to -260) were also mapped as important regulatory regions (Jump, 1989; Jump et al., 1990; 1988).

The PRR (-1.6 to -1.4) has been implicated as the carbohydrate and insulin responsive region. This region is also responsive to glucocorticoids and retinoic acid in adipocytes although this gene is not responsive to these compounds in hepatocytes (unpublished observations by A. Thelen and D. Jump). Towle et al., 1996, reported that -1476 to -1422 region was required for the glucose response of S14 in hepatocytes. These authors also reported that two other factors, upstream stimulatory factor (USF) and an unknown factor were involved in the carbohydrate response. USF binds E-boxes, two of which are present in the S14 PRR.

Although mutation of the E-boxes did not modify the glucose response, altering the spacing between them did change the responsiveness. These authors concluded that while USF may not be the carbohydrate response factor, it may be necessary for binding or modification by another factor to allow for the carbohydrate response of S14. However, recent experiments in the our laboratory have indicated that removal of the S14 PRR (-1600 to -1400bp) does not diminish the glucose response in hepatocytes (unpublished data).

The proximal promoter region spans the region from +19 to -290bp with +1 being the transcription start site. This area contains a TATA box (-27 to -21), an NF-1 binding site (-63 to -48) and two regions designated the Y box and C regions (see Figure 1.9). The Y box contains an inverted CCAAT box (-83 to -104) which binds C/EBP, NF-1 and NF-Y. Recent experiments have determined that T3 induction of S14 requires NF-Y binding to the Y box, implicating an interaction between the TRR and the proximal promoter for transcriptional regulation of S14 (Jump et al., 1997b).

NF-Y, or CCAAT box binding factor (CBF), is a trimeric protein that binds DNA. Two of the subunits (B and C) bind to DNA in a "handshake" motif, similar to that seen in histones H2a and H2b. NF-Y functions to recruit histone acetylases (e.g. GCN5, CBP and P/CAF) to the DNA (Currie, 1998). Histone transferases transfer the acetate from

acetyl CoA to the amino group of lysine. Histones within nucleosomes are acetylated at lysine residues at their N-terminal end leading to changes in DNA/histone interaction. Such modifications are thought to be involved in local remodeling of chromatin to enable additional transcription factors to bind DNA. The S14 Y box and entire proximal promoter region is within a DNase I hypersensitive region, a structure indicative of local chromatin remodeling (Jump et al., 1997b).

The proximal promoter region has been implicated as the PUFA response region. Analysis using hepatocytes transfected with various promoter deletion S14 CAT constructs treated with EPA showed that the region between -80 and -220 is sensitive to this PUFA (Jump et al., 1993). S14 mRNA in primary hepatocytes is suppressed by several PUFAs including linoleic acid (18:2n-6), α and γ linolenic acids (18:3n-6 and 18:3n-3), arachidonic acid (20:4n-6) and EPA (20:5n-3) (Jump et al., 1994; Ren et al., 1997).

It is not known how PUFAs control the S14 gene transcription. However, the Y box is involved in transcriptional regulation of S14 (Jump et al., 1997b) and the Y box is located in the PUFA-RR, an element expected to participate in the PUFA control. This thesis will test the hypothesis that NF-Y, per se, is the target for PUFA action.

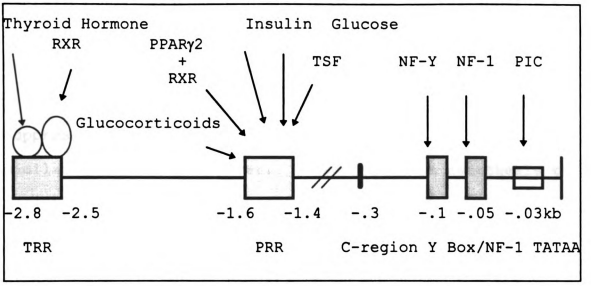


Figure 1.9. The S14 Promoter. The S14 promoter and its major binding domains are shown above. TRR= thyroid hormone response region and PRR= pluripotent response region. The different factors which bind S14 are listed with an arrow to its appropriate binding location.

Concluding Statements and Hypothesis

Regulation of lipid metabolism has proven to be extremely complex. Dietary, hormonal and tissue type can affect the transcription of the genes involved. A main goal of our laboratory is to determine how PUFAs regulate lipogenic gene transcription. We have used the S14 gene as a model to define the molecular basis of PUFA regulation of gene transcription. Previous research in our laboratory has demonstrated that PUFA suppress S14 gene transcription in both adipose tissue and liver and target the proximal promoter region (-220 to -80bp) of this gene (Jump et al., 1993). Furthermore, Ren et al. (1996; 1997) showed that EPA

suppression of S14 was not through a PPAR mediated pathway in vivo. Very recently, a Y-box has been found in the PUFA-RR of the S14 promoter (Jump et al., 1997).

These results lead to the following hypothesis: PUFA suppression of lipogenic gene expression in adipocytes is similar to that seen liver. To address this hypothesis the following questions are asked: 1) how does AA regulate lipogenic gene expression in adipocytes? 2) Is the adipocyte pathway of PUFA regulation of lipogenic gene expression also utilized by hepatocytes? 3) Is the Y box the target for PUFA regulation of S14 gene transcription?

CHAPTER 2

CHAPTER 2

ADIPOCYTES

Nearly a third of American adults are considered obese. Obesity, an imbalance in metabolism, is a result of hyperplasia and hypertrophy of adipocytes as a result of consuming too many calories. Fat in the diet contributes to this caloric load. The typical American diet contains 37% of the calories as fat although FDA recommendations suggest consuming under 30%. Obesity is correlated to serious health implications including heart disease, hypertension, insulin resistance and diabetes. Controlling dietary fat intake may help manage these health risks (Food Fats and Health, 1991).

Dietary fat includes saturated, monounsaturated and polyunsaturated fatty acids (PUFA). A diet high in saturated fat is linked to heart disease, cancer and diabetes. In contrast, polyunsaturated fats, especially n-3 fatty acids, are thought to lower the risk of heart disease by lowering serum triglycerides (Zemel, 1995; Rocchini, 1995; Food, Fats and Health, 1991). A major focus of

research is how these different fats cause or prevent health risks.

Much of the research on dietary fat has focused on rodent models. Dietary saturated fat has been shown to increase fat cell mass in rats at a higher rate than PUFA (Shillabeer and Lau, 1994). In rats, dietary polyunsaturated fats lower hepatic de novo lipogenesis. In the liver, n-3 polyunsaturated fats decrease the synthesis of fatty acids and triglycerides and lower enzyme activity and message for malic enzyme, fatty acid synthase and glucose-6-phosphate dehydrogenase (Clarke et al, 1990a; Clarke and Jump, 1994).

Unlike the liver, the role of PUFA in the adipocyte is not as well understood. Previously, our laboratory reported that rats fed a diet of n-3 fatty acids had lower S14 and FAS expression in both the liver and epididymal fat tissue (Jump et al., 1993). Several possible PUFA regulatory mechanisms were proposed. A PPAR dependent mechanism was studied in the liver as the S14 PUFA regulator. However, this pathway was found not to be operative in S14 suppression (Ren et al., 1996; 1997). Understanding how PUFAs regulate lipogenic enzymes, lipogenesis and triglyceride synthesis in the adipocyte is the focus of this chapter. These findings may be important both

physiologically and pharmacologically in the understanding and treatment of obesity or diseases related to obesity.

This chapter examines the effect of polyunsaturated fatty acids (PUFA) on lipogenic gene expression in cultured 3T3-L1 adipocytes. Preliminary work indicated that arachidonic acid (20:4,n-6; AA) and eicosapentaenoic acid (20:5,n-3; EPA) suppressed mRNAs encoding fatty acid synthase (FAS) and S14, but had no effect on β-actin. A clonal cell line containing a stably transfected S14CAT fusion gene was established to evaluate the transcriptional control of S14 by PUFA. Given the high potency of 20:4,n-6 on S14 mRNA expression and CAT activity and previous reports that dietary n-3 fatty acids lowered S14 expression in both liver and epididymal fat in rats, the following hypothesis was developed: "Arachidonic acid suppression of adipocytes is similar to the PUFA suppression of S14 in the liver". Several aims were developed to address how arachidonic acid suppressed S14 expression in adipocytes.

AIMS

Aim 1: Where is the adipocyte PUFA response region in the S14 promoter?

Aim 2: Do peroxisomal proliferator activated receptors (PPARs) bind the S14 pluripotent response region (PRR)?

Aim 3: Are PPARs involved in the PUFA control of S14 in adipocytes?

Aim 4: Does arachidonic acid regulate S14 gene expression through a prostanoid pathway?

Aim 5: Are PUFAs altering lipid metabolism in adipocytes?

INTRODUCTION

Polyunsaturated fatty acids (PUFA), particularly the highly unsaturated n-3 fatty acids, when fed to rodents inhibit hepatic *de novo* lipogenesis, triglyceride synthesis and secretion and induce peroxisomal and microsomal fatty acid oxidation (Clarke and Jump, 1994; Jump et al., 1996; 1993; 1994; Neat et al., 1980, Thomassen et al., 1982, Flatmark et al., 1988; Aarsland et al., 1990; Rambjor et al., 1996; Rustan et al., 1988; Toussant et al., 1981; Clarke et al., 1990a, 1990b; Blake and Clarke, 1990). PUFA effect on these metabolic pathways is controlled by the transcription of specific genes involved in these pathways. For example, fish oil rapidly inhibit the transcription of genes encoding the fatty acid synthase (FAS) and the S14 protein, while inducing expression of genes encoding acyl CoA oxidase (AOX) and cytochrome P450 4A2 (Cyp4A2), enzymes involved in peroxisomal and microsomal fatty acid oxidation, respectively (Ren et al., 1996; 1997).

The molecular basis for PUFA-mediated control of

hepatic gene expression involves at least two distinct pathways. One pathway requires the peroxisome proliferator activated receptor (PPAR), a member of the steroid-thyroid supergene family. In liver, PPARα is the principal PPAR subtype accounting for the fatty acid control of AOX and CYP4A2 (Ren et al., 1997). PPARα is activated by peroxisome proliferators, including fatty acids. The second pathway is independent of PPARα and is involved in the PUFA-mediated suppression of S14, FAS and L-pyruvate kinase (Ren et al., 1996; 1997; Liimatta et al., 1994). Specific fatty acid regulated transcription factors have not yet been identified for the PUFA-mediated control of FAS, S14 or L-PK. While these genes are subject to complex control by insulin, T_3 and glucose (Clarke and Jump, 1994, Jump et al., 1996; Liimatta et al., 1994), the cis-regulatory targets for PUFA control of S14 and L-PK do not converge with the principal targets for endocrine or carbohydrate control. Instead, the PUFA-regulatory elements converge with targets that serve an ancillary role in hormone/nutrient control of gene transcription (Jump et al., 1993; Liimatta et al., 1994).

While the liver serves as a major target for fatty acid regulated gene expression, recent studies indicate that white adipose tissue might also be a target for fatty acid control of gene expression (Jump et al., 1993; Ailhaud et

al., 1995; 1996). Fatty acids promote differentiation of preadipocytes to adipocytes, a process that involves PPARγ2. PPARγ2 is activated by thiazolidinediones, a class of insulin sensitizing drugs, as well as fatty acids and prostanoids (Tontonoz et al., 1994a; 1994b, 1994c; Spiegelman and Flier, 1996; Kliewer et al., 1995; Kliewer et al., 1995; 1997; Forman et al., 1995; 1997). Prostanoids, products of arachidonic acid metabolism, have also been reported to promote adipocyte differentiation in culture and to affect gene expression (Ailhaud et al., 1992; Kliewer et al., 1995; 1997; Forman et al., 1995).

Since feeding rats for 5 days on diets containing fish oil leads to a 50% suppression of $mRNA_{FAS}$ and $mRNA_{S14}$ in epididymal fat (Jump et al., 1993), I was interested in determining whether the mechanism of PUFA-mediated suppression of lipogenic gene expression in adipocytes is similar to that found in liver. Accordingly, I examined the effects of specific mono- and polyunsaturated fatty acids on lipogenic gene expression in 3T3-L1 cells.

L1 cells differentiate *in vitro* from preadipocytes (fibroblasts) to adipocytes and this differentiation is accompanied by the induction of lipogenic genes as well as receptors that bind lipogenic and lipolytic hormones (Ailhaud et al., 1992). My results show that while specific PUFA inhibit S14 and FAS gene expression in cultured

adipocytes, the mechanism for control involves a prostanoid pathway. This mechanism of control differs from the one previously described in liver (Jump et al., 1993).

METHODS AND MATERIALS

CELL CULTURE 3T3-L1 cells were grown to 2 days post confluence in Dulbecco's Modified Eagles Medium (D-MEM) supplemented with 10% calf serum, penicillin (100 units/l) and streptomycin (0.1 mg/l). Differentiation was initiated with D-MEM containing 10% fetal bovine serum (FBS), 1 μM insulin, 1 μM dexamethasone (DEX) and 250 μM isobutylmethyl xanthine (IBMX) for 48 hours. After initiating differentiation, media was replaced with D-MEM supplemented with 10% FBS and insulin and changed every 2-3 days. All cells were maintained at 37°C and 5% CO_2/95% O_2 (See Appendix A for further cell culture solutions.)

Several 3T3-L1 cell lines containing stably transfected S14CAT fusion genes have been previously described (MacDougald and Jump, 1992). The monoclonal C11 cell line contains S14CAT124 (CAT: chloramphenicol acetyl transferase) reporter gene and the S14 promoter to extending from -4315 to +19 bp fused upstream from CAT. The monoclonal cell line was derived from the original polyclonal line by differential dilution. Cell lines were

selected based on ability to differentiate and express detectable CAT activity. Cell line #11 was chosen (hence C11 cells) and all experiments were done using this cell line. The cells transfected with TKCAT208, MamNeoCAT or RSVCAT are pools of G418-resistant cells. TKCAT208 contains the region extending from -1.6 to -1.4 kb upstream from the S14 transcription start site fused upstream from the thymidine kinase (TK) promoter. The -1.6/-1.4 kb region contains the glucocorticoid and adipocyte-specific elements required for S14 expression in adipocytes (MacDougald and Jump, 1992). MamNeoCAT (Clontech) contains the glucocorticoid inducible MMTV promoter while RSVCAT (from S. Conrad, Michigan State University) contains the Rous Sarcoma Virus promoter. With the exception of MamNeoCAT, these stably transfected cell lines were prepared by co-transfection with SV2Neo and colonies were selected and maintained in 0.4mg/ml G418 (Geneticin, Life Technologies) until confluent.

Other monoclonal cell lines were derived using the calcium phosphate transfection method then selected by differential dilution under G418 (geneticin) pressure. Cells were plated at 600,000 cells per 100mm plate in 10ml growth media the day before transfection. On day of transfection, 30µg of CsCl prepared vector and 3µg of CsCl prepared selection vector (SV2Neo) were mixed with 0.5ml 2X-

BSS and 0.5ml $CaCl_2$. This mixture was incubated at room temperature for 20 minutes then added drop-wise to plate of cells. The plate was swirled and replaced into incubator at 37 C at 3% CO_2. Media was removed the next day and replaced with growth media and placed in normal incubator conditions (10% CO_2). When cells reached 70-80% confluence, they were rinsed with PBS, trypsinized and split 1:5 in media containing 400µg/ml geneticin. Media was changed as necessary until colonies appeared (2 weeks or more) and cells were further cloned with 96 well plates for monoclonal cell line establishment or pooled for polyclonal cell line establishment (Chen and Okayama, 1987).

From storage, all cell lines were initially plated with 0.4mg/ml G418 and maintained until confluence in 0.2mg/ml G418. After initiating differentiation, cells were maintained in the absence of G418. Cells were treated with fatty acids at concentrations indicated in figures and always at a 5:1 ratio with fatty acid-free bovine serum albumin. Other treatment concentrations are indicated in the figure legends. Following treatments, cells were assayed for CAT activity and protein content as previously described (Jump et al., 1993). CAT Units: ^{14}C-acetylated chloramphenicol CPM/100 µg protein/hour.

RNA ANALYSIS Total RNA was isolated from cells using RNA STAT-60 (Tel-Test B, Friendswood, Texas) or Gibco's

Trizol. RNA (20 µg/lane) was electrophoretically separated in 1% agarose-formaldehyde gels (Jump et al., 1994). Northern blots were prepared and probed with radiolabeled cDNA (Jump et al., 1994) for S14 (S14ExoPEII6), FAS (Fas-1; from H.S. Sul, Univ. Calif.-Berkeley) and β-actin (L. Kedes, Stanford, Palo Alto, CA).

RNA was electrophoresed in a 1% agarose, 1X MOPS, 1.7% formaldehyde gel (10-20µg/lane). After running the gel, gels were rinsed in water for 15 minutes and transferred to nitrocellulose membrane using the turbo-blotter system with 10X SSC as transfer buffer (Schleicher and Schuell, Keene, NH). After transfer blots were marked with pencil for lanes, allowed to dry then baked for 2 hours in a vacuum oven (nylon blots were UV linked). Blots were prehybridized for at least 2 hours in prehybridization buffer at 42°C then hybridized overnight with desired cDNA probe. Probes were synthesized using Gibco's Random Primers DNA Labeling System and cleaned with spin columns from 3' Prime 5' Prime. Blots were washed in .1X SSC, .1% SDS at 55-60°C. Blots were then exposed to film for 24-48 hours at -80°C before development or exposed to the phosphoimager screen for 1-24 hours. This protocol is a combination of Fourney et al., 1988, and Jump et al., 1984.

GEL SHIFT ANALYSIS Gel shift analysis involves four steps: labeling of the oligonucleotide, transcription/translation of the receptor, binding of receptor(s) to oligonucleotide and gel preparation.

Oligonucleotide Labeling: Oligonucleotides were annealed by heating an equal amount of both oligonucleotides in 1X TEN to 85°C for five minutes and cooling to room temperature slowly. Oligos were end labeled by incubating the following mix for 30 minutes at 30°C: 2µl polynucleotide kinase buffer, 1µl 100mM DTT, annealed oligos (100-200ng), 1µl polynucleotide kinase, 5µl ^{32}-P γATP and water to 20µl. The reaction was stopped with 5µl 200mM EDTA. One microliter was counted for specific activity determination. Oligos were cleaned by a spin column or with TCA precipitation and recounted for cpm/µl.

Transcription/translation of receptors was done using Promega's Transcription/translation kit according to the manufacturer's protocol. One or two micrograms of plasmid was required for this reaction. Receptors were stored at -80°C and kept on ice at all times after synthesis.

The binding reaction was set up after receptors were made and oligos labeled. Each reaction contained 4.5µl dialysis buffer, 1µl DTT, 0.5µl 100mM $MgCl_2$ and 1µl dI:dC, 2µl of receptor or receptor mix and 5000cpm labeled oligo

(usually 1 µl). As a control, one tube contained no receptor (only oligo) and another contained 2 µl unprogrammed cell lysate instead of receptor. The reaction incubated at room temperature for 20 minutes. Before loading, 5 µl of stop buffer was added to each tube.

Gel: 8% polyacrylamide gels were used for gel shift analysis. These were prepared or the Biorad precast gels were used. The precast gels were less likely to separate bands as clearly as the poured gels. In a .25X TBE buffer, the gels were run at 350 volts for about 1 hour at 4°C. The precast gels were run at half this speed for the same amount of time. After electrophoresis, gels were dried for 1-2 hours (the precast gels take at least 2 hours) and exposed to film overnight.

TRIGLYCERIDE AND FATTY ACID ANALYSIS Total triglycerides were analyzed using a Sigma Triglyceride Assay Kit and Oil Red O staining. For triglyceride extraction, cells were rinsed with PBS and then scraped into 1ml methanol in a corex tube and 2ml of chloroform was added to each tube. The sample was homogenized and 1ml of .15M acetic acid was added. The tube was vortexed and centrifuged for 10 minutes at 1000rpm. The lower layer was removed and aspirated under nitrogen. The remaining triglyceride was resuspended in 95% ethanol and analyzed

using the Sigma's triglyceride kit according to their protocol.

Oil Red O stains triglycerides. After staining as described previously (Mater, 1994), 1ml of 100% ethanol was added to the plate to remove the stain. The absorbance of each sample was determined at 540nm on a Beckman Spectrophotometer.

For fatty acid analysis, cells were rinsed with phosphate buffered saline then scraped into microtubes in methanol. Cell extracts were frozen until assayed. Fatty acid analysis of adipocyte fatty acids involved conversion of fatty acids to methyl esters of total cell lipids by direct trans-esterification using boron trichloride/methanol (14% w/v, Sigma). After methylation, extracts were extracted twice with hexane then dried under nitrogen. The fatty acids were then resuspended in 100µl hexane. The composition of the fatty acid methyl ester was determined by a capillary gas liquid chromatography using a Hewlett-Packard 5890 gas chromatograph fitted with a 50 m x 0.025 mm (id) CP-Sil 88 capillary column (Chrompack, Middleburry, The Netherlands) and a flame ionization detector. A temperature gradient program from 150°C to 250°C at 1°C/min was used. Injection port and detector temperatures were 240°C. The fatty acid methyl esters were identified by comparing their retention times versus those of authentic standards.

ISOLATION OF ADIPOCYTE NUCLEI Nuclei were isolated from both preadipocytes and adipocytes with this protocol. Media was removed from 100mm plates of cells and rinsed with PBS. Three milliliters of Solution A was added to the plates and swirled. Cells were allowed to sit and swell for 5 minutes at room temperature and then the solution was removed. Three milliliters of Buffer A were added to the cells, cells were scraped and placed into a sterile homogenizing flask. After a brief homogenization, the cells were layered over 5ml of Buffer B and centrifuged at 3000 x g for 10 minutes. The solution was decanted, cells washed in 3ml of Buffer C and again centrifuged at 1000 x g for five minutes. The solution was decanted and nuclei were resuspended in 200µl of Buffer D. After removing 5µl for absorption reading at 260nm, the remaining was stored at -80°C.

Nuclear proteins were obtained from isolated nuclei with this procedure. All procedures are carried out on ice. Nuclei were resuspended in Buffer D (from Nuclei isolation procedure) at 100 A_{260} units per milliliter. The nuclei were placed in a polyallomer tube (65V-Dupont vertical rotor, 13.5ml tubes) and nuclear lysis buffer was added to 7ml. 1.0ml 4M $(NH_4)_2SO_4$ was added and adjusted to 12ml with nuclear lysis buffer containing 0.5M $(NH_4)_2SO_4$. After capping and mixing, the tube sat on ice for one hour. The

chromatin was then sedimented for 80 minutes at 40,000rpm. The volume of the supernatant was determined and placed into a new tube. Solid $(NH_4)_2SO_4$ was added at .3mg/ml to tube and mixed on ice overnight. The extract was again spun for 30 minutes at 45,000 rpm. The supernatant was removed and the pellet carefully resuspended in dialysis buffer and allowed to shake overnight at 4°C. The extract was then dialyzed against 100 volumes of dialysis buffer overnight with one buffer change after 4 hours. Nuclear proteins were recovered into sterile tube, labeled and stored at -80°C after 2µl was removed for protein concentration.

RESULTS

PRELIMINARY RESULTS

PUFA Suppress S14 and FAS Gene Expression in L1 Adipocytes. Treatment of primary hepatocytes with 20:4, n-6 or 20:5, n-3 lead to a suppression of S14 and FAS mRNA with an ED_{50} < 100 µM (Jump et al., 1993). To determine if PUFA inhibited the expression of these mRNAs in cultured 3T3-L1 adipocytes, cells were treated with vehicle, 18:1, n-9; 20:4, n-6; or 20:5, n-3 for 48 hrs (Figure 2.1). 18:1,n-9 had no significant effect on mRNAs encoding S14, FAS or β-actin. In contrast, both 20:4, n-6 and 20:5, n-3 suppressed

S14 mRNA by 85 and 70%, respectively. FAS mRNA was suppressed by 70% following 20:4 treatment and ~40% following 20:5 treatment. Actin mRNA was unaffected by these treatments. These results indicated that the previously reported effects of dietary PUFA on adipocyte FAS and S14 gene expression may be due to direct effects of PUFA on fat cells. This effect is fat type specific and can not be attributed to a generalized fatty acid effect since 18:1 did not affect any mRNA examined. Moreover, the lack of a PUFA effect on actin mRNA suggested that the inhibition of S14 and FAS was gene specific.

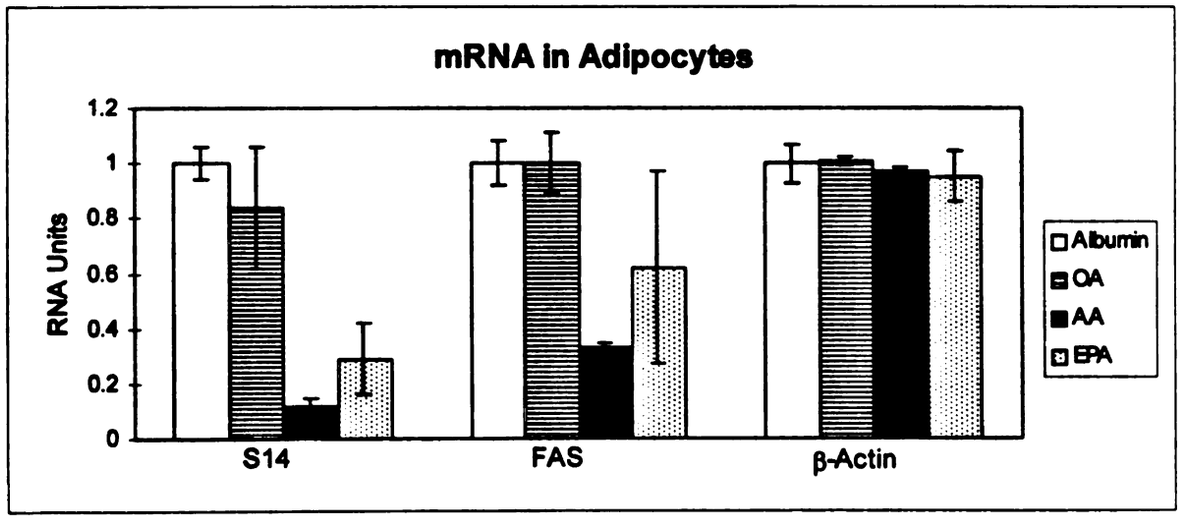


Figure 2.1. The effect of fatty acids on adipocyte mRNA levels. RNA was isolated from fully differentiated adipocytes after 48 hours of treatment with 50μM albumin and 250μM fatty acid. Samples were in triplicate and standard deviations of the mean are shown. This is a representative graph of at least 3 experiments.

PUFA regulates S14 at the transcriptional level. In an effort to establish the mechanism for control, a stable cell line containing the S14CAT fusion gene was used. This S14CAT fusion gene contains the cis-regulatory elements required for the adipocyte-specific glucocorticoid-mediated activation of transcription of this gene. Accordingly, basal and DEX-mediated induction of CAT activity was examined in preadipocytes and adipocytes receiving vehicle, 18:1, 20:4 and 20:5 (Figure 2.2).

In preadipocytes, CAT activity was expressed at low levels and was not induced by DEX or affected by any fatty acid treatment (Fig. 2.2A). In contrast, DEX induced S14CAT ~18-fold in adipocytes (Fig. 2.2B). While 18:1 treatment had no effect, 20:4,n-6 and 20:5,n-3 both inhibited CAT activity by >70%. The effect of the fatty acid treatment on CAT activity by both basal (no DEX treatment) and induced (DEX treatment) was comparable (Fig. 2.2b) indicating that 20:4,n-6 and 20:5,n-3 acted on the basal expression and not on DEX-mediated transactivation.

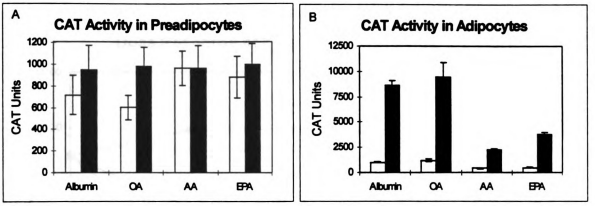


Figure 2.2. Effect of fatty acids on S14CAT Activity in Preadipocytes and Adipocytes. Cells were treated with 250μM fatty acid and 50μM albumin for 48 hours and CAT activity was measured. Solid bars are also treated with 1μM dexamethasone. Means of triplicate samples with standard deviations are shown. These graphs are one representative of several experiments are shown. A: Preadipocytes B: Adipocytes

Dose Response Analysis. In primary hepatocytes, both 20:4,n-6 and 20:5, n-3 are equipotent inhibitors of S14CAT activity (Jump et al., 1993). To determine if adipocytes responded to PUFA like hepatocytes, L1 adipocytes containing the stably integrated S14CAT gene were treated with fatty acids ranging from 50 to 1000μM (Figure 2.3). While treatment of cells with 18:1,n-9 up to 500μM had no significant effect on CAT activity; 1mM 18:1,n-9 reduced CAT activity by ~60%. Both 20:4, n-6 and 20:5, n-3 inhibited S14CAT activity. However, ED_{50} for 20:4,n-6 was 6-fold lower than 20:5,n-3. In contrast to liver (Jump et al., 1993), 20:4, n-6 is a more potent inhibitor of S14 gene expression than 20:5,n-3 in adipocytes.

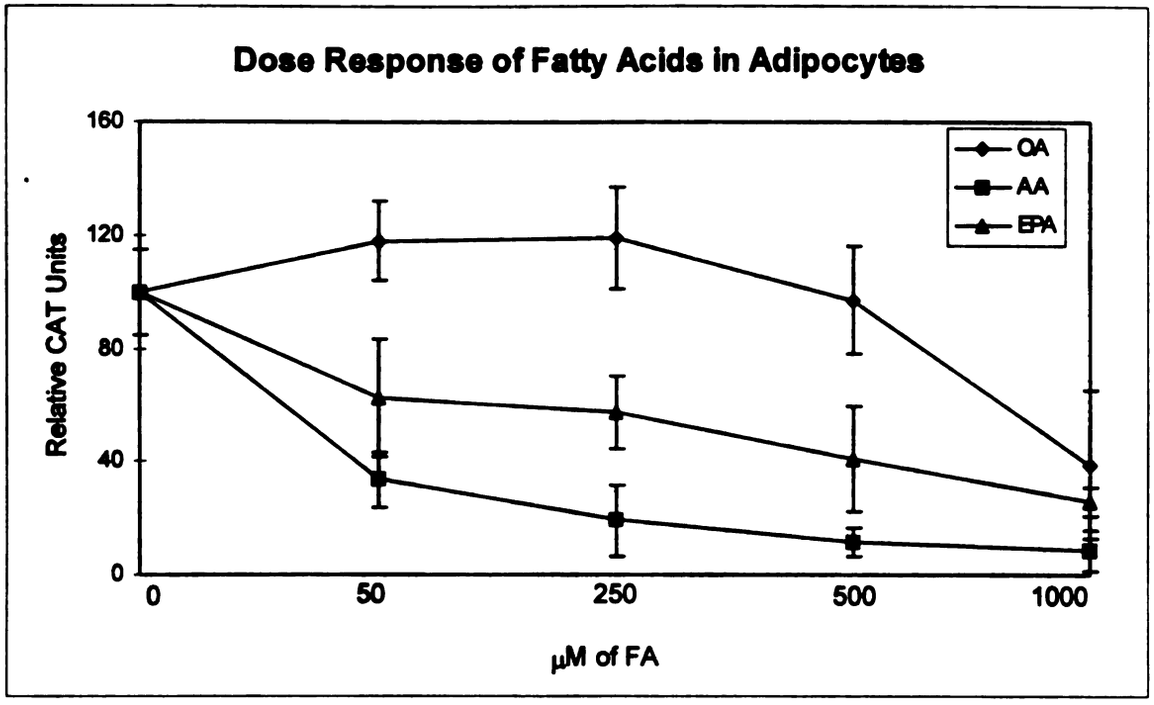


Figure 2.3. Dose Response of Fatty Acids in Adipocytes on S14CAT activity. Fully differentiated adipocytes were treated with varying doses of fatty acids and 1µM dexamethasone for 48 hours and CAT activity was measured. Values were normalized to the albumin control. Standard deviations are shown.

Continuous fatty acid treatment analysis. Saturated and polyunsaturated fatty acids have been reported to stimulate adipocyte differentiation in OB1771 cells (Ailhaud et al., 1995; 1996; 1992). To determine whether PUFA affected adipocyte differentiation, specific fatty acids were added to the medium after removal of the differentiation medium and maintained in the medium for 8 days at 50µM (Figure 2.4). While 18:1 had no effect on DEX-induced S14CAT expression, 18:2,n-6; 18:3,n-6 and 20:5,n-3 suppressed CAT activity by <30%. In contrast, cells treated

with 20:4,n-6 showed a >90% suppression of both basal (Figure 2.4) and DEX-induced S14CAT activity (not shown).

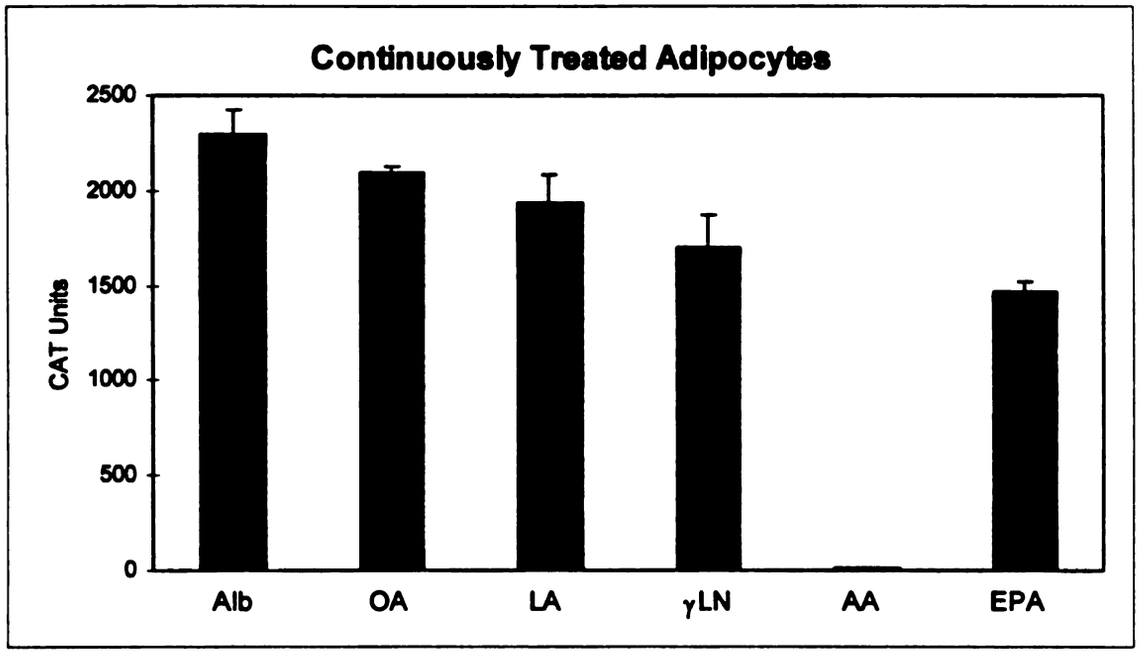
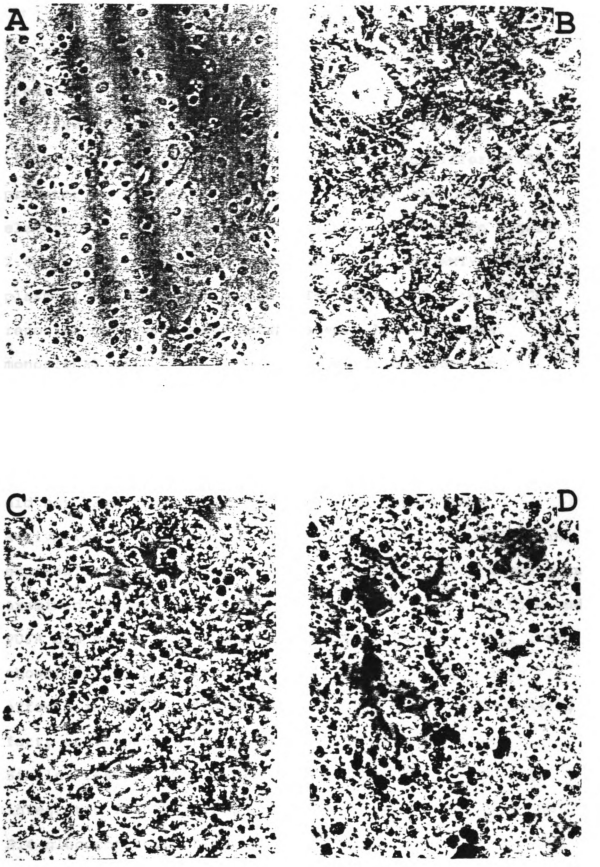


Figure 2.4. Continuous Fatty Acid Treatment on S14CAT Activity in Adipocytes. C11 cells were treated with 50μM fatty acid and 10μM albumin for 8 days and CAT activity was determined. Alb = albumin, OA = oleic acid (18:1n-9), LA = linoleic acid (18:2n-6), αLN = α-linolenic acid (18:3n-6), AA = arachidonic acid (20:4n-6) and EPA = eicosapentaenoic acid (20:5n-3). This is a representative of at least 2 separate experiments and each bar represents an average of three samples with standard deviation shown.

Fatty Acid Treatment has little effect on lipid accumulation in adipocytes. Cells treated with various fatty acids were stained with the lipid specific stain, Oil Red O. As shown in Figure 2.5, cellular lipid accumulated fairly equally among all treatments. This figure shows the adipocytes before, during and after differentiation. Even

with the various fatty acid treatments, the cells looked similar microscopically and after Oil Red O staining.

Figure 2.5. Oil Red O Stained Preadipocytes and Adipocytes. Adipocytes were fixed and stained with Oil Red O. A: Confluent preadipocytes before differentiation mix was added, B: Adipocytes not differentiated but kept in culture for 12 days with the usual media present in all treatments, C: Adipocytes differentiated and treated with 50μM AA for 5 days post-differentiation media, D: Adipocytes differentiated and treated with 50μM EPA for 10 days post-differentiation media. There were no differences visually detectable before or after staining in all the treatments, regardless of fatty acid present.


A
B
C
D

AIM 1 RESULTS

Aim 1: Where is the PUFA response region in the S14 promoter?

Several stable cell lines were established from transfection of reporter vectors containing different S14 promoter constructs to determine the PUFA response element(s). The different constructs are shown in Figure 2.6. As controls, cell lines containing the MMTV (MAMNeo plasmid) and thymidine kinase (TK) promoters with the CAT reporter gene were also established. In some cases, monoclonal cell lines were used (selection based on adipocyte phenotype and CAT activity levels).

PUFA Effects are specific to the S14 gene. To show the specificity of PUFA action on S14 expression and that PUFA did not have generalized effects on glucocorticoid activation of gene transcription, the effect of PUFA on the expression of the glucocorticoid inducible MMTV promoter was examined (Figure 2.7). DEX induced CAT activity ~8-fold and PUFA did not affect either the basal or induced level (not shown) of CAT activity. There were also no fatty acid effects in RSVCAT adipocytes on CAT activity, again showing fatty acid specificity to different promoters (Fig 2.7). Similar results were obtained in cells containing stably integrated TKCATPAN (Fig 2.7). This plasmid contains the S14 glucocorticoid response region fused upstream from the

thymidine kinase basal promoter (TKCATPAN, Methods and Materials). These results argue against the S14 glucocorticoid response region (between -1069 and -1588 bp) as the cis-regulatory target for PUFA-mediated suppression of S14 gene expression in adipocytes. Based on previous reports of the functional elements controlling S14 gene expression in liver and preadipocytes (Jump et al., 1993; MacDougald and Jump, 1992), these findings implicated the S14 proximal promoter (*i.e.* -290 to -1 bp) as a likely target for PUFA action. However, additional studies will be required to localize the cis-regulatory target for PUFA action.

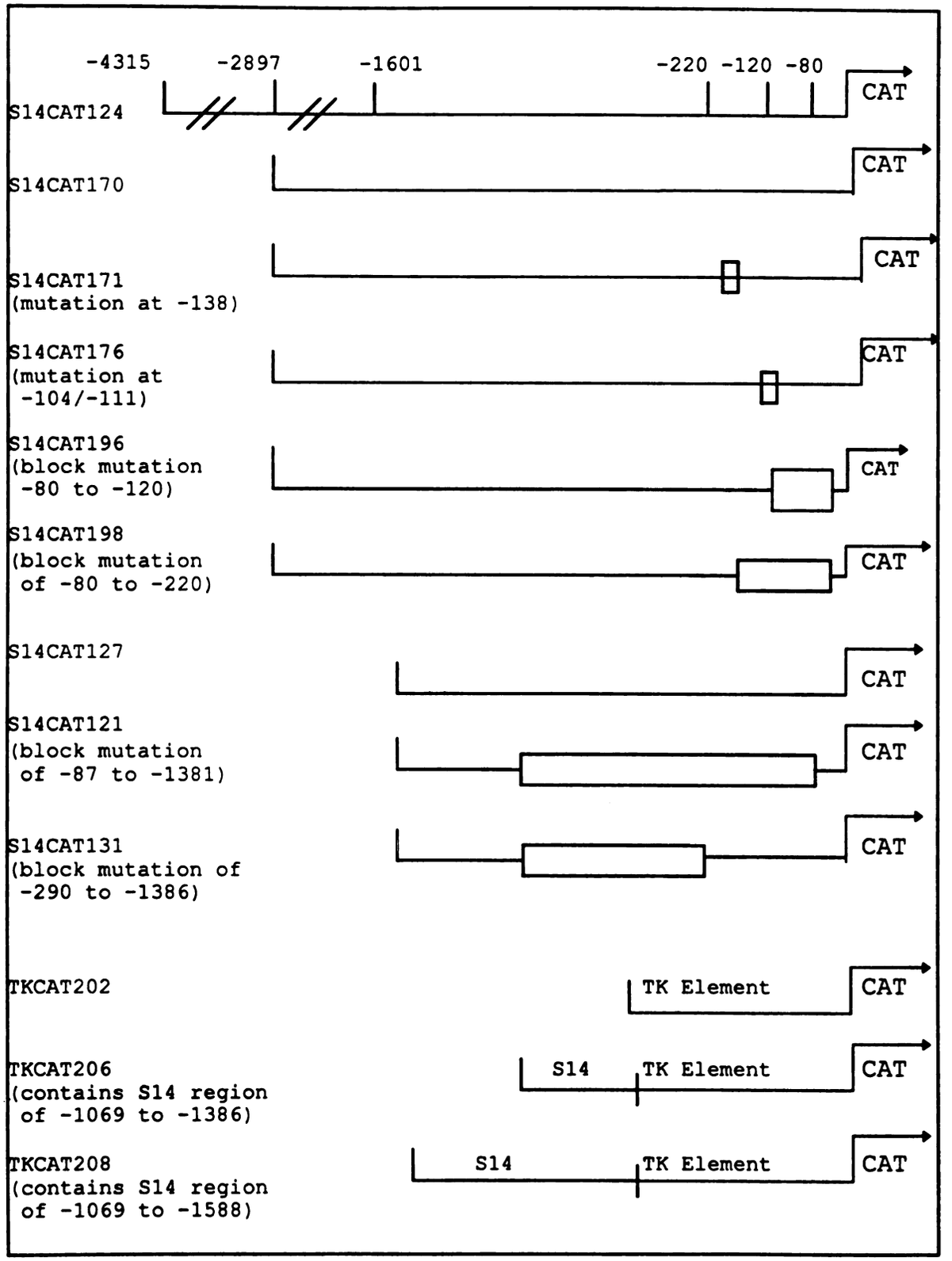


Figure 2.6. S14 Plasmid Construct Maps. Each plasmid used in the adipocyte transfections are shown above. The S14 promoter region included in each plasmid is described and shown. All contain CAT as the reporter gene.

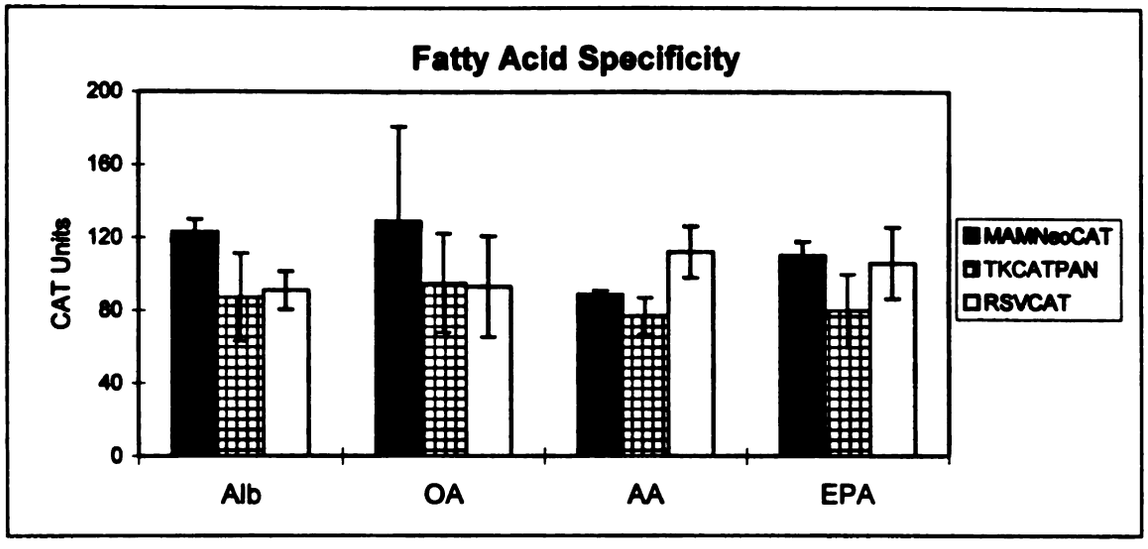


Figure 2.7. Fatty Acid Specificity. The fully differentiated cell lines shown above were treated with the usual fatty acid (250μM) and albumin (50μM) for 48 hours and CAT activity was determined. The RSVCAT data was divided by 100 for making this graph. Each of these shown is a representative of 3 separate experiments. Each bar represents a triplicate sample with standard deviation error bars.

Localization of PUFA-RR was unsuccessful in adipocytes. Several different S14 promoter constructs were tested in order to determine which region was sensitive to arachidonic acid suppression. Some of the cell lines had previously been established in the our laboratory and monoclonal cell lines were created from these cell lines. Other cell lines were established as described in Materials and Methods. As shown in Figure 2.7, the pluripotent response region (-1069 to -1588) of the S14 promoter was not sensitive to fatty acids in TKCATPAN adipocytes. Therefore, other cell lines

containing constructs with more proximal regions of the S14 promoter were established and tested for this project.

Table 2.1 summarizes the PUFA localization results in adipocytes. Each cell line was treated with AA and compared to OA treatment. The data confirmed that the PUFA response region was located between -220 and -80 bp of the S14 promoter in cell lines containing S14CAT131 and 5 of 6 clones of S146CAT198. S14CAT127 was not sensitive to AA and should have been with 1600 bp of the S14 promoter present. This cell line was developed many years ago, and efforts to develop a monoclonal cell line were unsuccessful as was creating a new cell line with this plasmid. One clone of the S14CAT198 cell lines was also sensitive to AA. This was unexpected since it is missing the -220 to -80bp region. It is unclear why this one clonal cell line would be sensitive to AA while 5 clones were not, although the site of integration by the plasmid into the genome of the cell could affect sensitivity.

The RSV constructs were all established as polyclonal cell lines. CAT activity was measured but results were variable and not identical to the S14 plasmids. For example, CAT activity in cells containing RSVCAT139 should have been sensitive to only AA, not both AA and OA.

Even with these exceptions, overall, the results confirmed that AA is suppressing S14 through the promoter

region between -220 and -80bp as expected from previous reports of the PUFA response region (Jump et al., 1993). Efforts to localize the PUFA RR more precisely in adipocytes were not successful. Chapter 3 will re-examine this issue in transfected primary hepatocytes.

Table 2.1. FA Sensitivity of S14 Constructs in Adipocytes

CONSTRUCT	PROMOTER REGION	SUPPRESSED BY AA
S14CAT124 (C11)	-4315 to +19	Yes
S14CAT171	-2897 to +19 w/mutation at -138	Yes (3 clones)
S14CAT196	-2897 to +19 with a block mutation of -120/-80	No (3 clones)
S14CAT198	-2897 to +19 with a block mutation of -220/-80	Yes (1 clone) No (5 clones)
S14CAT127	-1601 to +19	No
S14CAT121	-1588 to +1381 and -87 to -8	ND*
S14CAT131	-1588 to -1386 and -290 to -8	Yes
TKCAT208 (TKCATPAN)	-1588 to -1069 with TK promoter	No
TKCAT206	-1386 to -1069 with TK promoter	No**
RSVCAT136	TK element + RSV	No
RSVCAT137	-1400 to -1200 kb and -220 to -120 + RSV	No
RSVCAT138	1400 to -1200 and -120 to -80 + RSV	No
RSVCAT139	1400 to -1200 and -220 to -80 + RSV	Yes (both OA & AA)
RSVCAT140	1400 to -1200 and RSV	No
RSVCAT141	1400 to -1200 + TK element + RSV	No
RSVCAT148	-1309 to -1069 + RSV	No

Table 2.1. Fatty Acid Sensitivity of S14 Constructs. This tables lists the plasmids stably transfected into adipocytes and their respective promoter regions. All cells were treated with 250μM AA and 50μM albumin or albumin alone for 48 hours and CAT activity was determined. Each cell line was tested at least 3 times and a summary of the results are given. Some cell lines had very little or no CAT activity and this is indicated on the table.

*CAT counts at or below background
**CAT counts barely above background

AIM 2 RESULTS

Aim 2: Do peroxisomal proliferator activated receptors (PPARs) bind the S14 pluripotent response region (PRR)?

PPARγ2 binds the TSE-1 Region but TSE-2. Using gel shift analysis, the tissue specific elements (TSE-1 and 2) were tested for PPAR binding. TSE-1 and TSE-2 are regions in the S14 promoter which bind transcription factors. Binding to TSE-1 is more active after differentiation, while binding to TSE-2 is active during the preadipocyte stage (MacDougald and Jump, 1992). As shown in Figure 2.8, only PPARγ2 and not PPARα and FAAR bound to TSE-1. HNF-4 also did not bind this region (not shown). The AOX-PPRE was used as a positive control for PPAR binding.

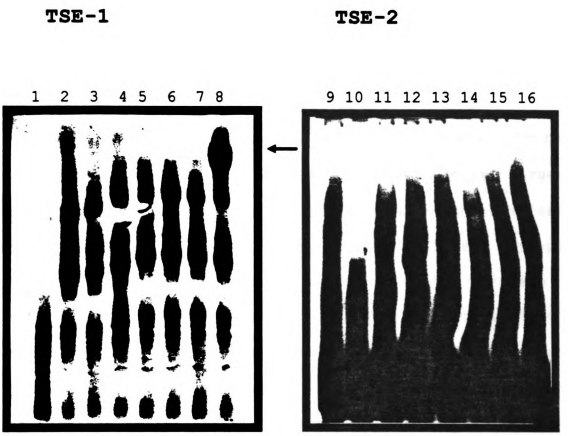


Figure 2.8. Gel Shift Analysis of TSE-1 and TSE-2. TSE-1 and TSE-2 were shifted with translated receptors. Lanes 1 and 10 contain the labeled TSE-1 or TSE-2 oligonucleotides only. Lanes 2 and 9 include TSE-1 or TSE-2 with the control translation cocktail. Lanes 3 and 11 contain 2μg RAR, Lanes 4 and 12 contain 2μg RXR, Lanes 5 and 13 contain 2μg PPARγ2, Lanes 6 and 14 contain 1μg each of RXR and RAR, Lanes 7 and 15 contain 1μg each of RAR and PPARγ2 and Lanes 8 and 16 contain 1μg each of RXR and PPARγ2. The arrow indicates the site of RXR/PPARγ2 heterodimer shifting TSE-1 but not TSE-2. This experiment was repeated at least 3 times and a representative example is shown above. Other receptors tested included FAAR, PPARα and HNF4, none of which bound to TSE-1 or TSE-2.

AIM 3 RESULTS

Aim 3: Are PPARs involved in the PUFA control of S14 in adipocytes?

PPARγ2 and PUFA do not interact in adipocytes. The region of PPARγ2 binding (Aim 2) did not correlate with the proposed PUFA target (Aim 1). Because the promoter targets for these two factors did not bind the same regions (PPARγ2 bound a region between -1550 and -1530bp while the PUFA-RE is between -220 and -80bp), it is unlikely that PUFA are acting through a PPAR pathway. To further substantiate this finding, adipocytes were treated with the PPARγ2 activator, pioglitazone. Pioglitazone, a thiazolidinedione, is known to increase differentiation. Treatment of adipocytes during the 48 hour differentiation period with pioglitazone did indeed increase lipid accumulation as seen microscopically. CAT activity ten days after differentiation was enhanced in cells treated during differentiation with pioglitazone (not shown). This was probably a result of better differentiation. In general the fatter the cells and the more fat cells present, the higher the CAT activity (observation). In contrast, activation of PPARγ2 by pioglitazone in the last 48 hours of culture in fully differentiated cells had no effect on S14 CAT activity (Fig. 2.9).

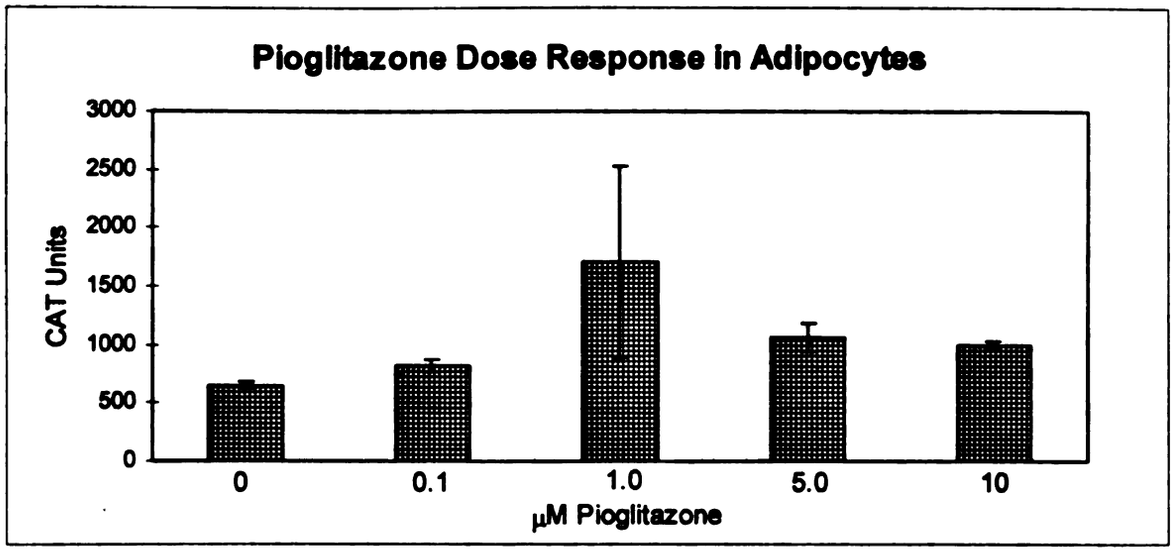


Figure 2.9. Pioglitazone Treatment of Adipocytes. Fully differentiated adipocytes were treated for 48 hours with the indicated dose of pioglitazone and CAT activity was determined. The bars are means of three samples with standard deviations shown. Several other experiments using 1-10μM pioglitazone duplicated these results.

In conclusion of Aims 2-3, PUFA and PPARγ2 target different sites in the S14 promoter. Pioglitazone had no effect on CAT activity (Figure 2.9) while PUFA had a suppressive effect (preliminary results). PPARγ2 plays a role in adipocyte differentiation and may also be involved in S14 expression early in adipocyte differentiation. However, in these studies, at the time when S14 shows PUFA sensitivity, it does not show sensitivity to pioglitazone as measured by CAT activity. These results do not indicate any interaction between PUFA and PPARγ2 in regulation S14 gene transcription in fully differentiated adipocytes.

AIM 4 RESULTS

Aim 4: Does arachidonic acid regulate S14 gene expression through a prostanoid pathway?

Arachidonic Acid Inhibits S14CAT Expression Through a Prostanoid Pathway. The goal has been to determine the mechanism of PUFA regulation of adipocyte lipogenic gene expression. The dose response studies have already shown that in contrast to liver, 20:4,n-6 is a 6-fold more potent inhibitor of lipogenic gene expression than 20:5, n-3. When compared to 20:4,n-6, 20:5,n-3 is a poor substrate for the synthesis of prostaglandins by cyclooxygenase 1 and 2 (Laneuville et al., 1995). Thus, the differential potency of 20:4,n-6 and 20:5,n-3 suggest that 20:4,n-6 may be converted to prostanoids which, in turn, induce changes in adipocyte lipogenic gene expression. This is in keeping with others who have established that adipocytes convert 20:4,n-6 to the prostaglandins, PGE_2, PGF_2 and PGI_2 (Hyman et al., 1982; Shillabeer et al., 1996; Smas and Sul, 1995; Shillabeer and Lau, 1994).

To determine if 20:4, n-6 requires metabolism to prostanoids, we used the cyclooxygenase inhibitor flurbiprofen. Cells were also treated with the nordihydroguaiaretic acid (NDGA) and clotrimazole, inhibitors of lipoxygenase and monooxygenase activity, respectively.

While flurbiprofen blocked the 20:4,n-6 inhibitory effect on CAT activity, NDGA and clotrimazole had no effect (Figure 2.10). Thus, 20:4,n-6-mediated inhibition of S14 gene expression requires cyclooxygenase and implicated a role for prostanoids in regulating adipocyte lipogenic gene expression.

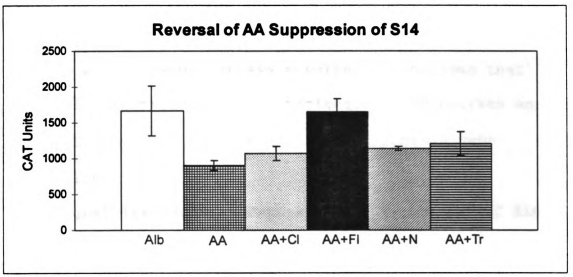


Figure 2.10. Flurbiprofen blocks the Arachidonic Acid Suppression of S14CAT Activity. Fully differentiated adipocytes were treated with the indicated compounds and 250μM arachidonic acid (except for albumin (50μM) control) for 48 hours and CAT activity was determined. This experiment is a representative of at least three experiments and each bar represents a mean of 3 samples with standard deviation error bars. Cl= clotrimazole (10μM), Fl= flurbiprofen (100μM), N=NDGA (50μM) and Tr= Triacsin C (10μM).

PGE_2 suppresses both S14CAT activity and S14 mRNA. To further examine this prostanoid dependent pathway of S14 gene suppression, the effect of specific prostanoids on

adipocyte lipogenic gene expression was evaluated by treating L1 adipocytes with PGE_2 or $PGF_2\alpha$ at 10μM (Figure 2.11). Both PGE_2 and $PGF_2\alpha$ (not shown) inhibited CAT activity. Dose response analyses show that PGE_2 inhibits S14CAT expression with an ED_{50} of ~5μM, a concentration well below the ED_{50} of ~50μM for 20:4,n-6. Analysis of S14 mRNA following PGE_2 treatment showed a similar decline (Fig 2.11). FAS mRNA showed a like results with PGE_2 treatment (not shown). Based on these results, I concluded that 20:4,n-6 is converted to prostaglandin in adipocytes and that PGE_2 and $PGF_{2\alpha}$ inhibit adipocyte lipogenic gene expression.

Signal Transduction Pathway for PGE_2 Control of S14 Gene Transcription. PGE_2 and PGF2α regulate cell function through G-protein linked plasma membrane receptors (Smith, 1989; Asboth et al., 1996; Hamon et al., 1993; Nagai et al., 1996; Uehara et al., 1994; Danesch et al., 1994). Depending on the G-protein linkage, PGE_2 can increase or decrease cellular cAMP levels or elevate IP_3 and Ca^{+2} levels.

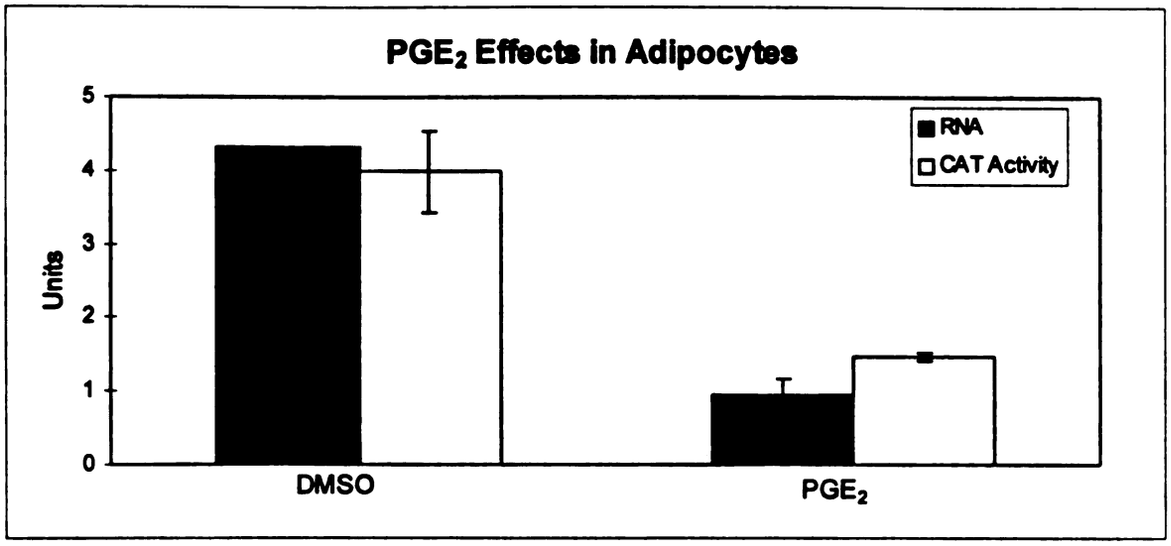


Figure 2.11. PGE_2 Inhibition of S14CAT Activity and RNA in adipocytes. Fully differentiated adipocytes were treated with DMSO or 10μM prostaglandin for 48 hours and CAT activity was determined or S14 RNA was measured. CAT Units were divided by 1000 for presentation. The CAT data is representative of several experiments. RNA results are representative of 2 separate experiments. The bars are means of triplicate samples and include standard deviations. $PGF_2\alpha$ effects on CAT activity are identical but are not shown.

To determine if changes in intracellular Ca^{+2} or cAMP was involved in prostaglandin-mediated suppression of S14 gene expression, L1 adipocytes containing the stably integrated S14CAT fusion gene were first treated with A23187 (a calcium ionophore) or 8-CTP-cAMP and isobutylmethyl xanthine (IBMX) to elevate intracellular calcium or cAMP respectively (Figure 2.12). Treatment of cells with 8-CTP-cAMP plus IBMX or the A23187 inhibited CAT activity by $\geq$50%. These studies demonstrated that alterations in intracellular cAMP or Ca^{+2} markedly suppress S14 gene transcription in fully differentiated adipocytes (Figure 2.12).

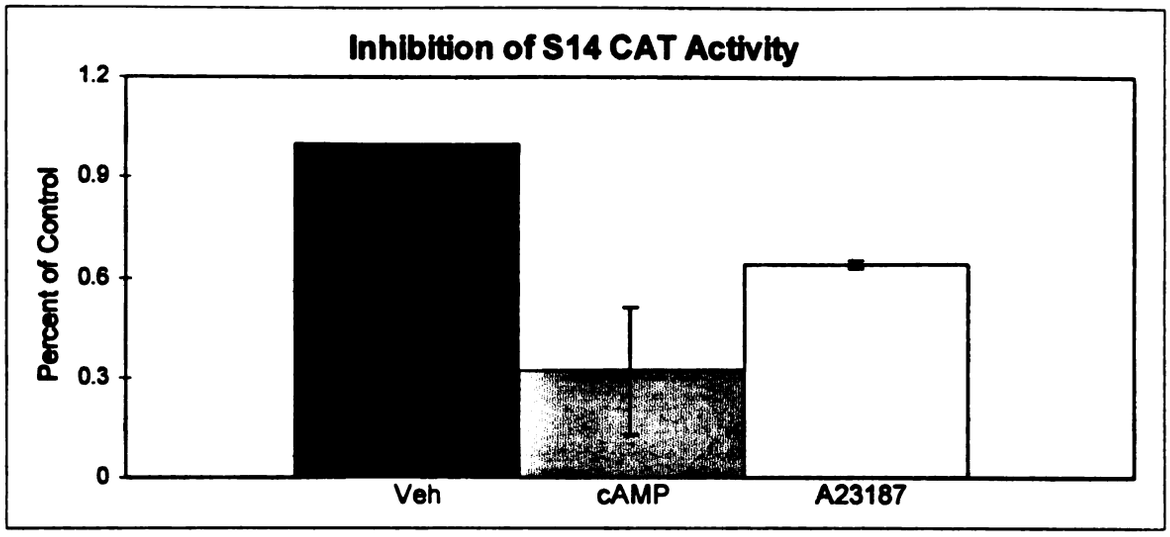


Figure 2.12. Inhibition of S14 CAT Activity by cAMP and A23187. Fully differentiated adipocytes were treated with 50μM 8-CPT-cAMP + 50μM IBMX or 1μM A23187 for 48 hours and CAT activity was determined. This graph is 5 experiments combined and normalized to vehicle. Percent errors are shown for each mean.

Depending on the G-protein linkage, PGE_2 can activate protein kinase A, protein kinase C or calcium-regulated mechanisms. To determine which pathway affects S14 gene expression, PGE_2-treated adipocytes were treated with H7 and staurosporin, inhibitors of A and C-kinases, or pertussis toxin an inhibitor of G_i /G_o-linked processes (Figure 2.13). G_i/G_o-linked processes promote a decrease in intracellular cAMP or an activation of phospholipase C_β and elevation in intracellular Ca^{+2} through release of inositol 1,4,5-phosphate [IP_3] (Uehara et al., 1994; Danesch et al., 1994). A rise in cAMP is associated with inhibition of S14CAT expression, therefore, pertussis toxin will provide a means to evaluate Ca^{+2} -regulated processes. While PGE_2 treatment

of cells inhibits CAT by ~60%, co-treatment with H7 or staurosporin at doses sufficient to inhibit both A and C-kinases did not block the PGE_2 effect (Fig. 2.13). Treatment of cells with the α_1 calcium channel blocker, verapamil, also failed to block the PGE_2 effect (Fig. 2.13). Only pertussis toxin blocked the PGE_2 effect on CAT activity (Figure 2.13). Pertussis toxin also partially reversed the PGE_2 inhibition of S14 mRNA (not shown). Treatment of cells with pertussis toxin, H7 or verapamil in the absence of PGE_2 had no effect on S14CAT expression (not shown). Taken together, these studies suggest that PGE_2 operates through a G_i/G_o-linked signaling system which may involve changes in intracellular calcium.

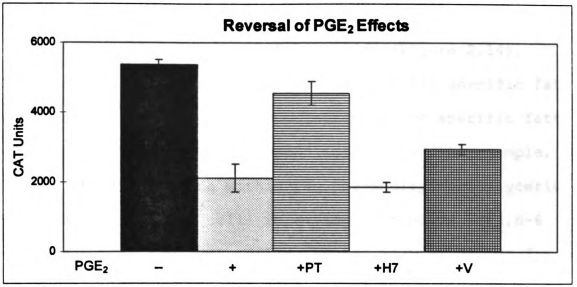


Figure 2.13. Effects of PGE_2 and PT, H7 or Verapamil on S14CAT Activity. Fully differentiated adipocytes were treated without (--) or with (+) 10μM PGE_2 alone or with 100ng/ml pertussis toxin (PT), 10μM H7 or 10μM Verapamil (V) for 48 hours before CAT activity was determined. This experiment is a representative of at least 3 separate experiments and each bar is a mean of 3 samples with standard deviation error bars indicated.

AIM 5 RESULTS

Aim 5: : Are PUFAs altering lipid metabolism in adipocytes?

Effect of PUFA on Adipocyte Differentiation and Lipid Accumulation. Saturated and polyunsaturated fatty acids have been reported to stimulate adipocyte differentiation in OB1771 cells (Ailhaud et al., 1992; 1995; 1996). To determine if PUFA affected adipocyte differentiation, specific fatty acids were added to the medium after removal of the differentiation medium and maintained in the medium for 10 days at 50μM. Continuous treatment of cells with 50μM fatty acid had no obvious effect on differentiation as

assessed by triglyceride accumulation (Table 2.2), oil red staining (Fig 2.5) or total fatty acids (Figure 2.14). Fatty acid analysis of cells treated with the specific fatty acids showed an expected accumulation of the specific fatty acid in the triglyceride/phospholipid pool. For example, 20% of the fatty acids within the phospholipid/triglyceride pool was 20:4,n-6 in cells receiving continuous 20:4,n-6 treatment. In contrast, cells treated with either no fat or 18:1,n-9 show less than 3% 20:4,n-6 (Table 2.2).

A corresponding decline in S14 and FAS mRNAs was also found following continuous fatty acid treatment like that seen in Figure 2.1 (not shown). The decline in FAS and S14 gene expression would suggest that *de novo* lipogenesis was inhibited in these cells. However, the triglyceride levels and the level of 16:0 were unaffected by these treatments. Presumably, these fatty acids accumulate from the fetal calf serum supplemented in the culture medium. Thus, in the presence of a continuous supply of exogenous PUFA, inhibition of de novo lipogenesis may have little impact on triglyceride accumulation in adipocytes.

Another observation is that in cells treated with 18:2n-6 or 18:3n-6 (Figure 2.14) show no change in 20:4n-6 levels. This indicates a lack of subsequent desaturation and elongation of the exogenously supplied 18-carbon PUFA.

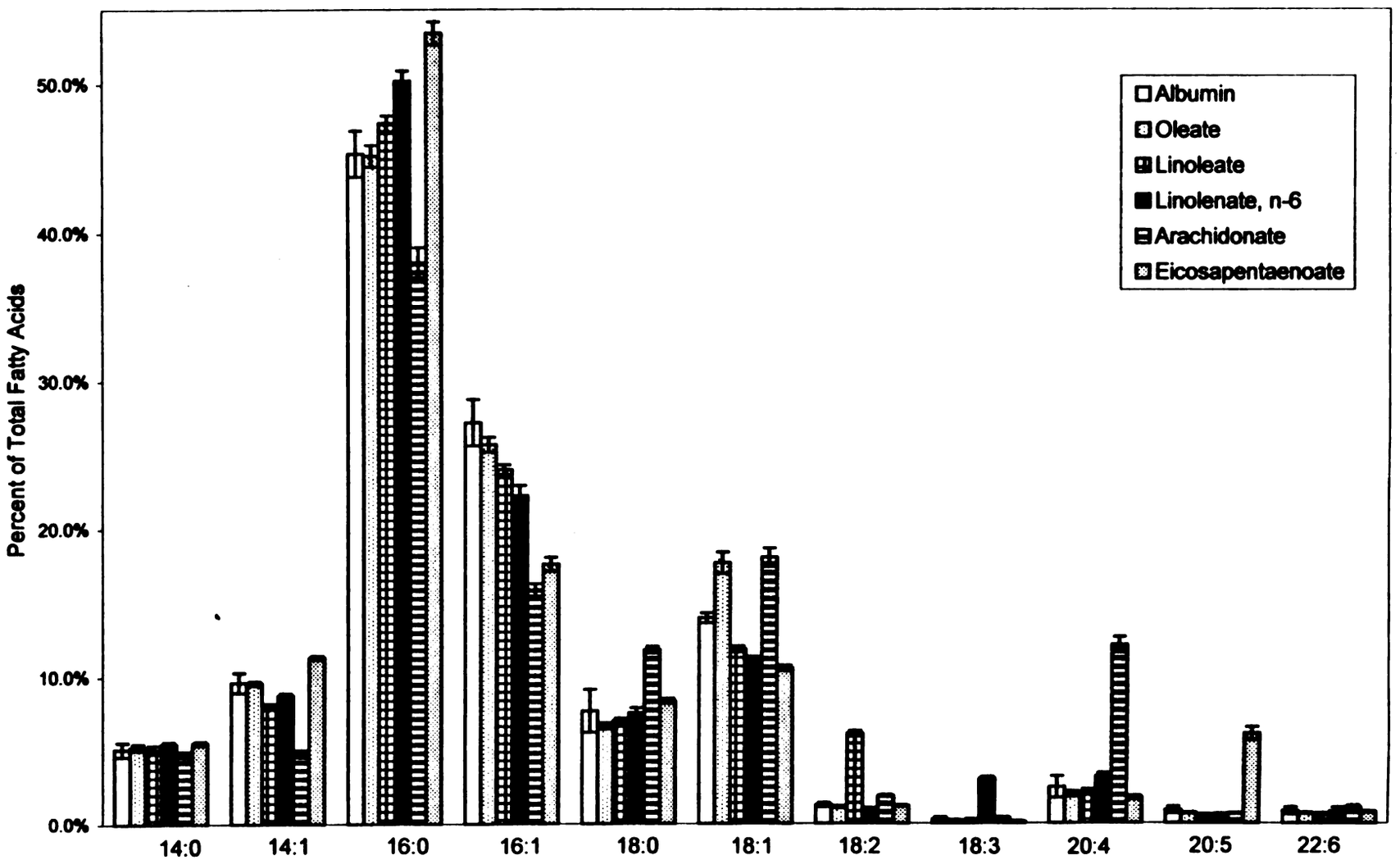
Thus, the exogenous FA enter either the TG/phospholipid pool for storage or are not further remodeled.

Table 2.2. Total Triglycerides in Adipocytes

Treatment:	**FCS**	**Alb**	**18:1**	**18:2**	**18:3**	**20:4**	**20:5**
Total TG:		34±5	37±1	33±1	40±4	35±8	39±7
Fatty Acid	**Percent of total fatty acid**						
16:0	59.8	55.3	46.7	44.4	46.3	45.3	46.2
18:0	10.4	9.7	8.6	8.8	8.3	10.5	8.0
18:1n-9	20.9	24.5	32.9	19.1	17.9	18.2	19.7
18:2n-6	1.9	2.0	2.1	16.0	4.1	1.8	1.8
18:3n-6	0.7	1.0	0.5	0.9	11.0	0.6	0.4
18:3n-3	0.4	0.8	1.5	1.6	4.0	0.4	0.5
20:4n-6	3.3	3.5	3.0	3.8	4.2	20.0	2.7
20:5n-3	0.7	1.1	2.9	3.4	2.8	1.2	19.0
22:6n-3	1.8	2.2	1.7	2.0	1.4	2.0	1.6

Table 2.2. Total Triglycerides in Adipocytes. Total triglycerides were determined as described in Materials and Methods. FCS:fetal calf serum, Alb:albumin. There was no statistical differences between treatments for total TG. The lower part of the table lists the percent of total fatty acids after continuous treatment with 50μM of the indicated fatty acid throughout differentiation.

Figure 2.14. Fatty Acid Profiles in Adipocytes. Immediately following removal of the differentiation mix, cells were treated with the fatty acids at 50μM continuously until harvest for fatty acid analysis (10 days). All fatty acid treatments contained 10μM albumin in DMEM containing FBS. The fatty acid treatments are as acids which were analyzed are shown on the graph and are all n-6 fatty acids except 20:5 and 22:6 which are n-3. Each bar represents 7 samples and standard error are shown. The Y-axis shows the percent of all the fatty acids in sample.


Fatty Acid Profiles in Adipocytes
Percent of Total Fatty Acids
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
Albumin
Oleate
Linoleate
Linolenate, n-6
Arachidonate
Eicosapentaenoate
14:0
14:1
16:0
16:1
18:0
18:1
18:2
18:3
20:4
20:5
22:6

DISCUSSION

Treatment of adipocytes with fatty acids have profound effects on differentiation and gene expression (Ailhaud et al., 1992; 1995; 1996; Tontonoz et al., 1994c; Forman et al., 1995; Long and Pekala, 1996; Vassaux et al., 1992). The principal mechanisms for the effects of fatty acids on adipocyte gene expression were thought to involve either prostanoid or PPAR-mediated pathways. Our studies show that the dominant negative suppression of lipogenic gene expression by PUFA in 3T3-L1 cell line is mediated by a prostanoid pathway and not through a PPAR-mediated pathway. PUFA suppress S14 and FAS mRNA levels and S14CAT expression in cultured 3T3-L1 adipocytes and this effect occurs only in fully differentiated adipocytes. The suppression of S14 gene transcription is 6-fold more sensitive to 20:4,n-6 than 20:5,n-3 and can be blocked by the cyclooxygenase inhibitor, flurbiprofen. These results are consistent with a prostanoid-mediated pathway for the control of lipogenic gene expression. Both PGE_2 and $PGF_{2\alpha}$ inhibit lipogenic gene expression and this effect is blocked with pertussis toxin implicating an G_i/G_o-coupled signaling pathway.

Cyclooxygenases convert 20:4,n-6 to prostanoids and prostanoids elicit a wide range of biological effects through plasma membrane receptors coupled to G-proteins.

Prostaglandins are produced locally and act in a paracrine fashion (Smith, 1989). PGE_2, PGI_2 and PGF_2 are the most predominant prostaglandins produced in 3T3-L1 cells, although more prostaglandins are produced in preadipocytes than adipocytes (Hyman et al., 1982; Long and Pekala, 1996; Vassaux et al., 1992; Richelsen et al., 1992; Christ and Nugteren, 1970). Long and Pekala showed that GLUT4 expression was depressed by 20:4,n-6 and PGE_2 and further showed that cAMP was produced in adipocytes upon treatment with PGE_2. While activation of protein kinase A by cAMP has a strong suppressive effect on S14CAT expression in L1 adipocytes (Fig. 2.13), treatment of adipocytes with the phorbol ester, PMA, an activator of PKC (not shown), had no effect on CAT activity. Treatment of cells with inhibitors of both protein kinase A and C, *i.e.* H7 and staurosporin, failed to block the PGE_2-mediated inhibition of CAT activity. Therefore, it is unlikely that the PGE_2-mediated inhibition of CAT activity is due to an increase in cAMP and/or subsequent activation of protein kinase A or C. In contrast, treatment of cells with pertussis toxin, which blocks G_i/G_o-linked pathways, was the only agent found to reverse the PGE_2 inhibition on S14CAT activity (Figure 2.14). G_i/G_o-linked signaling pathways inhibit G_s-linked activation of adenylate cyclase or promote an elevation in inositol 1,4,5-phosphate (IP_3) and intracellular Ca^{+2}

(Richelsen et al., 1992). As a decrease in cAMP would be expected to induce S14CAT expression, the involvement of the G_i/G_o-linked pathway implicates activation of phospholipase C_β, release of IP_3 and elevation of intracellular free Ca^{+2}. Treatment with the calcium ionophore A23187 also suppresses S14CAT expression (Figure 2.13). The control of S14 gene transcription by calcium will be addressed in future research.

Some studies suggest PGE_2 action on adipocytes is anti-lipolytic and acts via blocking an increase in cAMP (Vassaux et al., 1992; Christ and Nugteren, 1970; Chatzipanteli et al., 1992). Others suggest that PGE_2 acts through protein kinase C via a receptor coupled to phospholipase C in Swiss 3T3 fibroblasts (Danesch et al., 1994). $PGF_{2\alpha}$ treatment of 3T3-L1 preadipocytes leads to an increase in intracellular calcium, activation of calcium/calmodulin dependent protein kinase (CaM kinase) and inhibition of differentiation (Miller et al., 1996). Ntambi and Takova (1996) reported that A23187 inhibited differentiation of 3T3-L1 cells but had no effect when used two days after differentiation. These confusing reports make it difficult to determine how prostaglandins act on only differentiated adipocytes. In contrast to the Long and Pekalas' (1996) studies, my results suggest that the inhibition of S14CAT expression is not due to an increase in cAMP but through a separate pathway,

perhaps calcium linked. Further research is required to definitively show that PGE_2 suppresses S14 gene transcription via a calcium linked pathway.

Another possibility is that prostaglandins are working through a nuclear receptor pathway. Prostaglandins are reported to bind PPARγ2, a nuclear receptor (Spiegelman and Flier, 1996; Kliewer et al., 1997; 1995; Forman et al., 1997). While PPARγ2 plays an important role in adipogenesis, which includes the induction of lipogenic enzymes, these results show that pioglitazone-activated PPARγ2 has no effect on S14CAT expression in fully differentiated adipocytes. Though PPARγ2 did bind to the S14 promoter, this data does not indicate that PGE_2 or arachidonic acid are acting through a PPAR mediated pathway to suppress S14 in fully differentiated adipocytes.

Finally, my results would appear to conflict with reports by others documenting that rats fed vegetable oils do no show significant changes in adipose tissue lipogenesis (Clarke et al., 1976; 1977). One explanation is that the dose of AA used here is higher than in vivo levels. Our results do agree with these in vivo studies by showing that linoleic (18:2n-6) and α-linolenic (18:3n-3) and γ-linolenic (18:3n-6) acids, found in vegetable oils, were ineffective inhibitors of S14CAT activity (Figure 2.4). Interestingly,

epididymal fat mRNAs encoding S14 and FAS were suppressed 50% after 5 days on a diet containing 10% fish oil (Jump et al., 1993). EPA, a component of fish oil, effectively suppressed S14 and FAS mRNAs (Figure 2.1) and CAT activity (Figure 2.2) in adipocytes. When compared to 18-carbon PUFA, the highly unsaturated fatty acids found in fish oil were more potent suppressers of lipogenic gene expression (Clarke and Jump, 1994; Jump et al., 1996; 1994). Thus any conflict with earlier reports can be explained by the use of fish oil vs. vegetable oil for in vivo studies and the dose of AA or EPA used in adipocyte cell culture.

CONCLUSIONS

Aim 1 of this chapter was to determine the PUFA response region in the S14 promoter. Previously, this laboratory reported the PUFA response region (PUFA-RR) was located in the proximal promoter region)-220/-80 bp). The work presented here suggests that in adipocytes the same PUFA-RR is targeted by 20:4,n-6/PGE_2. However, I was unable to define further the specific PUFA target in adipocytes. This aim will be addressed again in the next chapter.

The second and third aims assessed the involvement of PPAR as the mediator of PUFA action on. Using gel shift analysis, PPARγ2 was found to bind the S14 promoter in the carbohydrate/glucocorticoid region, far upstream of the

proximal promoter. However, pioglitazone, a PPARγ2 activator, had no effect on S14 gene expression in fully differentiated adipocytes. Therefore, I concluded that although PPARγ2 does bind the S14 promoter, PPARγ2 does not mediate the PUFA control of this gene.

Aim 4 determined how arachidonic acid suppressed S14 gene expression. These results show that S14 is suppressed by AA in cultured 3T3-L1 adipocytes, and this effect is reversed with flurbiprofen, a cyclooxygenase inhibitor. Furthermore, the PGE_2 suppression of S14 was reversed by pertussis toxin, linking the suppression to a Gi/G_o signaling cascade and not a PPAR mediated pathway.

Aim 5 was originally intended to address the visible media changes in fatty acid treated cells. However, analysis of total triglycerides and staining of treated cells showed no changes, regardless of treatment or treatment time in adipocytes. Fatty acid analysis of continuously treated adipocytes indicated that whatever fat was fed to the cells, the cells contained ("you are what you eat" hypothesis). There was no indication that triglyceride levels changed even when lipogenic gene expression was suppressed by >80% by the fatty acid treatment. These observations indicate that while a specific fatty acid can alter lipogenic gene expression, adipocytes still accumulate fat from the diet.

In summary, my results show that lipogenic gene expression is suppressed by PUFA in cultured 3T3-L1 adipocytes. The results presented here are consistent with a requirement for AA conversion to prostanoids. The prostanoids may activate a G_i/G_o linked signaling cascade that leads to the inhibition of S14 gene transcription. This mechanism for control is different for PUFA-mediated suppression of lipogenic gene expression in the liver.

CHAPTER 3

CHAPTER 3

HEPATOCYTES

Like the adipocyte, the liver is a major site for lipid metabolism. Results in Chapter 2 indicated that arachidonic acid suppressed S14 gene transcription through a prostanoid pathway in adipocytes. How PUFA regulate S14 in the liver is unknown, although a PPAR-dependent pathway has been ruled out (Ren et al., 1996; 1997). Flick et al. (1977) reported that treatment of rats on an n-6 fatty acid diet with indomethacin failed to block the n-6 PUFA suppression of fatty acid synthase (FAS) activity, indicating a prostanoid independent pathway of PUFA suppression. Therefore, the hypothesis for Chapter 3 is "PUFA regulation of hepatic lipogenic gene expression is through a PPAR and prostanoid independent pathway".

As in the adipocyte, PUFA suppress hepatic S14 gene expression. In the rat, the liver rapidly responds to a diet containing n-3 PUFA by suppressing S14 and FAS expression (Jump et al., 1993). Several hepatic genes are regulated by PUFA including FAS, malic enzyme (ME), glucokinase (GK), citrate lyase, acetyl CoA carboxylase

(ACC), pyruvate kinase, Δ^9 desaturase, S14 (Clarke and Jump, 1994; Clarke and Abraham 1992; Clarke et al., 1990a), AOX and CYP4A (Ren et al., 1996; 1997). In rats fed a fish oil diet for five days, the hepatic expression FAS, S14, ME, pyruvate kinase and glucokinase are all depressed compared to olive oil fed rats (Jump et al., 1994). In contrast, AOX and CYP4A mRNA expression in the liver increases in mice fed n-3 PUFA compared to chow fed mice (Ren et al., 1997).

As discussed in Chapter 1, S14 gene transcription is controlled by a variety of factors in the liver, namely insulin, thyroid hormone and dietary components. Because PUFA control of adipocyte S14 gene expression was found to require cyclooxygenase, Aim 1 will address this requirement in hepatocytes. Subsequently, Aim 2 will address the prostanoid control of hepatic S14 expression.

As discussed in Chapter 2, results suggested that the PUFA-RR was located between -220 and -80bp in the S14 promoter. Results in adipocytes were not conclusive with further efforts to locate a more specific PUFA-RR. Aim 3 will use transfected hepatocytes to further narrow the promoter region targeted by AA and other suppressive compounds.

AIMS

Aim 1: Does arachidonic acid regulation of S14 gene expression in hepatocytes require cyclooxygenase?

Aim 2: Are PG regulating S14 in hepatocytes and if so, how?

Aim 3: Where is the cis-regulatory element required for AA and PGE_2 regulation of S14 gene transcription?

INTRODUCTION

The effect of dietary n-6 polyunsaturated fatty acids (PUFA) on hepatic de novo lipogenesis was first reported by Allman and Gibson (1965) while studying the effects of essential fatty acid deficiency on hepatic lipid metabolism. Those studies showed that removal of n-6 PUFA from the diet led to a rise in de novo lipogenesis. In subsequent studies, Flick, et al. (1977) reported that administration of indomethacin in vivo failed to block the n-6 PUFA suppression of fatty acid synthase (FAS) activity. N-6 PUFA are precursors of prostaglandins and indomethacin inhibits prostaglandin synthesis. These observations led investigators to suggest the n-6 PUFA regulation of hepatic lipogenesis did not involve prostaglandins.

However, recent studies on the effects of n-6 PUFA on lipogenic gene expression in cultured adipocytes indicated 20:4,n-6 suppressed (ED_{50} ~ 50 μM) mRNAs encoding FAS and the S14 protein. The 20:4,n-6 effect on S14 CAT activity could be blocked by flurbiprofen, a non-specific cyclooxygenase inhibitor (Chapter 2). PGE_2 and $PGF_{2\alpha}$ also suppressed S14 mRNA (ED_{50}<10μM). Using a cell line containing a stably transfected S14CAT fusion gene as a monitor of transcriptional effects, PGE_2 was found to suppress S14CAT activity through a pertussis-sensitive G_i/G_o-linked regulatory pathway. Thus, the 20:4, n-6 control of lipogenic gene expression in 3T3-L1 adipocytes required cyclooxygenase.

Based on these findings and the fact that primary hepatocytes have prostanoid receptors and are responsive to prostanoids, I reexamined the requirement of cyclooxygenase in the n-6 PUFA regulation of hepatic lipogenic gene expression. Here, results show that the 20:4, n-6 suppressed lipogenic gene expression in the hepatic parenchymal cell does not involved metabolism through a cyclooxygenase. However, PGE_2 suppresses mRNAs encoding FAS, S14 and L-pyruvate kinase (L-PK). Using the S14CAT as a monitor for transcriptional control, these results show that the cis-regulatory targets for PGE_2 and 20:4, n-6 map to the PUFA-response regions within S14 proximal promoter (-

220 to -80bp). These studies indicated that prostanoids also affect hepatic lipogenic gene expression and that PUFA and prostanoids might utilize a common pathway at the genomic level to control S14 gene transcription.

MATERIALS AND METHODS

HEPATOCYTE PREPARATION AND CULTURE A complete protocol for hepatocyte preparation is given in Appendix B. Briefly, male Sprague-Dawley rats were starved overnight then anesthetized using Nembutol (interperitoneal injection) or methoxyflurane (inhalation). After shaving and washing, the skin and muscle layers were cut back to reveal the hepatic vein. The vein was catheterized and the liver perfused with a wash buffer then a digestion buffer containing either collagenase or Liberase™ (Boehringer Mannheim). The liver was removed and hepatocytes were separated through a Percoll cushion. Hepatocytes were plated at 3 million cells per 60mm plate for CAT assays or 10 million cells per 100mM plate for RNA. Cells were plated in Williams E media containing 10% fetal bovine serum, 10mM lactate, 200nM insulin and 10nM dexamethasone. Transfection (4-6 hours later) with reporter plasmids (2µg/plate), thyroid hormone receptor expression vector (1µg/plate) and Lipofectin™

(6.6µl/µg DNA) was performed in the same media as plating media but without serum. The next morning media was changed Williams E media containing 25mM glucose, 1µM insulin and 10nM dexamethasone unless otherwise noted. Media was changed after 24 hours; all experiments were terminated after 48 hours.

RNA ISOLATION AND NORTHERN BLOTTING Total RNA was isolated using Triazol™ (Gibco) according to the manufacturer's protocol. RNA was electrophoresed, transferred to nitrocellulose membranes and probed with radiolabelled cDNA as described in Chapter 2 (Materials and Methods). Radiolabelled probes used here include those listed in Chapter 2 as well as AOX (T. Osumi, Japan), cyclooxygenase I and II (D. Dewitt, Michigan State University) and CYP4A2 (A. Thelen, Michigan State University).

CAT ASSAYS Following treatments, cells were assayed for CAT activity and protein content as previously described (Jump et al., 1993). CAT Units: ^{14}C-acetylated chloramphenicol CPM/100 µg protein/hour.

RESULTS

AIM 1 RESULTS

Aim 1: Does arachidonic acid regulation of S14 gene expression in hepatocytes require cyclooxygenase?

S14 suppression by arachidonic acid is not reversible by flurbiprofen. Based on the observation in adipocytes S14 CAT activity suppression by AA could be reversed by the cyclooxygenase inhibitor, flurbiprofen, reversal of S14CAT activity in hepatocytes by flurbiprofen was also tested. Primary hepatocytes were transfected with S14CAT124 and treated with 50μM Albumin or 250μM AA with or without 100μM flurbiprofen for 48 hours. As expected, AA inhibited CAT activity approximately 60% but flurbiprofen could not reverse this inhibition. The lipoxygenase inhibitor, NDGA, was also unable to reverse the AA suppression of S14 CAT activity (data not shown). These results indicate that AA suppression of S14 gene expression does not require metabolism through the cyclooxygenase or lipoxygenase pathway (Figure 3.1).

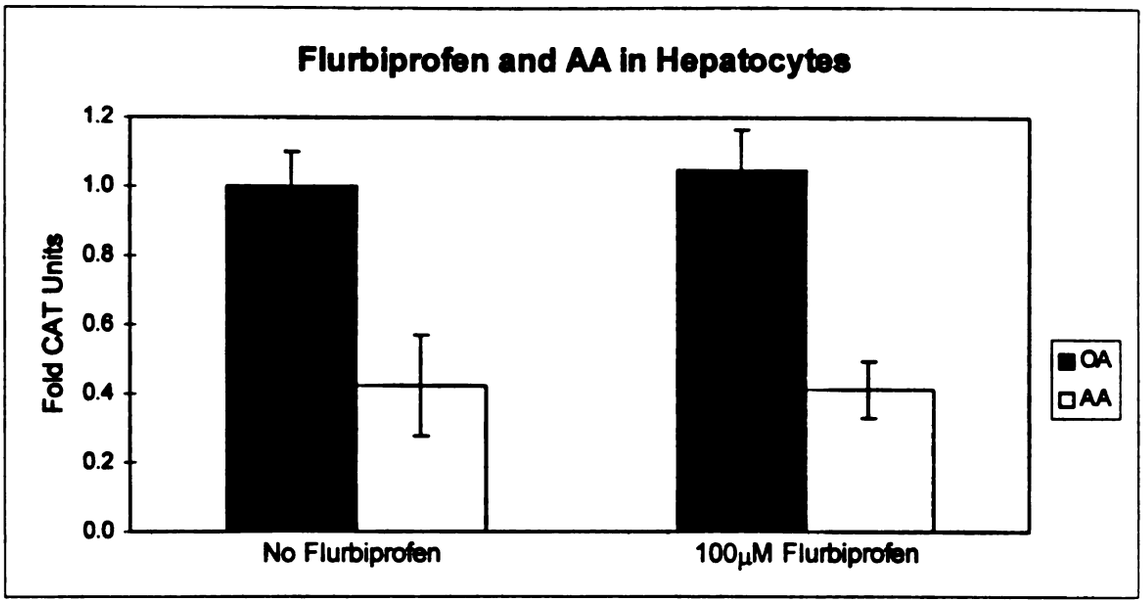


Figure 3.1. AA Suppression of S14CAT Activity is Not Reversed by Flurbiprofen. Hepatocytes transfected with S14CAT124 (-4315/+19) were treated with 250μM OA or AA alone or with 100μM flurbiprofen for 48 hours. CAT activity was determined and all data normalized to the OA treatment. The bars represent 3 pooled experiments and standard error is included. No differences existed between treatments with or without flurbiprofen.

AIM 2 RESULTS

Aim 2: Are PG regulating S14 in hepatocytes and if so, how?

PGE_2 suppresses S14 message and CAT activity. PG suppressed S14 mRNA and CAT activity in adipocytes (Chapter 2). Therefore, similar experiments were set up with hepatocytes to test suppression of S14 mRNA and CAT activity. Figure 3.2 shows that S14 message in hepatocytes is suppressed 66% by 10μM PGE_2 after a 48 hour treatment. Other mRNAs were also measured in response to this treatment. FAS mRNA levels were suppressed ~60% and PK

~35%. AOX and CYP4A2 were not effected by the treatment. These results indicate that like PUFA, PGE_2 can depress mRNA levels for the lipogenic genes FAS, PK and S14. However, unlike EPA which induces AOX and CYP through PPAR (Ren et al., 1996; 1997), the oxidative genes AOX and CYP are not effected by PGE_2 treatment in hepatocytes. Interestingly, like PGE_2, AA also has no consistent effect on AOX and CYP4A2 mRNA in hepatocytes (data not shown).

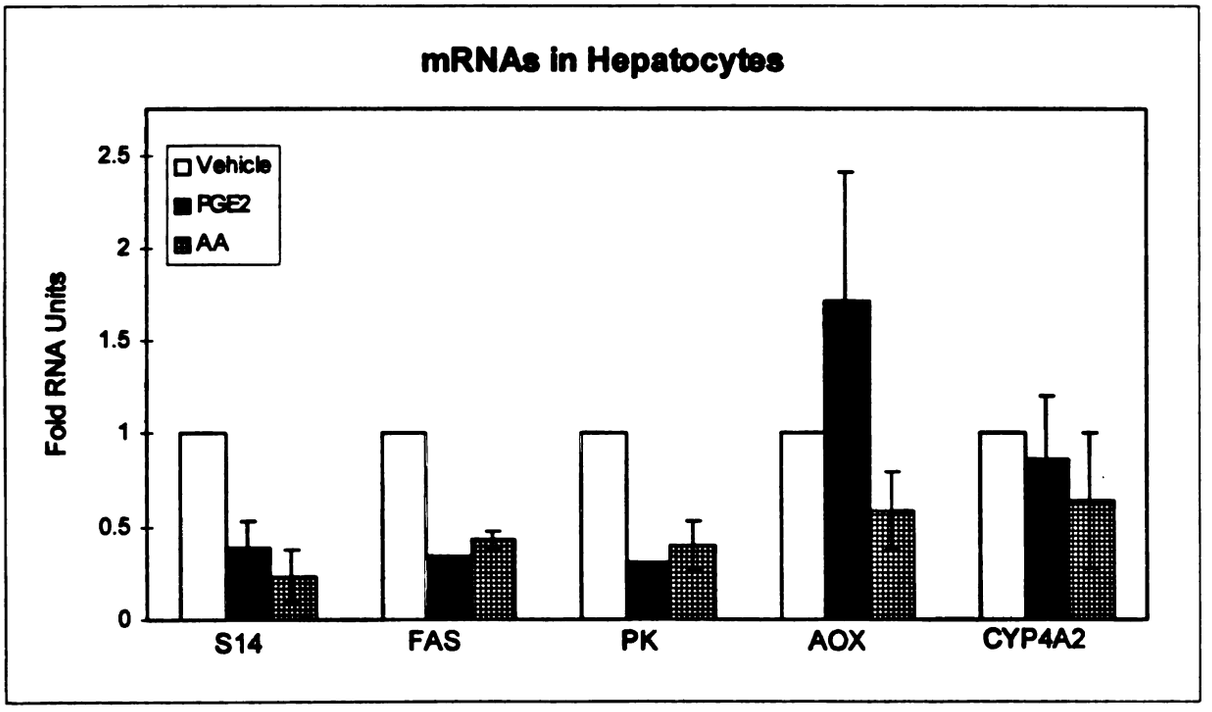


Figure 3.2. PGE_2 Effects on Hepatic Gene Expression. Hepatocytes were treated with DMSO or 10μM PGE_2 for 48 hours. Total RNA was isolated, transferred and probed for S14, FAS, PK, AOX and CYP4A2. This graph represents pooled data from 2 separate experiments of triplicate plates each. The data was normalized to the control (DMSO). Standard error of the pooled PGE_2 data is indicated.

As described in Chapter 2, S14 CAT activity in adipocytes was inhibited by PG treatment. To examine the effects of prostanoids on hepatic gene transcription, hepatocytes were treated with various prostaglandins to determine if CAT activity was also suppressed in these cells transfected with S124 (-4315/+19 bp). The results are shown in Figure 3.3. Both PGE_2 and $PGF_2\alpha$ inhibit S14 CAT activity by about 54% and 43% respectively in transfected hepatocytes. PGI_2 is very unstable and was inhibitory only when freshly prepared (data not shown). These results indicate that PG can suppress S14 transcription in hepatocytes.

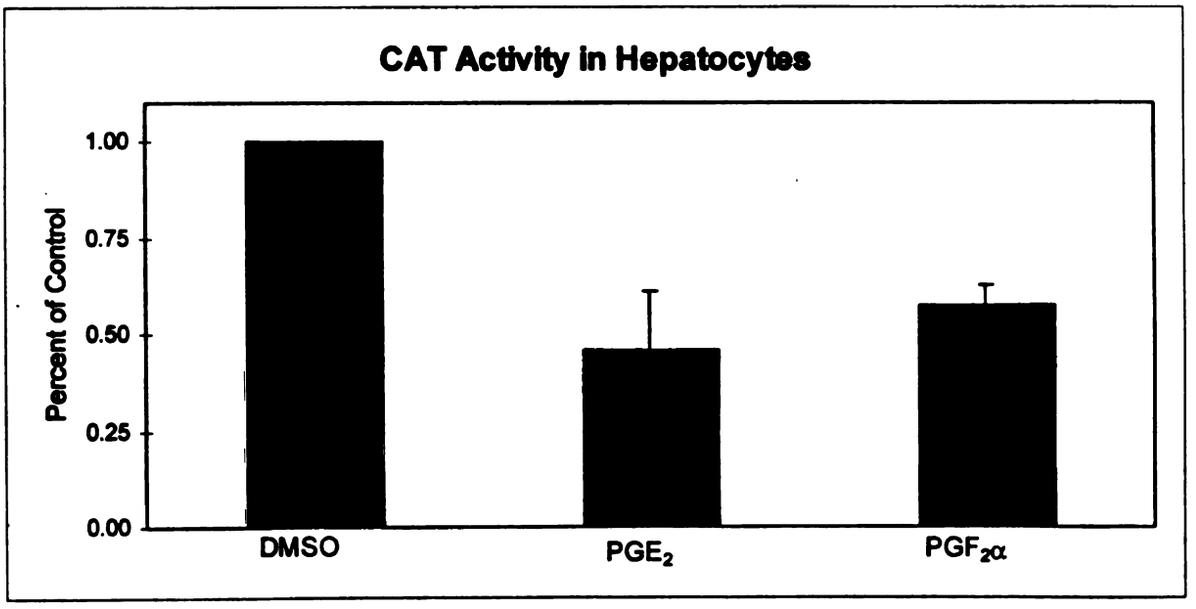


Figure 3.3. Inhibition of S14 CAT activity by PG. Primary hepatocytes transfected with S124 and treated with the indicated PG at 10μM for 48 hours. CAT activity was determined and data from several experiments were pooled and normalized to the DMSO control. Standard error is shown.

PGE_2 suppression of CAT activity was not affected by inhibitors of signal transduction pathways. In adipocytes, the PG suppression of S14 CAT activity was reversed by pertussis toxin. Several inhibitors were also used here in attempts to block the PGE_2 inhibition of S14 CAT activity in hepatocytes (inhibitors used in both cell types are summarized in Table 3.1). The results of the hepatocyte studies are shown in Figure 3.4. The PGE_2 suppression was reversed by pertussis
toxin treatment in adipocytes, however, in hepatocytes this was not the case. As shown in Figure 3.4, the PGE_2 suppression was not reversed by pertussis toxin. Pertussis toxin blocks G_i, a G-protein which inhibits cAMP formation. Pertussis toxin was used at 100ng/ml and the cells treated with the PGE_2 concurrently. Cells were also pretreated with pertussis toxin from 15 minutes to 12 hours. However, pretreatment of hepatocytes with pertussis toxin also had no effect and could not consistently block the inhibition by PGE_2 or AA (not shown).

H7, a PKA/PKC inhibitor at 10μM, also had no effect. Other PKA or PKC inhibitors were unable to reverse the PGE_2 effect as well. These are shown in Table 3.1. Verapamil, an α_1 calcium ion channel blocker, also was unable to reverse the inhibition by PGE_2. Verapamil was used at 10μM and had no effect. Chelating extracellular calcium with

EGTA was toxic to the cells (not shown). Each of these compounds used were tested alone and used at the highest nontoxic dose, measured both by protein levels and CAT activity.

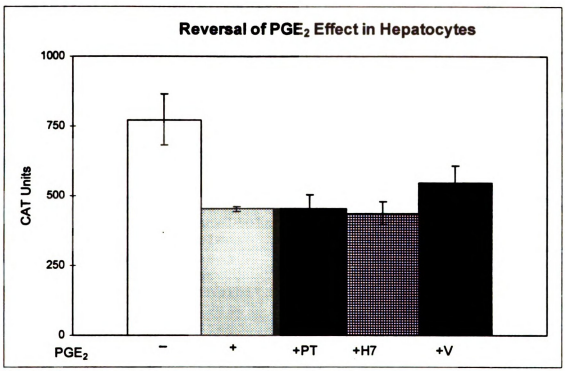


Figure 3.4. Effects of PGE_2 and Inhibitors on S14 CAT activity. Hepatocytes were transfected with S14CAT124 and treated for 48 hours without (--) or with (+) 10μM PGE_2 and also plus 100ng/ml pertussis toxin (PT), 10μM H7 or 10μM verapamil. CAT activity was determined. This is a representative graph of at least three separate experiments and includes the standard error of each triplicate on each bar. CAT units = cpm/hour/100μg of protein.

Table 3.1. PGE_2 SIGNAL TRANSDUCTION INHIBITORS

DRUG (SOLVENT)	ACTION	ADIPOCYTE RESULT	HEPATOCYTE RESULT
H7 (water)	inhibits PKA and PKC at conc. used	no effect at 10μM	no effect at 10μM
H89 (water)	inhibits PKA specifically	no effect at 10μM	no effect at 10μM
Staurosporin (DMSO)	inhibits PKA and PKC	no effect at 10nM	no effect at 10nM
KN-62 (DMSO)	inhibits Ca^{2+} calmodulin kinase II	inhibitory but slight rev. of PGE_2 at 25μM	NA
Verapamil (DMSO)	blocks α1 Ca ion channels	some reversal of PGE inhibition at 10μM	slight to no reversal of PGE inhib. at 10μM
BAPTA-AM (DMSO)	blocks intracellular Ca release	no effect at 10μM	no effect at 20μM
Thapsigargin (DMSO)	releases intracell. Ca, and inhibits Ca ATPase	toxic at 170nM	NA
TMB-8 (water)	Ca antagonist and PKC inhib. -blocks Ca release from ER	NA	no effect at 15μM
Pertussis Toxin (glycerol)	blocks Gi/q/o proteins	reverses PGE_2 effect at 25 to 100ng/ml	no consistent effect even with pretrt.
PD980059 (DMSO)	blocks MEK, or p44 (a MAPK)	NA	no reversal of PGE effect up to 50μM w/ pretreatment

Table 3.1. PGE_2 Signal Transduction Inhibitors. The listed agents were used at the stated concentrations with 10μM PGE_2 in either adipocytes or hepatocytes. CAT activity was determined and results are described in the table. All experiments were done at least twice. NA= not applied to that cell system.

PGE_2 acts through an EP3 cell surface receptor. In adipocytes, all three PG receptor agonists were effective in suppressing S14 CAT activity (not shown). In order to determine which prostaglandin receptor was active in hepatocytes, agonists to each of the PGE_2 receptors were used to treat cells. As shown in Figure 3.5, only sulprostone, an EP3 receptor agonist, was able to mimic the suppression shown by PGE_2. This indicates that EP3 receptors are present and active in these hepatocytes while EP1 and EP2 are not active in suppressing S14 CAT activity. In contrast, the adipocytes appeared to be sensitive to all the agonists, indicating each signal transduction pathway (cAMP increase, cAMP decrease and IP3/calcium levels) was operative in this cell type. A summary of the prostaglandin receptor agonist actions and responses of both cell types are also shown in Table 3.2. The structure of the different prostaglandins and PG agonists are shown in Figure 3.6.

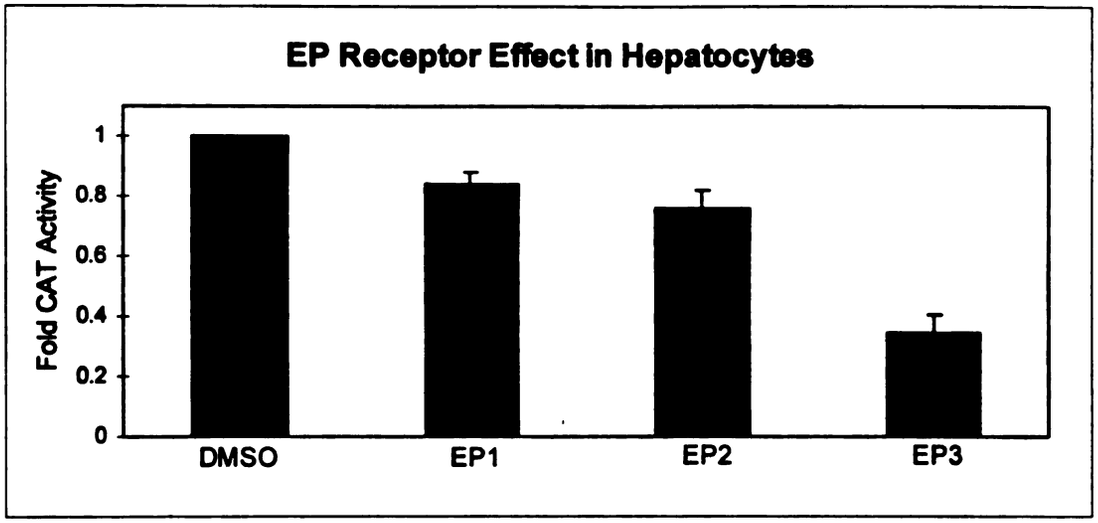


Figure 3.5. PGE_2 Receptor Agonist Effects on S14 CAT Activity. Hepatocytes, transfected with S14CAT124, were treated with DMSO or 10μM EP1, EP2 or EP3 agonists. EP1=17-phenyl trinor PGE_2, EP2=11-deoxy 16,16 dimethyl PGE_2, EP3=sulprostone. The CAT data was pooled from several experiments and normalized to the control (DMSO). Standard errors of the pooled data is included on the graph.

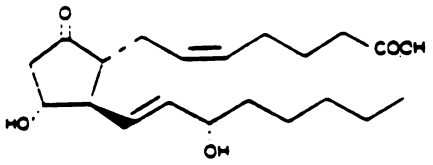
Table 3.2. PROSTAGLANDIN AGONISTS EFFECTS ON S14 CAT ACTIVITY IN ADIPOCYTES AND HEPATOCYTES

DRUG (SOLVENT)	**ACTION**	**ADIPOCYTE RESULT**	**HEPATOCYTE RESULT**
PGE_2 (DMSO)	prostaglandin	inhibitory at 10μM	inhibitory at 10μM
$PGF_2\alpha$ (DMSO)	prostaglandin	inhibitory at 10μM	inhibitory at 10μM
PGI_2 (DMSO)	prostaglandin	inhibitory at 10μM or unstable	inhibitory at 10μM or unstable
17-phenyl trinor PGE_2 (DMSO)	EP1 receptor agonist	inhibitory at 10μM	no effect at 10μM
11-deoxy 16,16 dm PGE_2 (DMSO)	EP2 receptor agonist	inhibitory at 10μM	no effect at 10μM
Sulprostone (DMSO)	EP3 receptor agonist	inhibitory at 10μM	inhibitory at 10μM
17-phenyl trinor $PGF_2\alpha$ (DMSO)	FP receptor agonist	inhibitory at 10μM	inhibitory at 10μM

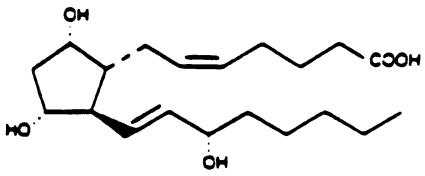
Table 3.2. Prostaglandin Agonists Effects on S14 CAT Activity in Adipocytes and Hepatocytes. A summary of the different prostaglandins used in both cell types are shown here. Cells were treated with 10μM PG and CAT activity was determined. Each of the above PG were used in at least three different experiments in each cell type.

Prostaglandins

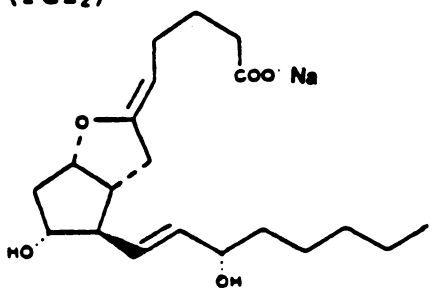
Prostaglandin E_2
(PGE_2)



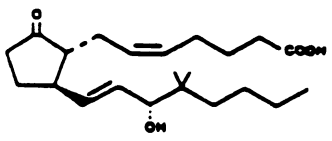
Prostaglandin $F_{2\alpha}$
($PGF_{2\alpha}$)



Prostaglandin I_2
(PGI_2)

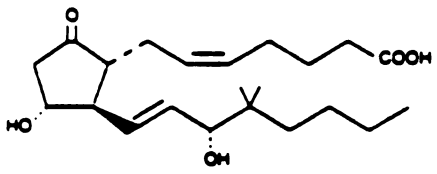


16,16-dimethyl PGE_2

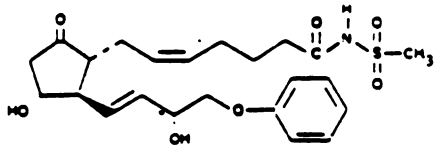


11-deoxy-16,16-dimethyl PGE_2
(EP1 Agonist)

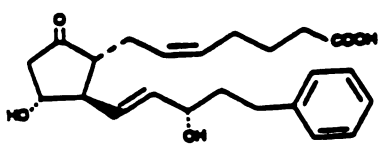
Sulprostone
(EP3 agonist)



17-phenyl trinor PGE_2
(EP2 Agonist)



17-phenyl trinor $PGF_{2\alpha}$
(FP Agonist)



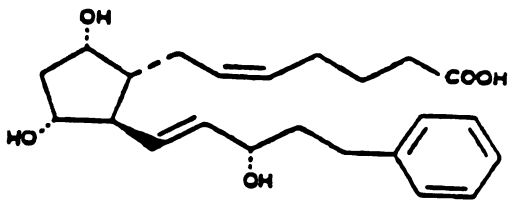


Figure 3.6. PG and Agonist Structures. The chemical structure of each prostaglandin and agonist is depicted above. These structure models were copied from Cayman Chemical (Ann Arbor, Michigan) product literature.

AA Suppression of S14 CAT activity could not be reversed in hepatocytes. No tested inhibitor could reverse the PG inhibition of S14 CAT activity in hepatocytes. Also, although both AA and PG inhibited S14 expression and CAT activity, AA suppression is not dependent upon PG production in hepatocytes (Figure 3.1). With these facts, I tested some other agents with the intention of trying to reverse the AA inhibition of CAT activity. These are shown in Table 3.3. In most cases, the highest non-toxic dose was used. In all cases, this table represents CAT activity in hepatocytes or adipocytes transfected with S14CAT124, which contains the entire S14 promoter (-4300bp).

As shown in Table 3.2, only flurbiprofen reversed the inhibition of S14CAT activity and only in adipocytes. Although the efficacy of the drugs were not tested, each was used at the highest nontoxic dose and at levels comparable to reports in the literature. Perhaps arachidonic acid itself rather than a specific metabolite can regulate S14 gene expression in hepatocytes. Further research will be required to determine how both AA and PG are acting to suppress S14 gene transcription in hepatocytes.

Table 3.3. AA METABOLISM INHIBITOR EFFECTS ON S14 CAT ACTIVITY

DRUG (SOLVENT)	ACTION	ADIPOCYTE RESULT	HEPATOCYTE RESULT
Flurbiprofen (DMSO)	blocks COX I and II	100μM, reverses AA inhibition of CAT activity	no effect at 100μM
NS-398 (DMSO)	blocks COXII	toxic	NA
Clotrimazole (DMSO)	inhibits P450 group 2 epoxygenases	slight reversal of AA inhibition at 50μM	can be toxic at 15μM but removes FA effects
Aminobenzotriazole (DMSO)	suicide inhibitor of P450 mono-oxygenases	NA	no effect at 15μM, may increase CAT activity
NDGA (DMSO)	inhibitor of lipoxygenases but also COX, EPOXYs	no effect at 100μM	no effect at 50μM
Triacsin (DMSO)	inhibitor of FA -CoA formation, PPAR activator	no effect at 10μM	no effect at 10μM
Vitamin E (DMSO)	anti-oxidant	NA	no effect up to 100μM
b-Mercaptoethanol (water)	stops peroxide formation from FA	NA	no effect up to 100μM
Nembutol (pentobarbital)	induces CYP2A1 & 2B1	NA	no effect up to 100μM
Miconazole (DMSO)	blocks P450 monooxygenases	inhibitory to CAT activity at 15μM	NA

Table 3.3. AA Metabolism Inhibitor Effects On S14 Cat Activity. The listed agents were used to treat adipocytes or hepatocytes also treated with 250μM AA at the concentrations listed. CAT activity was measured and the result is listed the table. Most experiments were done at least twice. NA = not applied to that cell system.

AIM 3 RESULTS

Aim 3: Where is the cis-regulatory element required for AA and PGE_2

regulation of S14 gene transcription?

PGE_2 inhibition is specific to the S14 promoter. To examine the effects of PGE_2 on parenchymal cell gene transcription, primary hepatocytes were transiently transfected with either an S14CAT reporter gene (S170) or RSVCAT. S170 contains the S14 promoter extending from +19 bp to -2.8 kb. This promoter contains a proximal promoter region required for proper initiation of gene transcription (MacDougald and Jump, 1991) and two enhancers. One enhancer located between -1.6 and 1.4 kb is a target for insulin and glucose induction of S14 gene transcription. A second enhancer located between -2.8 and -2.5 kb contains 3 thyroid hormone response elements (TRE) which are targets for thyroid hormone receptors (TR) binding in association with the retinoid X receptor (RXR). Cells transfected with S170 and treated with T_3, insulin and glucose express high levels of CAT activity. RSVCAT contains the RSV promoter/enhancer fused to CAT and cells transfected with this plasmid constitutively express high levels of CAT activity. PGE_2 (10 μM) suppressed S170 CAT activity by ~50% (Figure 3.5).

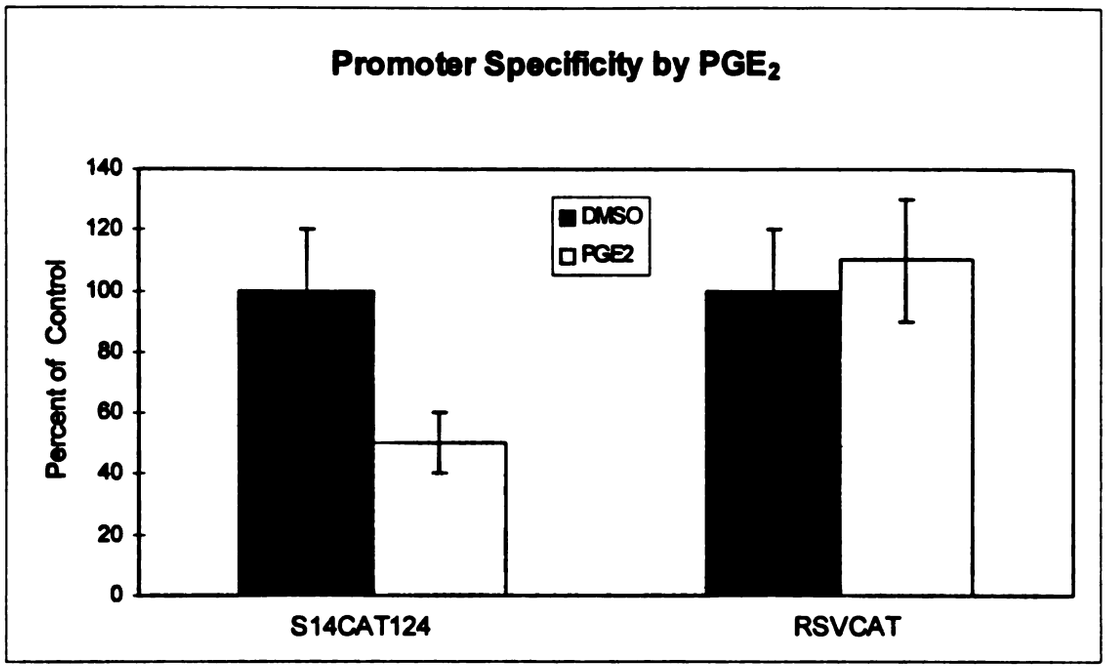


Figure 3.7 Specificity of PGE_2 on the S14 Promoter. Hepatocytes were transfected with the above plasmids and treated without or with 10μM PGE_2 for 48 hours. CAT activity was determined and normalized to the control. This is a representative graph of at least 3 separate experiments.

Arachidonic acid and PGE_2 target the same regions of the S14 promoter. Although both PGE_2 and AA both inhibit S14 CAT activity, AA does not require conversion to PG to be inhibitory to this gene. This experiment was designed to determine if PGE_2 and AA targeted the same site in the S14 promoter in hepatocytes. Several S14 promoter constructs were transfected into primary hepatocytes which were treated with either arachidonic acid or PGE_2. As shown in Figure 3.8, arachidonic acid progressively shows less inhibition in CAT activity as the promoter is truncated from -2897 to -80

base pairs. The greatest inhibition (78%) is with S170, and no inhibition is detectable with S158, which only includes -80 base pairs and the TRR. About 50% inhibition is seen with S14155, approximately 2/3 of that seen with S170. PGE_2, is less inhibitory even in S170 than arachidonic acid (50% vs. 78%). There is approximately 20% inhibition with both S155 and S16 and none with S158 by PGE_2. These results indicate that 1)arachidonic acid and PGE_2 are targeting at least 2 sites to suppress S14: a region between -220 and -2897, the region between -220 and -80, and 2) PGE_2 is somewhat less inhibitory than arachidonic acid. While there is overlap of the targets between these two compounds, there are also some differences in specificity and level of inhibition. PGE_2 is metabolized very quickly by hepatocytes and may explain why inhibition was not always as strong as AA.

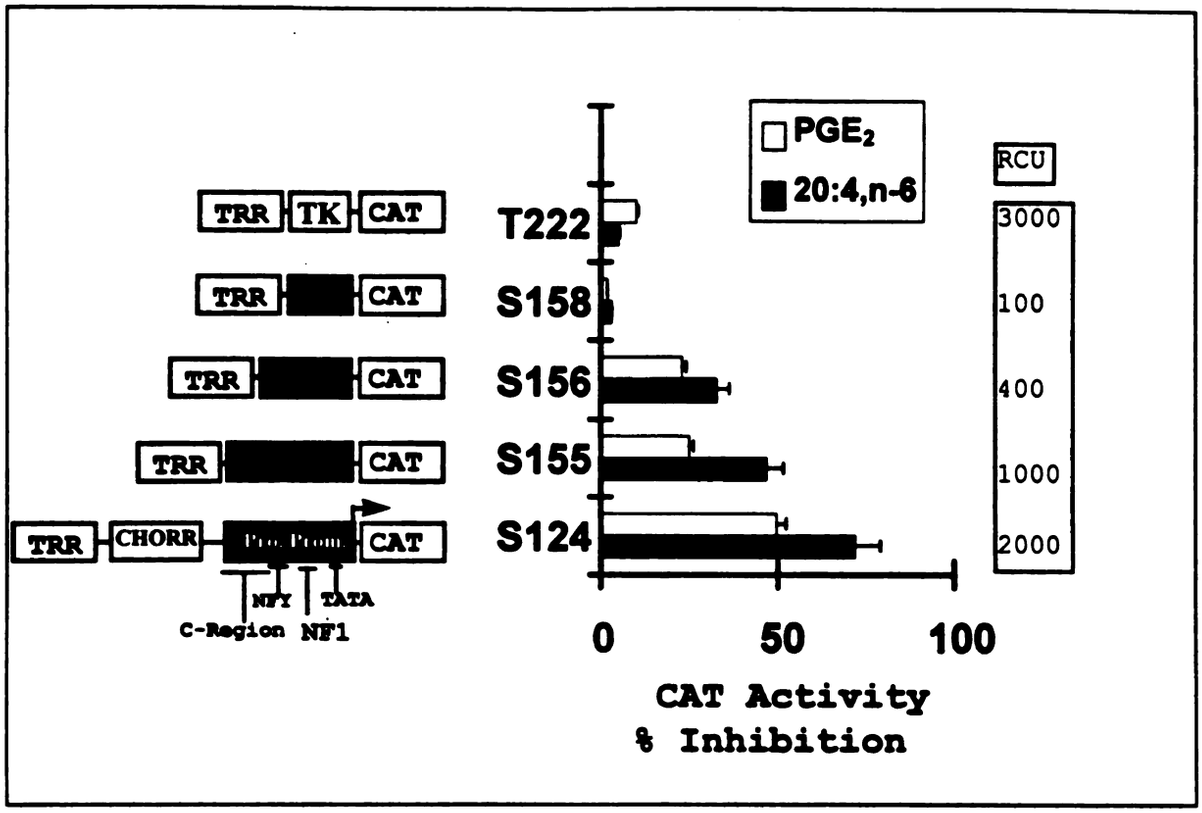


Figure 3.8. Deletion Analysis of the S14 Promoter with AA and PGE_2. The plasmids were transfected into hepatocytes with TRβ1 and then treated with 250μM arachidonic acid or 10μM PGE_2 and the corresponding controls (albumin + OA and DMSO respectively) for 48 hours. The data from at least 3 separate experiments was pooled. The results are presented as percent of control with standard errors. RCU = relative CAT Units.

The Y box and the C-region are necessary for S14 suppression by AA and PGE_2. Results from the above experiments indicated that the region between -220 and -80 is responsible for at least 2/3 of the inhibition of S14 gene transcription by PGE_2 and AA. Accordingly, several other plasmids were used to more specifically determine which regions were required for the inhibition by AA and PGE_2. This region contains a Y-box, which binds NF-Y and is

required for the T3 induction of S14 gene transcription (Jump et al., 1997b). To determine whether the Y-box or other elements within the -220/-80bp PUFA-RR, a series of plasmids were constructed (Figure 3.9). Each plasmid contains the RSV-TATA box (-60/+20 bp) and the S14 TRR (-2.8/-2.5 bp). Various components of the S14 proximal promoter and other promoters are inserted between the TRR and TATA-box.

Only S14 promoter constructs (R131 and R119) containing the S14 Y box and the C region (upstream of the Y box) showed inhibition by AA and PGE_2. These results indicate that regions surrounding the Y-box (-99 to -104) are important for regulation by both of these compounds. R157 and R117, containing either the albumin Y box or S14 Y box alone respectively, was only slightly sensitive to AA. R132 contains a Y box from the TK gene which is flanked by an Sp1 site on either side of the Y box. This plasmid (RSV132) was not sensitive to arachidonic acid. By comparing RSV119 and RSV159, which contain the Y box and either the upstream or downstream flanking region respectively, the AA suppression is similar to R131 with only R119, which contains the Y box and the upstream C region. Plasmids containing a Y box from another gene (R157) or the Y-box competitor C/EBP (R158) were not suppressed by AA. These results clearly point out that the S14 Y box and the C region alone are not effective

but that both are required for the full effect. Furthermore, suppression is specific to the S14 Y box (due to its flanking regions) and not to other Y boxes.

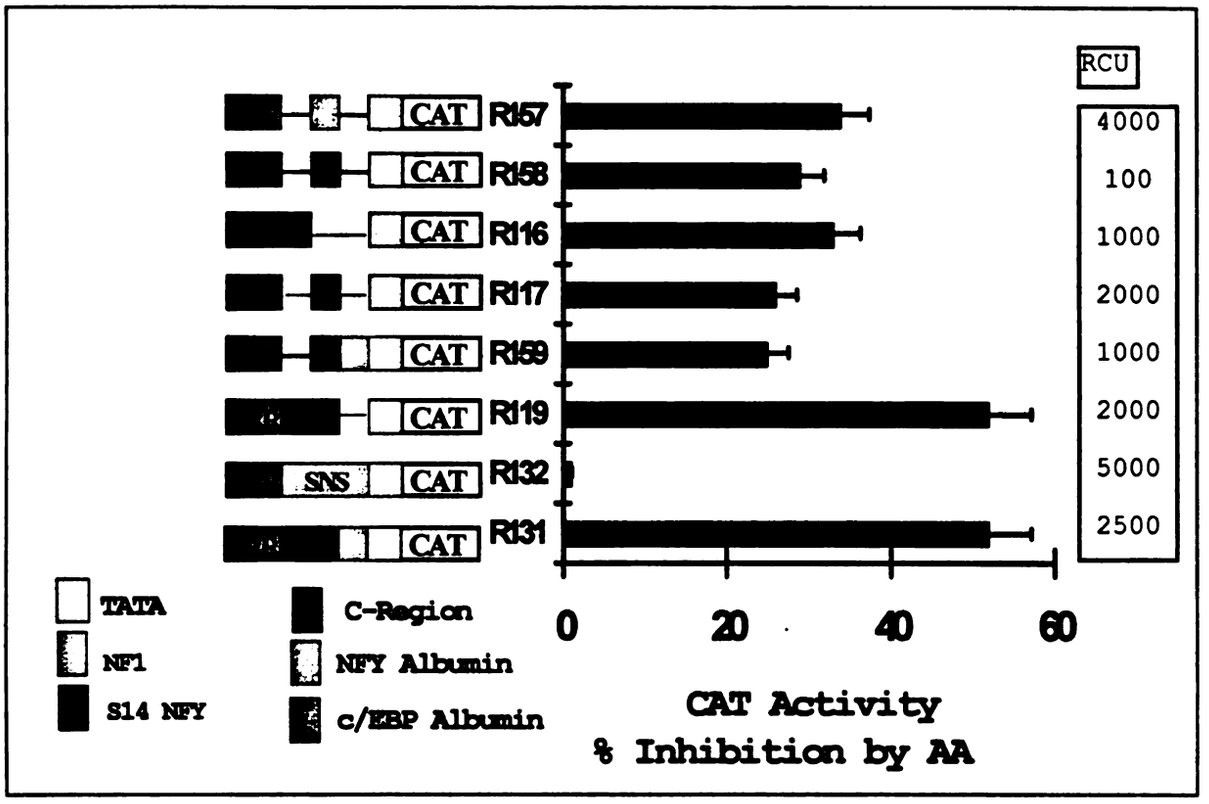


Figure 3.9. Y Box and C-Region are Required for PUFA Control of S14. The above S14 promoter constructs were treated with either 250μM OA or AA. CAT activity was determined and normalized to OA. This graph is combined data from three experiments and includes the percent error. RCU = relative cat units

PUFAs and PGE_2 regulation of S14 gene transcription is dictated by promoter context. Based on above results with AA, I wanted to determine if PGE_2 inhibition of S14 gene transcription also required the Y box and its flanking regions. The n-3 fatty acid, EPA was tested as well to determine if it too required this region for inhibition of

S14 CAT activity. Hepatocytes were transfected with the RSV plasmids R131 and R132 and treated with 10μM PGE_2 or 250μM AA or EPA. Figure 3.10 illustrates the effect of these compounds on CAT activity transfected with R131 and R132. R131 contains the S14 promoter region between -80 and -220 (includes the S14 Y box and flanking regions) while R132 contains the TK Y box flanked by Sp1 sites. Only R131, which contains the S14 Y box and the C-region, is suppressed by AA, EPA and PGE_2. This result again stresses the importance of the Y box and its requirement for the flanking regions for suppression of this gene.

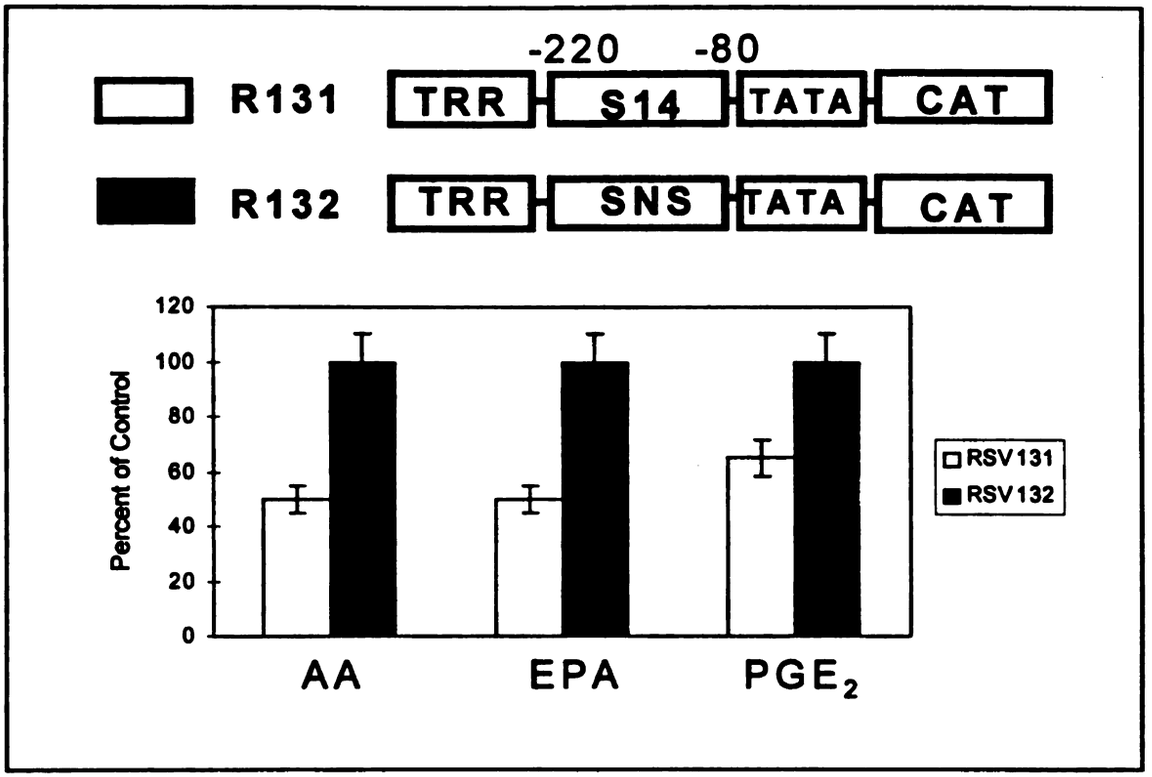


Figure 3.10. Promoter Context Dictates Control by PUFA and PGE_2. Hepatocytes transfected with RSV131 and RSV132 were treated with 250μM EPA or AA or 10μM PGE_2 for 48 hours and CAT activity was determined. Results were normalized to the control (albumin or DMSO) and percent of control was plotted on the graph. This graph depicts at least three combined experiments for each treatment and the standard error.

DISCUSSION

The analysis of prostanoid regulation of hepatic lipogenic gene expression was prompted by studies showing that 20:4,n-6 suppression of lipogenic gene expression in 3T3-L1 adipocytes required cyclooxygenase (Chapter 2). These studies suggested that 20:4, n-6 was converted to prostanoids in adipocytes. Further results indicated that

PGE_2 and $PGF_{2\alpha}$ suppressed mRNA encoding FAS and S14 through a pertussis-toxin sensitive G_i/G_o regulatory pathway in adipocytes. In the this chapter, results show that the 20:4, n-6 mediated inhibition of hepatic lipogenic gene expression does not require cyclooxygenase activity (Figure 3.1). Moreover, Northern analysis failed to detect mRNAs encoding PGHS1 or 2 in hepatic parenchymal cells (not shown). Thus, n-6 PUFA-mediated inhibition of lipogenic gene expression in hepatic parenchymal cells does not require a prostaglandin intermediate. This finding does not exclude the possibility that some other 20:4,n-6 metabolite might be generated in parenchymal cells and activate a signaling pathway. For example, cytochrome P450 mediated fatty acid metabolism can potentially generate PUFA metabolites that activate signaling cascades that affect gene transcription (Capdevila et al, 1990). Studies are currently in progress to evaluate the role these pathways play in dietary PUFA regulation of hepatic lipogenic gene expression.

While dietary PUFA does not require cyclooxygenase for its regulatory effects on hepatic gene expression, a number of reports have indicated that 20:4,n-6 cyclooxygenase products from non-parenchymal cells can act in a paracrine fashion on parenchymal cells. For example, prostaglandins produced by Kupffer cells in response to injury, sepsis or

other stimuli act in a paracrine fashion on the surrounding hepatocytes to alter specific functions (Billiar and Curran, 1992). PGE_2 increase hepatocyte proliferation through the EP3 receptor (Hashimoto et al., 1997) and induce glycogen breakdown (Garrity et al., 1987; 1989; Hespeling et al., 1995a; 1995b). Glucagon induces PGE_2, PGD_2 and $PGF_2\alpha$ synthesis and release from Kupffer cells. Interestingly, these prostaglandins counter glucagon-mediated glycogenolysis. Thus the effect of PGE_2 on hepatic metabolism *in vivo* is likely to be influenced by the physiological status of the animal.

Our studies show that PGE_2 acts on primary cultures of hepatic parenchymal cells to regulate mRNAs encoding genes involved in lipogenesis. PGE_2 suppresses mRNAs encoding proteins involved in lipid synthesis (FAS, S14 and LPK) but had no consistent effect on mRNAs encoding proteins involved in non-mitochondrial fatty acid oxidation (AOX and CYP4A2). Others have reported that PGE_2 effects on glycogen metabolism were linked to G_i-linked EP3 receptor that decreased cAMP levels (Garrity et al., 1987; 1989; Hespeling et al., 1995a; 1995b). I did not detect changes in hepatic cAMP levels in response to PGE_2 treatment (not shown). Depending on the subtype, EP3 receptors can augment or decrease intracellular cAMP as well as activate a PLC to change intracellular IP3 and calcium. $PGF_{2\alpha}$ is thought to

regulate intracellular IP3 and calcium levels. Based on this reasoning, the PGE_2 and $PGF_{2\alpha}$ suppression of hepatic lipogenic gene expression is consistent with an activation PLC. This finding is consistent with our earlier results with adipocytes (Chapter 2).

At the molecular level, both 20:4,n-6 and PGE_2 suppress S14 mRNA by inhibiting gene transcription (Figure 3.11). Efforts to map the cis-regulatory targets for PGE_2 action showed that the 20:4,n-6 and PGE_2 regulatory mechanism share similar elements. In contrast to the inhibition of S14 gene expression by peroxisome proliferator activated receptors (Ren et al., 1996), neither 20:4, n-6 or PGE_2 inhibited S14 gene expression through the thyroid hormone response region. Thus, PGE_2 does not have generalized effects on thyroid hormone regulation of this gene. Instead, the S14 proximal promoter (-220 to -80 bp) was the principal target for both PGE_2 and 20:4,n-6 suggesting that these two regulatory pathways converge on common elements within the S14 promoter to control its transcription. One key transcription factor regulating S14 within the PUFA/PGE_2 response region (-220 to -80 bp) is N-FY. NF-Y binds a Y-box at -104/-99 bp. It is a heterotrimeric transcription factor that is critical for the functioning of the 2 upstream enhancers (Jump et al., 1997b). Any mutation or substitution of this element essentially abrogates S14 gene transcription. Thus, factors

controlling NF-Y action impact on the transcriptional capacity of the S14 gene.

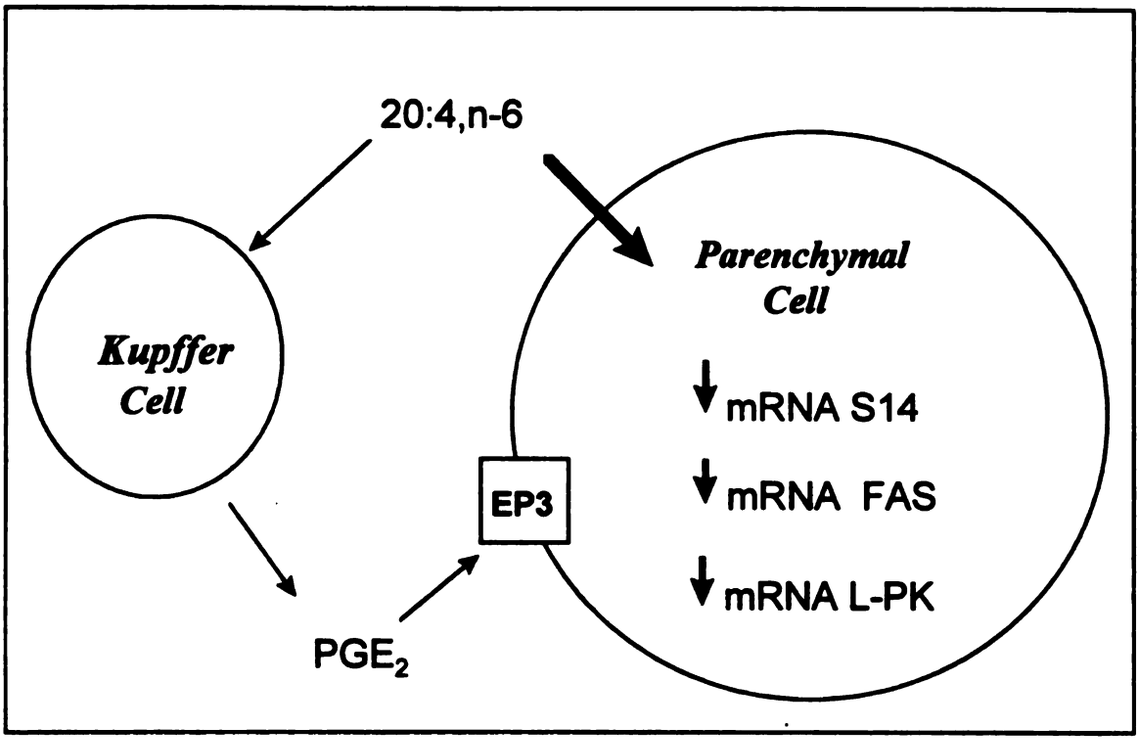


Figure 3.11. Hepatocyte and Kupffer Cell Interactions. This picture demonstrates the actions of PGE_2 and AA in the liver. Kupffer cells synthesize PGE_2 from AA. Both AA and PGE_2 can act independently on hepatocytes to change gene expression.

Here, I show that the promoter context dictates sensitivity of NF-Y to PUFA/PGE_2 control. NF-Y binds many promoters (Wright et al., 1995). The thymidine kinase promoter region at -115/-35 bp (Wagner et al., 1981), was used here as it binds NF-Y in the context of Sp1, to evaluate its role in PUFA/PGE_2 action. A reporter gene containing the TK NF-Y elements (R132) was insensitive to 20:4,n-6, 20:5,n-3 and PGE_2 (Figure 3.10). However, substituting the S14 PUFA-RR for the TK element conferred

both PUFA and PGE_2 control to the gene. The difference between these two elements is that in the TK promoter, NF-Y is flanked by Sp1 binding sites. No SP1 binding sites are found within the PUFA-RR. Thus, factors other than NF-Y that are present in the S14 PUFA-RR are critical for PUFA/PGE_2 control of S14 gene transcription. Studies are in progress to identify these other factors.

CONCLUSIONS

In summary, these studies have shown that 20:4,n-6 acts directly on hepatocytes to suppress lipogenic gene transcription. There is no requirement for cyclooxygenase activity for this control mechanism (Figure 3.1). However, specific prostanoids can act on the liver through EP3 and FP receptors to activate a G-protein linked signaling cascade that probably involves PLC, IP3 and calcium. In vivo, these prostanoids arise from non-parenchymal cells and act in a paracrine fashion on parenchymal cells to affect carbohydrate and lipid metabolism. Taken together with our previous studies on PPARs (Ren et al., 1997), fatty acids can regulate lipogenic gene transcription through 3 distinct pathways: one is PPAR-dependent, another is prostanoid-dependent and a third pathway is PPAR and prostanoid-independent. Given the paucity of PPAR in the human liver

(Gonzalez et al., 1997), this latter pathway is probably the operative pathway involved in the dietary PUFA suppression of hepatic lipogenic gene expression in both rodents and human under normal physiological conditions.

CHAPTER 4

CHAPTER 4

FUTURE EXPERIMENTS

PGE_2 was found to regulate lipogenic gene expression in both adipocytes (Chapter 2) and hepatocytes (Chapter 3). AA is converted to PG in adipocytes but not in hepatic parenchymal cells (hepatocytes). Thus, the PGE_2 must be derived from nonparenchymal cells to affect parenchymal cell gene expression. This finding led to interest in other non-parenchymal cell factors and how they might affect hepatic gene expression. Accordingly, I tested the TNFα and IL-1α in primary hepatocytes for suppression of S14 gene transcription.

This chapter will describe some preliminary experiments to answer the following questions:

1. Do the Kupffer cell products IL-1α and TNFα affect S14 expression in hepatocytes?

2. If so, where is the target in the S14 promoter for the effective compounds?

INTRODUCTION

Because prostaglandins were inhibitory to CAT activity in hepatocytes, this raised questions about the role other Kupffer cell products played in S14 gene expression. During sepsis or injury, Kupffer cells release a variety of interleukins, TNFα, prostaglandins, growth factors and nitric oxide, all of which can affect both glucose and lipid metabolism. The animal becomes hypertriglyceridemic and hyperglycemic then quickly hypoglycemic. Hepatic fatty acid synthesis and oxidation increase and lipolysis increases in adipocytes as a result of TNFα (Billiar and Curran, 1992). I was interested in determining how these products might change S14 expression in hepatocytes.

S14 promoter analysis indicated that the Y box and its flanking regions were necessary for inhibition by both AA and PGE_2 (Chapter 3). The second question will address the issue of what other compounds may target this same region in the S14 promoter. A similar target may be indicative of a similar co-factor involved in the suppression of S14 gene transcription.

MATERIALS AND METHODS

HEPATOCYTE PREPARATION AND CULTURE Hepatocytes were prepared as described in Chapter 3 and Appendix B.

RNA ISOLATION AND NORTHERN BLOTTING Total RNA was isolated, blotted and probed as described in Chapter 2 (Materials and Methods).

CAT ASSAYS Following treatments, cells were assayed for CAT activity and protein content as previously described (Jump et al., 1993). CAT Units: ^{14}C-acetylated chloramphenicol CPM/100 μg protein/hour.

RESULTS

TNFα, but not IL-1α, suppressed S14 gene transcription. Due to the inhibition of S14 by the Kupffer cell product, PGE_2, other Kupffer cell products were tested. TNFα, but not IL-1α, suppressed CAT activity in primary hepatocytes transfected with S14CAT124 (Figure 4.1). Because only TNFα showed an effect, a dose response curve was determined in hepatocytes treated with 0.5-10ng/ml of TNFα and CAT activity was measured. This result (Figure 4.2) determined that the ED_{50} = 1ng/ml TNFα in primary hepatocytes as

measured by CAT activity. However, RNA analysis indicated that 10ng/ml TNFα was required for 50% suppression of S14 mRNA (Figure 4.3). IL-1α had no effect on S14 mRNA levels (not shown). Other preliminary experiments indicate the mRNAs encoding PK and ApoC3 are also suppressed by TNFα at 10ng/ml (data not shown). Further effects of TNFα and its in vivo activator, LPS, on gene expression are currently underway in our laboratory.

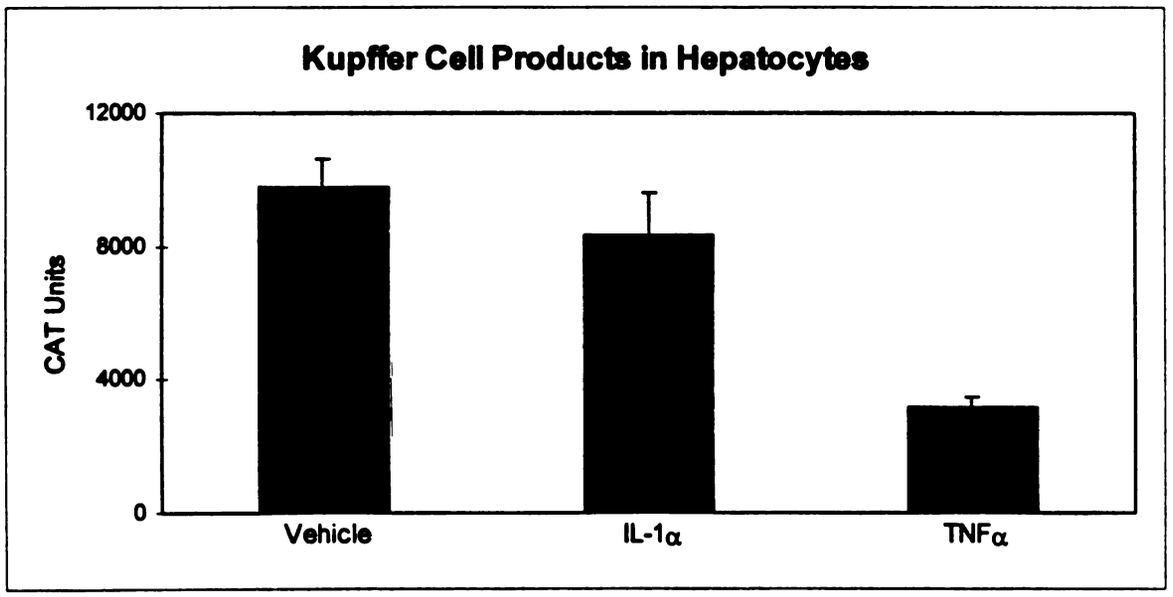


Figure 4.1. IL-1α and TNFα Effects on S14 CAT Activity. Primary hepatocytes transfected with S14CAT124 were treated with vehicle, 1ng/ml Il-1α or TNFα for 48 hours and CAT activity was determined. This is a representative graph of several experiments.

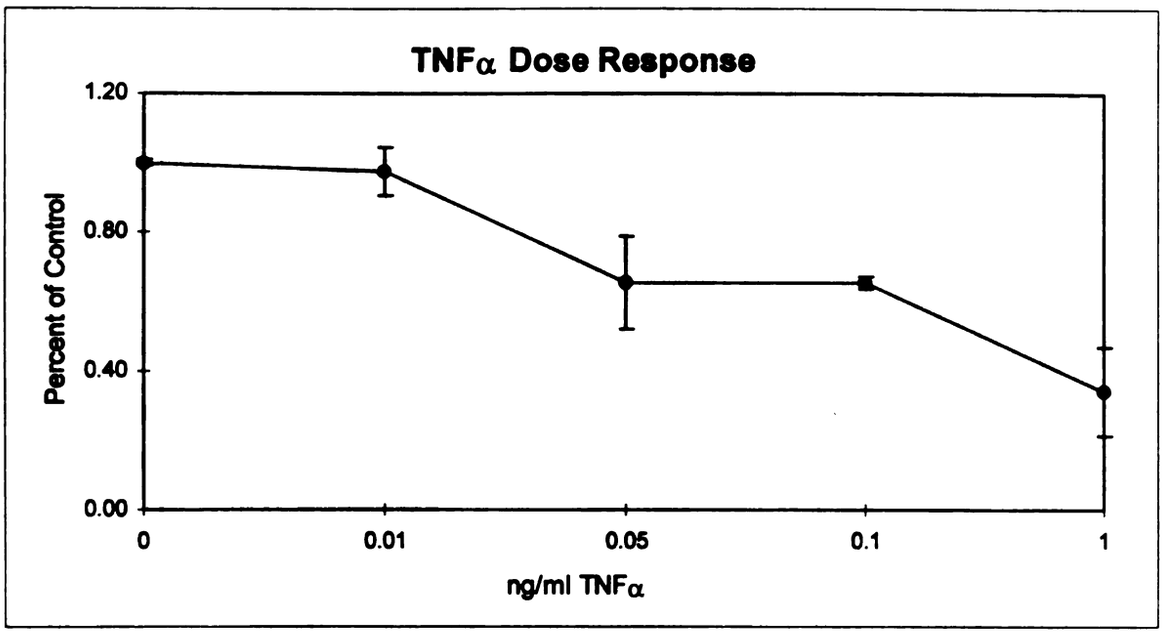


Figure 4.2. Dose Response of TNFα. Primary hepatocytes transfected with S14CAT124 were treated with TNFa at dose from 0 to 1ng/ml for 48 hours and CAT activity was determined. The data was normalized to the 0 dose value and percent error was determined. Each point represents the mean of 3 plates.

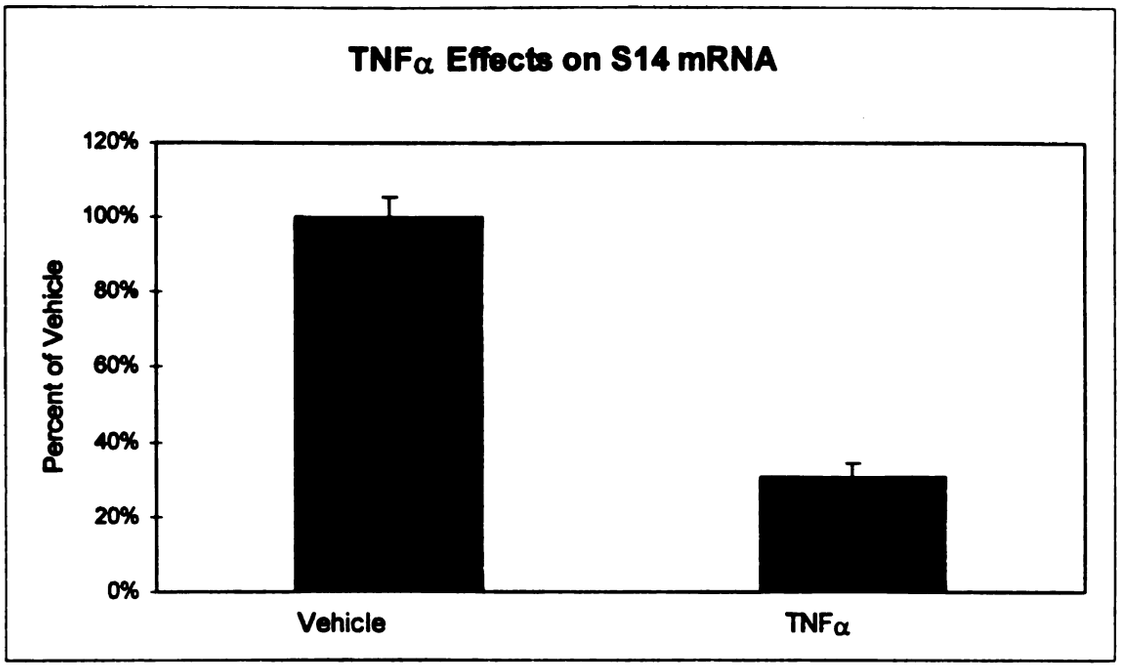


Figure 4.3. TNFα Effects on S14 mRNA. Primary hepatocytes were treated for 48 hours with vehicle or 10ng/ml TNFα and then total RNA was harvested and probed for S14 mRNA. This graph represents data pooled from 2 separate experiments each done with duplicate samples and percent error is shown.

TNFα targets the Y box. Because the target in the S14 promoter for both arachidonic acid and PGE_2 both involve the proximal promoter, I was curious about whether TNFα also targeted this region. TNFα was used to treat hepatocytes transfected with several different S14 promoter deletion constructs. These results are shown in Figure 4.4. TNFα showed good inhibition (70%) with S170 and 60% inhibition of both S155 and S156 and some effect on S158 (18%). There is also some inhibition of TK222, implying that the TRR and/or the TK element may be targeted by this compound. These results are complex but seem to indicate that the same

region targeted by AA and PGE_2, also is targeted by TNFα in the suppression of S14. However, further research will be necessary to determine if TNFα also targets other regions such as the TRR or the TK element.

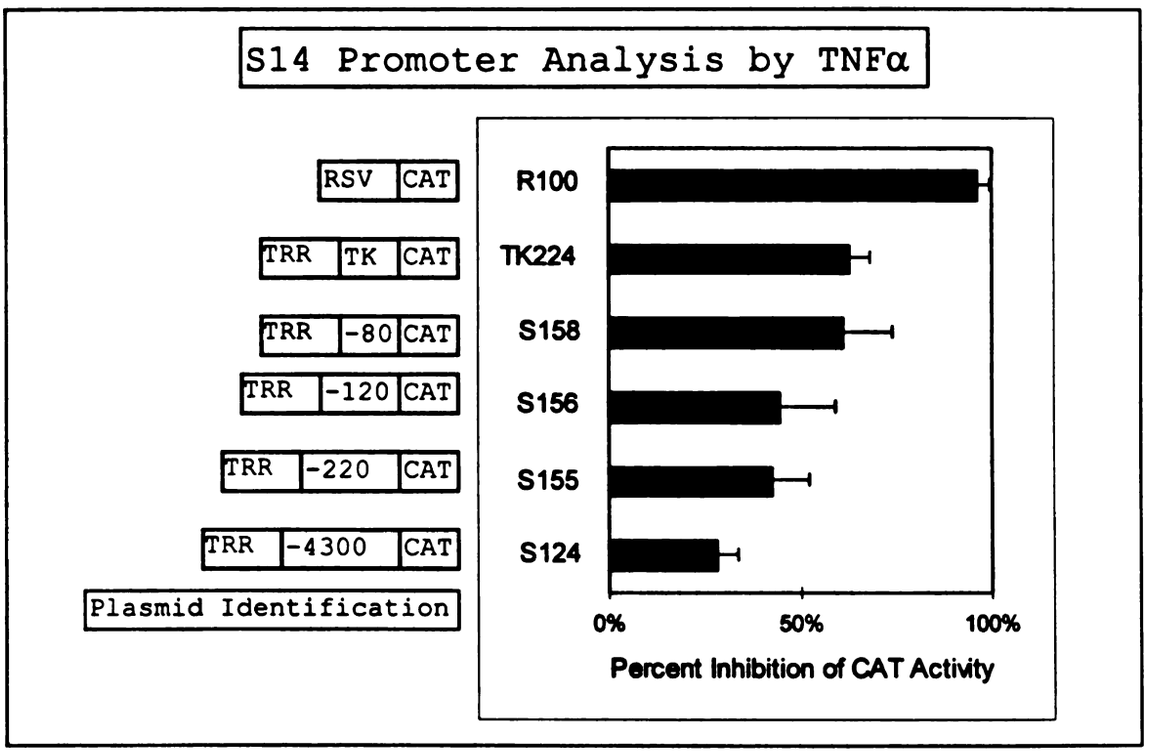


Figure 4.4. S14 Promoter Analysis on CAT Activity by TNFα. Hepatocytes were transfected with the above plasmids and treated for 48 hours with 1ng/ml TNFα. CAT activity was determined and normalized to the control treatment. This graph represents 3 separate experiments of combined data and percent error.

The RSV constructs used in Chapter 3 determined that PGE_2 and AA both required the Y box and the C-region and was promoter context specific. The same plasmids were used here to determine if TNFα also required the Y box and flanking regions. The results are shown in Figure 4.5. Unlike AA

and PGE_2, TNFα required only the Y box and not the flanking regions to give the full suppressive effect. R131, which contains the Y box and both the upstream and downstream regions, was suppressed 50% with TNF treatment as was R117, containing only the Y box. However, R116 also showed suppression by TNF (40%). Perhaps TNFα is acting through the Y box without requiring the flanking regions to aid in the inhibition. Both this result and Chapter 3 indicate that the Y box appears to play an extremely important role in the suppression of S14 transcription by a variety of factors.

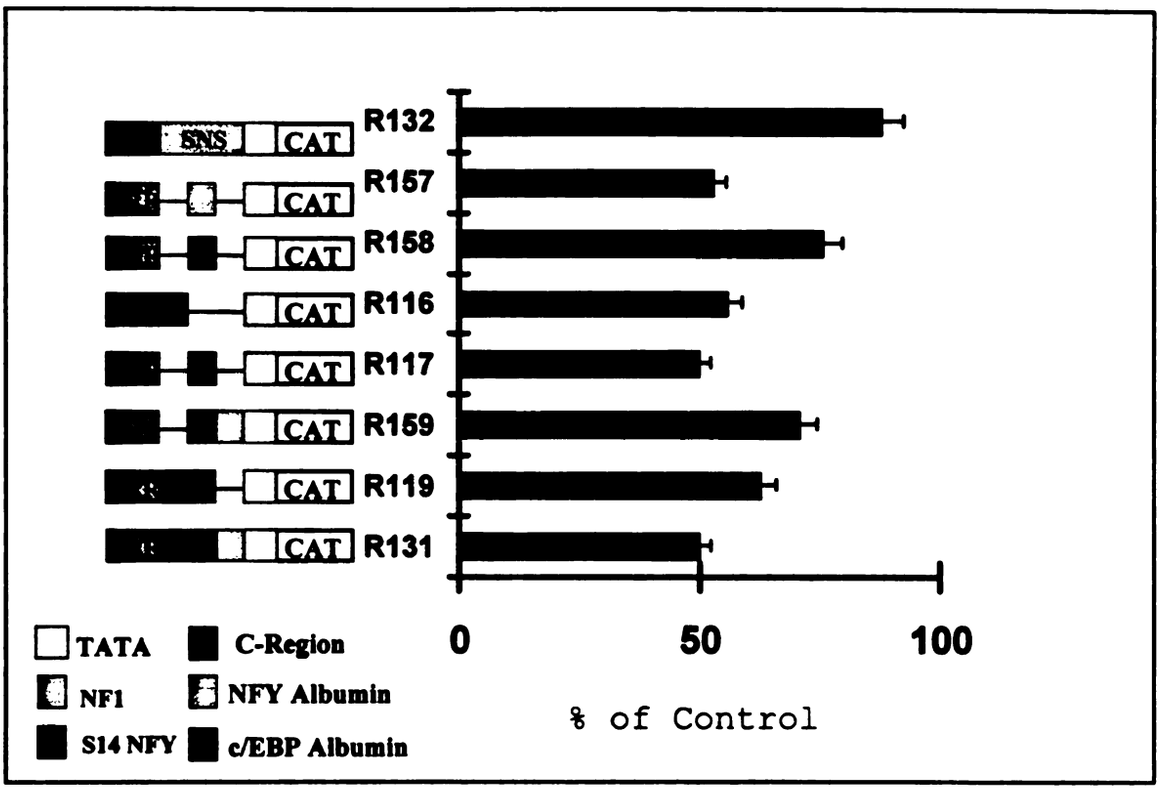


Figure 4.5. TNFα Effect on S14 Proximal Promoter Elements. Primary hepatocytes transfected with the above plasmids were treated for 48 hours with 1ng/ml TNFα. CAT activity was determined and normalized to the vehicle and presented as % of control. The above graph is pooled data from at least 3 separate experiments with percent error given. CAT activity was very low in cells transfected with R116 and R158.

DICUSSION

Question 1 asked what other Kupffer cell products change S14 expression. Of the cytokines tested, TNFα was the most inhibitory. This cytokine is also released in the liver as a result of sepsis or LPS stimulation. The actions of TNFα released from Kupffer cells and acting on the hepatocyte appear somewhat contradictory to the *in vivo* and

adipocyte data in the literature. In rats treated with TNFα, liver lipogenesis and serum triglycerides increase and LPL activity decreases (Grunfeld et al., 1990; Feingold et al., 1990). However, in hepatocyte cell culture, TNFα did not increase lipogenesis in experiments reported by Brass and Vetter (1994). I did not measure lipogenesis directly, rather, results shown in Figures 4.2 and 4.3 indicate that TNFα is inhibiting the lipogenic gene model (S14) mRNA expression in primary hepatocytes. My treatments are also chronic treatments (48 hours) compared to the above *in vivo* results, which are acute treatments. Thus, the reports that TNFα does not increase lipogenesis in hepatocytes and my results that S14 transcription is not increased by TNFα are similar.

The search for what mediates TNFα induction of lipogenesis has resulted in several reported possibilities: IL-1, IL-6, interferons and prostaglandins. Grunfeld et al. (1990) ruled out prostaglandins as a TNFα mediator but suggested that IL-6 could be involved. Brass and Vetter (1994) reported that IL-6 and a prostaglandin E agonist both increased hepatocyte lipogenesis. There also appears to be some interaction between the compounds released during sepsis.

Another mediator of TNFα is IL-1. IL-1 is a proinflammatory cytokine which binds a separate and distinct cell surface receptor from TNF. Both can cause similar effects, however. Several inducers of IL-1 such as LPS, phorbol esters, radiation and viruses also induce TNF. Kupffer cells also produce both of these cytokines, although monocytes produce much more IL-1. IL-1 binds a receptor which causes activation of a MAP kinase also activated by LPS. Blocking this MAP kinase phosphorylation can stop production of TNF and IL-1. There is some evidence that this MAP kinase phosphorylation involves a Janus kinase (JNK) or Stat pathway. IL-1 can also increase prostaglandin synthesis (Tocci and Schmidt, 1997). However, my results with IL-1 on S14 transcription do not indicate that this compound is mimicking TNFα or that IL-1α is a mediator of TNFα or PGE_2.

TNFα has a cell surface receptor belonging to the cytokine family. Although no attempts were made to reverse the TNFα suppression of S14 CAT activity, it is likely that this compound acts through its receptor to activate a JAK/STAT phosphorylation cascade, perhaps activating some mediator require for gene suppression. One such possible mediator is NF-κB, another is sphingomyelin (see literature review). It is not known if NF-κB binds the S14 promoter or

if ceramide is suppressive to S14. Further research will be necessary to determine how TNF suppresses S14.

These results show that AA, PGE_2, and TNFα have at least two separate targets each in the S14 promoter, one of which the Y box. I have focused on the Y-box region and determined that this suppression requires the surrounding regions and is promoter context specific. The Y-box alone does not result in more than 25% suppression of CAT activity by any compound tested but adding the flanking regions results in at least 2/3 of the total suppression seen with the full length promoter when using AA, PGE_2 or TNFα. The other portion in the S14 promoter necessary for the suppression by these agents remains to be determined.

During sepsis or liver injury, Kupffer cells release many different compounds. I did not test these compounds in any combinations except PGE_2 and TNFα. Separately they inhibited CAT activity about 50% and together inhibition was 75% (data not shown). This result indicates that they are additive and probably operating through distinct regulatory mechanisms. Other combinations of cytokines and prostaglandins have not been attempted with this system. There is an abundance of literature indicating several complex relationships between Kupffer cell products, and much more research will have to done before scientists can hope to understand these interactions.

CONCLUSIONS AND FUTURE DIRECTIONS

ADIPOCYTES: Although results show that in adipocytes, AA acts through prostaglandins which involve a G-protein signaling mechanism to suppress S14, what that G-protein system is regulating is still unclear. While calcium seems to be involved in the signaling, that has not been definitively proven here. Perhaps the inhibitors tried were not effective or perhaps there are other agents that would be better utilized for blocking intracellular calcium release. Intracellular calcium changes were not measured and experiments set up to do so may be necessary to verify calcium involvement.

I also did not test reversal of EPA suppression in adipocytes by the cyclooxygenase inhibitor. EPA can be metabolized to prostanoids, although this pathway is more prevalent with AA. It would be interesting to know if flurbiprofen could reverse S14 suppression in EPA treated adipocytes.

HEPATOCYTES: In the hepatocyte experiments, no inhibitor reversed the PGE_2 or AA inhibition of S14 gene transcription. Again, the inhibitors may not have been effective. Pertussis toxin has reversed PGE_2 effects in hepatocytes in other work (see literature review) but could not in my system. Perhaps the dose or time of treatment I

used was not correct, though several doses and times were attempted. However, if PGE_2 is acting through a different pathway than those reported in the literature for S14 suppression, pertussis toxin may not be an effective agent and other signaling pathway inhibitors should be used. While PG were inhibitory to S14 in hepatocytes, further research will have to be done to determine how they are acting to cause suppress of transcription.

Arachidonic acid also acts to suppress S14 transcription and does so independently of PG and PPARs. Yet the many inhibitors of arachidonic acid metabolism, oxidation and blockers of signaling pathways could never reverse this inhibition in hepatocytes. This seems to imply that the fatty acid is directly changing transcription, but how? Is AA changing binding of a co-factor, and if so, what is this co-factor? Is AA inhibiting the binding of a known transcription factor that normally binds and if so, which one? Perhaps it is an unknown transcription factor that has reduced binding when PUFA are present. Considering how complex transcriptional control has become as more research is done, it is likely that there is more than one factor involved. Again, further research will have to be completed to answer these questions about PUFA control of gene transcription.

Although preliminary, it is very interesting that the cytokine TNFα, which is released from Kupffer cells in the liver, also suppresses S14 gene transcription. LPS or sepsis is known to increase lipogenesis, yet TNFα depressed S14 gene transcription in hepatocytes. While this may be due to differences in treatment times and cell types, it is a very curious observation. For example, would diet change this effect or influence it? Is the sepsis induced hypertriglyceridemia in animals or humans harmful and could it be changed by drugs or diet in patients?

The promoter targeting results demonstrate importance of the Y box in controlling the transcription of S14. While the target in the S14 promoter has been narrowed to the Y box and its flanking region, the co-factor involved in the inhibition is still unknown. Do AA, PGE_2 and TNFα directly interact with the DNA binding at Y box or is there a common factor they interact with? Determination of this common factor, if indeed one exists, may prove to answer the above questions about how AA and PGE_2 are suppressing this gene. Current efforts are underway in our laboratory to address this question.

In conclusion, this dissertation has shown that in adipocytes, AA suppression is through a prostanoid pathway linked to a G-protein signaling system. I have also demonstrated that AA, PGE_2 and TNFα inhibit S14 gene

transcription via the Y-box and its flanking regions in hepatocytes, and that AA acts in a prostanoid and PPAR independent pathway.

APPENDIX A

APPENDIX A

Solutions for growth of 3T3-L1 cells

1. DMEM: Gibco (Gaithersburg, MD), 1 package DMEM or 13.37g, 3.7g $NaHCO_3$, 4.8g HEPES (20mM final concentration), dissolve, adjust to pH 7.0, volume to 1 liter, filter sterilize in hood, store at 4 C.

2. Calf Serum: Gibco, Heat inactivated for 45 minutes at 56 C.

3. Fetal Bovine Serum: Atlanta Biologicals (Atlanta, GA)

4. Penicillin/Streptomycin Solution: Sigma Chemicals (St. Louis, MO), 100KU/ml of penicillin and 10mg/ml streptomycin.

5. Insulin: Gibco, 1mM insulin in 0.2M HCl-0.1M NaCl, filter sterilized, stored at 4 C.

6. IBMX: Sigma Chemicals, 100mM IBMX in 0.1N NaOH, filter sterilized, stored at 4 C.

7. Dexamethasone: Sigma, 10mM dexamethasone in DMSO, filter sterilized, stored at 4 C.

8. Albumin: Boehringer Mannheim, 6.8 g of fatty acid free bovine serum albumin dissolved in 50 ml of DMEM, filter sterilize (.45micron filter), store at 4 C.

9. Phosphate Buffer Saline (PBS): see Molecular Cloning (Maniatis vol. 3.)

10. 10X Trypsin EDTA: Sigma, diluted to 1X in PBS.

11. Geneticin: Gibco, 100mg/ml geneticin in water, filter sterilized, stored at -20 C.

Solutions for transfection of 3T3-L1 cells

1. 0.25 M $CaCl_2$: 3.675 g $CaCl_2$ in 100 ml glass distilled water

2. 2X-BSS: 50mM BES, pH 6.95, 280 mM NaCl, 1.5mM Na_2HPO_4 For 100ml solution: add BES 1.066g and 1.64 g NaCl to 50 ml glass distilled water and adjust pH to 6.95 with HCl then fill to volume of 90ml. Make Na_2HPO_4 (0.212g/100ml water) and add 10ml to BES and NaCl solution to get 1.5mM Na_2HPO_4.

Solutions for RNA and Northern blots

1. 1% Large gel (250ml): Dissolve 2.5 g agarose, 25 ml 10X MOPS and 245 ml water by boiling then cool to 60 C. In the hood, add 7.5ml formaldehyde while stirring and immediately pour then allow to gel.

2. Running buffer: 1X MOPS

3. 10X MOPS: 0.2M MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0, filter sterilize and/or autoclave.

4. RNA Sample Preparation: Dissolve 10-20 RNA in 5µl TE-8 and add 25µl electrophoresis sample buffer (made fresh). Mix and incubate at 65 C for 15 minutes then add 1µl 1mg/ml EtBr (this is a 1:10 dilution of the usual EtBr). Mix and quick spin and load sample.

5. Electrophoresis Sample Buffer: 0.75ml deionized formamide, 0.15ml 10X MOPS, 0.24ml formaldehyde, 0.1ml water, 0.1ml glycerol, 0.08ml 10% (w/v) bromophenol blue.

6. 10X SSC: see Molecular Cloning (Maniatis) vol 3.

7. 10% SDS: see Molecular Cloning (Maniatis) vol 3.

8. Prehyb/hybridization solution: 50% deionized formamide, 0.5X Denhardts, 5X SSC, 0.1% SDS, 50mM sodium phosphate buffer pH 6.5, 0.1mg/ml salmon sperm DNA (boiled before addition), 0.25mg/ml tRNA (boiled before addition) and water to volume.

9. cDNA Probe was made according to Gibco's protocol, cleaned with spin columns from 3 Prime 5 Prime (Boulder, CO) and boiled for 10 minutes. The probes were added to hybridization solution at 2 million cpm/ml and allowed to hybridize overnight.

Solutions for Gel Shifts

1. TEN (Tris-EDTA-NaCl): see Molecular Cloning (Maniatis vol. 3).

2. Polynucleotide kinase buffer:500mM Tris-Cl, pH 7.5; 100mM $MgCl_2$; 1mM spermidine and 1mM EDTA. Store at -20 C.

3. 100mM DTT:Store at -20 C.

4. 100mM $MgCl_2$: Store at -20 C.

5. Poly dI:dC: resuspended in TE-8 and add NaCl to 0.1M at a final concentration of 2mg/ml. Heat to 90 C for 10 minutes and cool slowly to room temperature. Store at -20 C.

6. Nuclear protein dialysis buffer: 25mM Tris-Cl, pH 7.5; 40mM KCl, 0.1mM EDTA and 10% glycerol.

7. 8% acrylamide gel:16ml 30% acrylamide-bisacrylamide; 1.5ml 10X TBE, pH 8.3; 0.3ml 20% NP-40 and 41.9ml GD-water. Mix and degas. Add 300µl 20% ammonium persulfate (made fresh) and 60µl TEMED. Mix gently and pour into casting stand. Let the gels polymerize for 2 hours and store at 4 C wrapped in plastic wrap.

8. TBE (Tris-Borate-EDTA): see Molecular Cloning (Maniatis vol. 3).

Solutions of nuclei isolation & nuclear protein preparation

1. Solution A: 20mM Tris-Cl, pH 7.5; 2mM $MgCl_2$.

2. Buffer A: 10mM Tris-Cl, pH 7.5; 5mM $MgCl_2$; 320mM sucrose;
0.2%nonidet P-40; 1mM DTT*; 1mM PMSF*.

3. Buffer B: 10mM Tris-Cl, pH 7.5; 5mM $MgCl_2$; 880mM sucrose; 0.2%nonidet P-40; 1mM DTT*; 1mM PMSF*.

4. Buffer C: 10mM Tris-Cl, pH 7.5; 5mM $MgCl_2$; 250mM sucrose; 0.2%nonidet P-40; 1mM DTT*; 1mM PMSF*.

5. Buffer D: 50mM HEPES, pH 7.5; 5mM $MgCl_2$; 60µM EDTA; 40% glycerol; 1mM DTT*; 1mM PMSF*.

6. Nuclear lysis buffer: 10mM HEPES, pH 7.6; 100mM KCl; 3mM $MgCl_2$; 0.1mM EDTA; 10% glycerol; 1mM DTT*; 1mM PMSF*.

7. 4 M $(NH_4)_2SO_4$ pH 7.9

8. Nuclear protein dialysis buffer: 25mM Tris-Cl, pH 7.5; 40mM KCl, 0.1mM EDTA and 10% glycerol; 1mM DTT*; 1mM PMSF*.

* Autoclave all solutions for 20 minutes then add DTT and PMSF at time of use.

APPENDIX B

APPENDIX B

Hepatocyte Preparation Protocol

Required items

1. operating tools
2. gauze pads
3. 500ml beaker w/ stir bar (autoclaved)
4. 500ml beaker w/ funnel (autoclaved)
5. silastic tubing
6. hemacytometer
7. sutures (in ethanol)
8. elastic bands
9. bubblers (autoclaved)
10. Hepatocyte filter UV/ethanol sterilized
11. catheter
12. syringes/needles
13. heparin
14. Methoxyflurane
15. water bath at 42°C
16. peristaltic pump
17. O_2/CO_2 gas
18. Betadine™
19. Bottle top filters

Prepared Items

IT IS IMPERATIVE THAT THE BUFFERS ARE PERFECT AND THE pH IS CORRECT FOR GOOD HEPATOCYTE PREPS. ABSOLUTELY NO SOAP IS TOLERATED, SO WASH THE GLASSWARE CAREFULLY.

Perfusion Buffer I (PB-I)

For preparing 1 Liter of 10X Buffer

1.42 M NaCl	82.9 g
.067 M KCl	5.0 g
0.1 M HEPES	23.8 g

Dissolve in ddH_2O, pH to 7.4
Filter

Preparing 400 ml of 1X Buffer
Filter the following into sterile bottle
360 ml ddH_2O
40 ml 10X PB-I

Perfusion Buffer II (PB-II)

Preparing 2 Liters of PB-II

66.7 mM NaCl	7.8 g NaCl or 33.4 ml 4 M NaCl
6.7 mM KCl	1.0 g KCl or 4.6 ml 3 M KCl
100 mM HEPES	47.66 g HEPES
4.8 mM $CaCl_2 \bullet 2H_2O$	1.41 g $CaCl_2 \bullet 2H_2O$

Dissolve in ddH_2O, pH to 7.6
Filter sterilize in 400 ml aliquots into sterile bottles

Liberase-Albumin Solution

Stock Liberase comes in 70mg aliquots. Add 10ml PB-II to bottle, shake gently to dissolve, let sit on ice for 10 minutes then aliquot into 10 glass vials (1 ml each). Store at -20°C. On the day of hepatocyte preparation, remove 1 vial and let it thaw on ice for at least 10 minutes. Previously albumin was used in PB-II but this is optional. Liberase works without the albumin. Once the PB-II has gassed 30 minutes, pour PB-II into this beaker. Before filtering, add the 1ml of Liberase. If albumin is used, the Liberase should not be added until most of the albumin is dissolved to prevent loss of activity of the Liberase. The Liberase should always be on ice or left frozen until it is used.

Percoll

Preparing the Percoll Stock
90 ml Percoll
10 ml sterile 10X PBS
1 ml 1 M HEPES, pH 7.4

Store at 4°C.

Williams E Media

Preparing 1 Liter
1 package Williams E media powder
2.2 g sodium bicarbonate
5.5 g HEPES
12 ml penicillin/streptomycin antibiotic
1.5ml 5 N NaOH
one of the following:
- 1.43 ml lactate (for 10mM lactate)
- 4.5 g glucose (for 25mM glucose

Plating Media - 10mM lactate in Williams E Media + 10% FBS

Transfection Media - 10mM lactate in Williams E Media + 200nM insulin + 10nM dexamethasone

Treatment media - 10mM lactate or 25mM glucose in Williams E + 1μM insulin + 10nM dexamethasone

Protocol

The day before

1. Starve a cage of rats. Use the "do not feed" tape on the cage so ULAR doesn't feed them. Starve at least one more rat than what is planned on being used.
2. Turn on the blower on the hood. Place a filter (1 per rat) in the glass dish with 70% ethanol covering it. UV light should be ON.
3. Make sure all the proper equipment is autoclaved.
4. Prepare at least 500ml of 10% FBS/Williams E 10mM Lactate media per rat. Williams E 10mM media will also be needed for transfection as well as treatment media.
5. Make Perfusion buffers I and II are ready.

The day of preparation

1. Turn UV light to regular light in the hood. Turn on and light gas.
2. Spray off PB-I and PB-II bottles w/ ethanol and place a sterile bubbler in each bottle. Place weight around bottle neck. Wrap top w/ sterile tin foil. Place in the waterbath set at 42°C.
3. Gas each w/ 95%O_2/5%CO_2 for 30 minutes. The tubing with clamp should be used for PB-II.
4. Get 2 buckets of ice. Get the rat cage from ULAR.
5. Weigh out Albumin (1g/100ml PB-II) into glass beaker with the stir bar. Place it back in the hood on stir plate. (THIS IS NOT REQUIRED WITH THE LIBERASE PROTOCOL)
6. Prepare 0.5ml heparin in syringe. Prepare orange cap tube w/ methoxyflurane in dryer hose on surgery table. Prepare surgery table and utensils.
7. Rinse tubing on pump with 70% ethanol and drain.
8. By now the gassing should be complete, clamp off PB-II tubing, remove bottle and pour PB-II into beaker (w/ the albumin if using this) in the hood. Allow the albumin to dissolve, then add the Liberase aliquot (1ml). Once this is complete, sterile filter back into the same bottle. Place the weight back over the top, rewrap w/ tinfoil and put back into the water bath.

9. Wash the pump tubing carefully with 70% ethanol. DO NOT TOUCH WITH HANDS AFTER THE TUBING IS WASHED. Carefully place the tubing into the buffers. The longest tubing goes into PB-II bottle. Turn on pump and pull up PB-II to the joint, then pump PB-I through tubing. PB-I should be coming out the other end with no bubbles.
10. Weigh the rat. Place him in the sock. Wrap him up with the towel bending his tail up toward his head. This will prevent him from going backwards.
11. Place his head into the tubing about to his eyes. Gently but firmly hold his head in the tube until he is unconscious. Carefully remove towel and sock. Fasten legs w/ rubber bands to surgery table (another person to assist with this step is handy). Keep his head partially in the tube throughout procedure. Monitor his breathing and adjust tube to keep him alive but still unconscious.
12. Inject ~500µl heparin into the tail vein.
13. Shave belly from groin to sternum. (The better shaved he is the easier it is to cut the skin.)
14. Wash thoroughly with Betadine solution. Rinse with 70% ethanol. Be generous with each.
15. Make sure the rat is breathing and everything is still ready before cutting.
16. Make a small incision in the middle lower portion of his belly. Carefully detach skin from muscle layer below. Cut toward his sides from the middle and angle slightly superior as you cut transversely. The cut should resemble a "V". Once you have loosened the skin sufficiently, use the large hemostats to pull back the skin. Further loosen and cut skin until it is pulled back from the muscle layer all the way around the rib cage.
17. Rinse muscle layer with ethanol. Be careful! The ethanol is cold to the rat and he takes a deep breath. A deep breath means more anesthetic, which can kill him. Adjust tube until his breathing returns to normal.
18. Again, make a medial incision at the base of his belly. Don't go too deep, preventing any slicing of internal organs! Make a cut toward each side and up to his front legs, angling upward from the middle point (cut both sides). Use the small hemostats to hold back the muscle layer. It should slide over the ribs and stay there. Make sure the cut is low enough (toward the table) so blood can run out later.
19. Push back the organs with a piece of gauze. The hepatic vein should be apparent. Adjust organs and gauze so the vein is easy to access.

20. Using the small tweezers, place a piece of thread on the rat so one end is near the vein. Carefully make a hole with the tweezers in your left hand and pull the thread under the vein. Make a LOOSE knot.
21. Make sure the tubing is ready and not twisted. Lay it close to the vein, usually on the gauze covering the organs.
22. Cannulate the vein. Tighten the knot. Turn pump on to 1 and gently attach tubing to catheter. Turn up pump to 4, simultaneously clipping the main vein going down his backbone.
23. The liver should perfuse immediately. The muscle or skin layer may have to be cut further to allow good drainage. Using the spatula, carefully make sure all the lobes are perfusing. DON'T BUMP THE CATHETER!
24. After PB-I is mostly through the liver, turn pivot so PB-II is perfused through. Perfusion speeds and amounts depends on the rat and many other things. Watch the liver, don't let it get too mushy.
25. 4 100mM petri dishes will be needed. Place 3 in the hood and one next to the rat's head. Once perfusion is finished, remove liver, place it in the petri dish and carry it to the hood. Take the large forceps to the hood as well.
26. Add ice cold plating media to each dish and rinse the liver three times in successive dishes.
27. Use a comb to break up the liver in the 4^{th} dish. Hold the liver with the forceps and comb to until the liver is demolished.
28. Place filter over beaker with the funnel. Carefully place the combed liver solution in the filter and allow it to filter through. There will be some debris that will not go through. Rinse dish w/ a small amount of media. Rinse filter.
29. Place contents of beaker into a 50ml orange cap tube. Spin for 10 minutes at 50 g. Keep the tube on ice at all times from this point on.
30. Make 4 tubes of Percoll Stock-Media solution. (10mls of each in each tube = 20mls per tube). (Start cleaning up the mess.)
31. Dump off supernatant from tube and resuspend pellet in 10-15ml media. Once the pellet is COMPLETELY resuspended, fill tube to 40mls with media. Place 20mls in each of 2 Percoll-Media tubes on top of the Percoll-media mix. Do NOT mix the layers. Spin 10 minutes at 270 g.
32. Remove supernatant and interface, saving the pellet. Repeat step 31.
33. Remove supernatant and resuspend pellet in 25ml media. Centrifuge 5 minutes at 50 g.

34. Dump supernatant and resuspend pellet in 30mls.
35. Count the cells (# of plates = average # of cells per grid if 30mls was used to resuspend the pellet).
36. Plate cells (3 million/60mm plate or 10million/100mm plate)
37. Transfect about 4 hours later.

Transfection Protocol

1. Prepare transfections in blue capped tube = 1ml lactate media + 2µg reporter + 1µg receptor + 6.6µl lipofectin per plate.
2. Let preparation sit 20-30 minutes after mixing gently.
3. Rinse plates with 2ml PBS.
4. Add 2ml lactate media.
5. dd 1ml transfection mix.

REFERENCES

REFERENCES

Aarsland, A.M., M. Lundquist, B. Borreston and R.K. Berg. 1990. On the effect of peroxisomal β-oxidation and carnitine palmitoyltransferase activity by eicosapentaenoic acid in liver and hearts from rats. Lipids 25:546-548.

Adachi, T., S. Nakashima, S. Saji, T. Nakamura and Y. Nozawa. 1997. Possible involvement of pertussis toxin-sensitive G protein in hepatocyte growth factor-induced signal transduction in cultured rat hepatocytes: pertussis toxin treatment inhibits activation of phospholipid signaling, calcium oscillation, and mitogen-activated protein kinase. Hepatology 26:295-300.

Adachi, T., S. Nakashima, S. Saji, T. Nakamura and Y. Nozawa. 1995. Roles of prostaglandin production and mitogen-activated protein kinase activation in hepatocyte growth factor-mediated rat hepatocyte proliferation. Hepatology 21:1668-1674.

Adams, M., M.J. Reginato, D. Shao, M.A. Lazar and V.K. Chatterjee. 1997. Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. Journal of Biological Chemistry 272:5128-5132.

Adi, S., A.S. Pollock, J.K. Shinenaga, A.H. Moser, K.R. Feingold and C. Grunfeld. 1992. Role for monokines in the metabolic effects of endotoxin. Journal of Clinical Investigations 89:1603-1609.

Ailhaud, G., E. Amri, S. Bardon, S. Barcellini-Couget, B. Bertrand, R. M. Catalioto, C. Dani, A. Doglio, C. Forest, D. Gaillard, P. Grimaldi, A. Ibrahimi, R. Negrel, C. Vannier and G. Vassaux. 1991. Growth and differentiation of regional adipose tissue:molecular and hormonal mechanisms. International Journal of Obesity 15:87-90.

Ailhaud, G., E.-Z. Amri and P. Grimaldi. 1995. Fatty acids and adipose cell differentiation. Prostaglandins Leukotrienes and Essential Fatty Acids 52:113-115.

Ailhaud, G., E.-Z. Amri and P. Grimaldi. 1996. Fatty acids and expression of lipid-related genes in adipose cells. Proceedings of the Nutrition Society 55:151-154.

Ailhaud, G., P. Grimaldi and R. Negrel. 1992. Cellular and molecular aspects of adipose tissue development. Annual Review of Nutrition 12:207-233.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J.D. Watson. 1994. Molecular Biology of the Cell, 3rd ed., Garland Publishing, Inc., New York, New York.

Amri, E.-Z, G. Ailhaud and P. Grimaldi. 1991. Regulation of adipose cell differentiation. II. Kinetics of induction of the aP2 gene by fatty acids and modulation by dexamethasone. Journal of Lipid Research 32:1457-1463.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J.D. Watson. 1994. Molecular Biology of the Cell, 3rd ed., Garland Publishing, Inc., New York.

Allmann, D.W. and D.M. Gibson. 1965. Fatty acid synthesis during early linoleic acid deficiency in the mouse. Journal of Lipid Research 6:51-62.

Amri, E.-Z., G. Ailhaud and P.A. Grimaldi. 1994. Fatty acids as signal transducing molecules:involvement in the differentiation of preadipose to adipose cells. Journal of Lipid Research 35:930-937.

Amri, E.-Z., B. Bertrand, G. Ailhaud and P. Grimaldi. 1991. Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression. Journal of Lipid Research 32:1449-1456.

Amy, C.M., B. Williams-Ahlf, J. Naggert and S. Smith. 1990. Molecular cloning of the mammalian fatty acid synthase gene and identification of the promoter region. Biochemical Journal 271:675-679.

Amy, C.M., B. Williams-Ahlf, J. Naggert and S. Smith. 1992. Intron-exon organization of the gene for the multifunctional animal fatty acid synthase. Proceedings of the National Academy of Sciences 89:1105-1108.

Armstrong, M.K., W.L. Blake and S.D. Clarke. 1991. Arachidonic acid suppression of fatty acid synthase gene expression in cultured rat hepatocytes. Biochemical and Biophysical Research Communications 177:1056-1061.

Asboth, G., S. Phaneuf, G.N. Europe-Finner, M. Toth and A.L. Bernal. 1996. Prostaglandin E_2 activates phospholipase C and elevates intracellular calcium in cultured myometrial

cells:involvement of EP1 and EP3 receptor subtypes. Endocrinology 137:2572-2579.

Aubert, J., G. Ailhaud and R. Negrel. 1996. Evidence for a novel regulatory pathway activated by (carba)prostacyclin in preadipose and adipose cells. FEBS Letters 397:117-121.

Avasthy, N., J.Y. Jeremy and P. Dandona. 1988. The role of calcium in mediating phorbol ester- and insulin-stimulated adipocyte lipogenesis. Diabetes Research 9:91-95.

Beaumier, L., N. Faucher and P.H. Naccache. 1987. Arachidonic acid-induced release of calcium in permeablized human neutrophils. FEBS Letters 221:289-292.

Belury, M.A., K.E. Patrick, M. Locniskar and S.M. Fischer. 1989. Eicosapentaenoic and arachidonic acid:comparison of metabolism and activity in murine epidermal cells. Lipids 24:423-429.

Bennet, M.K., J.M. Lopez, H.B. Sanchez and T.F. Osborne. 1995. Sterol Regulation of fatty acid synthase promoter. Journal of Biological Chemistry 270:25578-25583.

Bernlohr, D.A., M.A. Bolanowski, T.J. Kelly and M.D. Lane. 1985. Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 260:5563-5567.

Berven, L.A. and G.J. Barritt. 1994. A role for a pertussis toxin-sensitive trimeric G-protein in store-operated Ca^{2+} inflow in hepatocytes. FEBS Letters 346:235-240.

Berven, L.A., M.F. Crouch, F. Katsis, B.E. Kemp, L.M. Harland and G.J. Barritt. 1995. Evidence that the pertussis toxin-sensitive trimeric GTP-binding protein G_{i2} is required for agonist-and store-activated Ca^{2+} inflow in hepatocytes. Journal of Biological Chemistry 270:25893-25897.

Berven, L.A., B.P. Hughes and G.J. Barritt. 1994. A slowly ADP-ribosylated pertussis-toxin-sensitive GTP-binding regulatory protein is required for vasopressin-stimulated Ca^{2+} inflow in hepatocytes. Biochemical Journal 299:399-407.

Billiar, T.R. and R.D. Curran, eds. Hepatocyte and Kupffer Cell Interactions. Boca Raton, Florida: CRC Press, 1992.

Billiar, T.R., T.W. Lysz, R.D. Curran, B.G. Bentz, G.W. Machiedo and R.L. Simmons. 1990. Hepatocyte modulation of Kupffer cell prostaglandin E_2 production in vitro. Journal of Leukocyte Biology 47:304-311.

Bjornsson, O.G., J.D. Sparks, C.E. Sparks and G.F. Gibbons. 1992. Prostaglandins suppress VLDL secretion in primary rat hepatocyte cultures:relationships to hepatic calcium metabolism. Journal of Lipid Research 33:1017-1027.

Blake, W.L. and S.D. Clarke. 1990. Suppression of rat hepatic fatty acid synthase and S14 gene transcription by dietary polyunsaturated fat. Journal of Nutrition 120:1727-1729.

Blobe, G.C., W.A. Khan and Y.A. Hannun. 1995. Protein Kinase C: Cellular target of the second messenger arachidonic acid? Prostaglandins Leukotrienes and Essential Fatty Acids 52:129-135.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Braissant, O., R. Foufelle, C. Scotto, M. Dauca and W. Wahli. 1996. Differential expression of peroxisome proliferator activated receptors (PPARs): tissue distribution of PPAR-α, -β and -γ in the adult rat. Endocrinology 137:354-366.

Brasier, A.R., D. Ron, J.E. Tate and J.F. Habener. 1990. A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1α induced, NFκB mediated trans-activation of the angiotensinogen gene acute-phase response element. EMBO Journal 9:3933-3944.

Brass, E.P. and W.H. Vetter. 1994. Interleukin-6, but not tumor necrosis factor-α, increases lipogenesis in rat hepatocyte primary cultures. Biochemical Journal 301:193-197.

Brown, S.B., M. Maloney and W.B. Kinlaw. 1997. "Spot 14" protein functions at the pretranslational level in the regulation of hepatic metabolism by thyroid hormone and glucose. Journal of Biological Chemistry 272:2163-2166.

Brun, R.P., J.B. Kim, E. Hu, S. Altiok, B.M. Spiegelman. 1996a. Adipocyte differentiation: a transcriptional

regulatory cascade. Current Opinion in Cell Biology 8:826-832.

Brun, R.P., P. Tontonoz, B.M. Forman, R. Ellis, J. Chen, R.M. Evans and B.M. Spiegelman. 1996b. Differential activation of adipogenesis by multiple PPAR isoforms. Genes and Development 10:974-984.

Butta, N., E. Urcelay, C. Gonzalez-Manchon, R. Parrilla and M.S. Ayuso. 1993. Pertussis toxin inhibition of α_1-adrenergic or vasopressin-induced Ca^{2+} fluxes in rat liver. Journal of Biological Chemistry 268:6081-6089.

Butterwith, S.C. 1994. Molecular events in adipocyte development. Pharmacological Therapeutics 61:399-411.

Camandola, S., G. Leonarduzzi, T. Musso, L. Varesio, R. Carini, A. Scavazza, E. Chiarpotto, P.A. Baeuerle and G. Poli. 1996. Nuclear factor κB is activated by arachidonic acid but not by eicosapentaenoic acid. Biochemical and Biophysical Research Communications 229:643-647.

Camp, H.S. and S.R. Tafuri. 1997. Regulation of peroxisome proliferator-activated receptor g activity by mitogen-activated protein kinase. Journal of Biological Chemistry 272:10811-10816.

Castan, I., P. Valet, N. Quideau, T. Viosin, L. Ambid, M. LaBurthe, M. Lafontan and C. Carpene. 1994. Antilipolytic effects of α2-adrenergic agonists, neuropeptide Y, adenosine, and PGE1 in mammal adipocytes. American Journal of Physiology 266:R1141-R1147.

Castelien, H., T. Gulick, R.E. Declercq, G.P. Mannaerts, D.A. Moore and M.L. Baes. 1994. The peroxisome proliferator activated receptor regulates malic enzyme gene expression. Journal of Biological Chemistry 269:26754-26758.

Chan, K.-M. and J. Turk. 1987. Mechanism of arachidonic acid-induced Ca^{2+} mobilization from rat liver microsomes. Biochimica et Biophysica Acta 928:186-193.

Chapman, A.B., D.M. Knight and G.M. Ringold. 1985. Glucocorticoid regulation of adipocyte differentiation:hormonal triggering of the developmental program and induction of a differentiation-dependent gene. Journal of Cell Biology 101:1227-1235.

Chatzipanteli, K., C. Head, J. Megerman and L. Axelrod. 1996. The relationship between plasma insulin level, prostaglandin production by adipose tissue, and blood pressure in normal rats and rats with diabetes mellitus and diabetic ketoacidosis. Metabolism 45:691-698.

Chatzipanteli, K., S. Rudolph and L. Axelrod. 1992. Coordinate control of lipolysis by prostaglandin E2 and prostacyclin in rat adipose tissue. Diabetes 41:927-935.

Chawla, A. and M.A. Lazar. 1994. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. Proceedings of the National Academy of Sciences 91:1786-1790.

Chawla, A., E.J. Schwarz, D.D. Dimaculangan and M.A. Lazar. 1994. Peroxisome proliferator-activated receptor (PPAR) γ: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology 135:798-800

Cheneval, D., R.J. Christy, D. Geiman, P. Cornelius and M.D. Lane. 1991. Cell-free transcription directed by the 422 adipose P2 gene promoter: Activation by the CCAAT/enhancer binding protein. Proceedings of the National Academy of Sciences 88:8465-8469.

Christ, E.J. and D.H. Nugteren. 1970. The biosynthesis and possible function of prostaglandins in adipose tissue. Biochimica et Biophysica Acta 218:296-307.

Christie, P.E., J.P. Arm and T.H. Lee. 1993. Leukotrienes. Mast Cell in Health and Disease, Marcell Dekker, Inc., New York, NY, 62:393-407.

Christy, R.J., K.H. Kaestner, D.E. Geiman and M.D. Lane. 1991. CCAAT/enhancer binding protein gene promoter: Binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences 88:2593-2597.

Christy, R.J., V.W. Yang, J.M. Ntambi, D.E. Geiman, W.H. Landschulz, A.D. Friedman, Y. Nakabeppu, T.J. Kelly and M.D. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes and Development 3:1323-1335.

Clark, J.D., A.R. Schievella, E.A. Nalefski, L.-L. Lin. 1995. Cytosolic phospholipase A_2. Journal of Lipid Mediators and Cell Signaling 12:83-117.

Clarke, S.D. and S. Abraham. 1992. Gene expression:nutrient control of pre- and posttranscriptional events. FASEB 6:3146-3152.
FAS, S14, review, PUFA

Clarke, S.D., M.K. Armstrong and D.B. Jump. 1990a. Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. Journal of Nutrition 120:225-231.

Clarke, S.D., M.K. Armstrong and D.B. Jump. 1990b. Nutritional control of rat liver fatty acid synthase and S14 mRNA abundance. Journal of Nutrition 120:218-224.

Clarke, S.D. and D.B. Jump. 1993. Regulation of gene transcription by polyunsaturated fatty acids. Progress in Lipid Research 32:139-149.

Clarke, S.D. and D.B. Jump. 1993. Regulation of hepatic gene expression by dietary fats:a unique role for polyunsaturated fatty acids. Nutrition and Gene Expression, CRC Press, pp. 227-246.

Clarke, S.D. and D.B. Jump. 1994. Dietary polyunsaturated fatty acid regulation of gene transcription. Annual Reviews of Nutrition 14:83-98.

Clarke, S.D. and D.B. Jump. 1996. Polyunsaturated fatty acid regulation of hepatic gene transcription. Lipids 31(Suppl):S7-S11.
Liver, PUFA, PPAR, review

Clarke, S.D., D.R. Romsos and G.A. Leveille. 1976. Specific inhibition of hepatic fatty acid synthesis exerted by dietary linoleate and linolenate in essential fatty acid adequate rats. Lipids 11:485-490.

Clarke, S.D., D.R. Romsos and G.A. Leveille. 1977. Differential effects of dietary methyl esters of long-chain saturated and polyunsaturated fatty acids on rat liver and adipose tissue lipogenesis. Journal of Nutrition 107:1170-1180.

Coleman, D.L. 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 14;141-148.

Coleman, R.A., B.C. Reed, J.C. Mackall, A.K. Student, M.D. Lane and R.M. Bell. 1978. Selective changes in microsomal

enzymes of triacylglycerol phosphatidylcholine, and phosphatidylethanolamine biosynthesis during differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 253:7256-7261.

Coleman, R.A., W.L. Smith and S. Narumiya. 1994. Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacological Reviews 46:205-229.

Cooper, A.D. 1997. Hepatic uptake of chylomicron remnants. Journal of Lipid Research 38:2173-2192.

Cornelius, P., O.A. MacDougald and M.D. Lane. 1994. Regulation of adipocyte development. Annual Reviews of Nutrition 14:99-129.

Cornelius, P., M. Marlowe, M.D. Lee and P.H. Pekala. 1990. The growth factor-like effects of tumor necrosis α. Journal of Biochemistry 265:20506-20516.

Cunningham, B.A., M. Maloney and W.B. Kinlaw. 1997. Spot 14 protein-protein interactions: evidence for both homo- and heterodimer formation *in vivo*. Endocrinology: 138:5184-5188.

Currie, R.A. 1998. NF-Y is associated with the histone acetyltransferases GCN5 and P/CAF. Journal of Biological Chemistry 273:1430-1434.

Danesch, U., P.C. Weber and A. Sellmayer. 1994. Arachidonic acid increases c-*fos* and *Egr*-1 mRNA in 3T3 fibroblasts by formation of prostaglandin E_2 and activation of protein kinase C. Journal of Biological Chemistry 269:27528-27263.

Danesch, U., P.C. Weber and A. Sellmayer. 1996. Differential effects of n-6 and n-3 polyunsaturatd fatty acids on cell growth and early gene expression in Swiss 3T3 fibroblasts. Journal of Cellular Physiology 168:618-624.

Darlington, G.J., N. Wang and R.W. Hanson. 1995. C/EBPα:a critical regulator of genes governing integrative metabolic processes. Current Opinion in Genetics and Development 5:565-570.

Demoz, A., H. Vaagenes, N. Aarsaether, E. Hvattum, J. Skorve, M. Gottlicher, J.R. Lillehaug, G.G. Gibson, J.-A. Gustafsson, S. Hood and R.K. Berge. 1994. Coordinate induction of hepatic fatty acyl-CoA oxidase and P4504A1 in

rat after activation of the peroxisome proliferator-activated receptor (PPAR) by sulphur-substituted fatty acid analogues. Xenobiotica 24:943-956

De Vos, P., A.-M. Lefebvre, S.G. Miller, M. Guerro-Millo, K. Wong, R. Saladin, L.G. Hamann, B. Staels, M.R. Briggs and J. Auwerx. 1996. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator activated receptor γ. Journal of Clinical Investigation 98:1004-1009.

Doglio, A., C. Dani, P. Grimaldi and G. Ailhaud. 1986. Growth hormone regulation of the expression of differentiation-dependent genes in preadipocyte Ob1771 cells. Biochemical Journal 238:123-129.

Duddy, S.K., G.E.N. Kass and S. Orrenius. 1989. Ca^{2+}-mobilizing hormones stimulate Ca^{2+} efflux from hepatocytes. Journal of Biological Chemistry 264:20863-20866.

Ek, B.A., D.P. Cistola, J.A. Hamilton, T.L. Kaduce and A.A. Spector. 1997. Fatty acid binding proteins reduce 15-lipoxygenase-induced oxygenation of linoleic acid and arachidonic acid. Biochmica et Biophysica Acta 1346:75-85.

Feingold, K.R., M. Soued, M.K. Serio, A. Adi, A.H. Moser and C. Grunfeld. 1990. The effect of diet on tumor necrosis factor stimulation of hepatic lipogenesis. Metabolism 39:623-632.

Feingold, K.R., W. Doerrler, C.A. Dinarello, W. Fiers and C. Grunfeld. 1992. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinology 130:10-16.

Fernando, K.C. and G.J. Barritt. 1994. Evidence from studies with hepatocyte suspensions that store-operated Ca^{2+} inflow requires a pertussis toxin-sensitive trimeric G-protein. Biochemical Journal 303:351-356.

Fernando, K.C., R.B. Gregory, F. Katsis, B.E. Kemp and G.J. Barritt. 1997. Evidence that a low-molecular-mass GTP-binding protein is required for store-activated Ca^{2+} inflow in hepatocytes. Biochemical Journal 328:463-471.

Fitzpatrick, F.A. and R.C. Murphy. 1989. Cytochrome P-450 metabolism of arachidonic acid:formation and biological actions of "epoxygenase"-derived eicosanoids. Pharmacological Reviews 40:229-241.

Flatmark, T., A. Nilsson, J. Kvannes, T.S. Eikhorn, M.H. Fukami, H. Kryvi and E.N. Christiansen. 1988. On the mechanism of induction of enzymes systems for peroxisomal β-oxidation of fatty acids in rat liver by diets rich in partially hydrogenated fish oil. Biochimica et Biophysica Acta 962:122-130.

Flick, P.K., J. Chen and P.R. Vagelos. 1977. Effect of dietary linoleate on synthesis and degradation of fatty acid synthetase from rat liver. Journal of Biological Chemistry 252:4242-4249.

Task Force Report. Food Fats and Health. Council for Agricultural Sciences and Technology, Ames, Iowa: 1991

Forman, B.M., J. Chen and R.M. Evans. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and γ. Proceedings of the National Academy of Sciences 94:4312-4317.

Forman, B.M., P. Tontonoz, J. Chen, R.P. Brun, B.M. Spiegelman and R.M. Evans. 1995. 15-Deoxy-^ 12,14-Prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 83:803-812.

Fourney, R.M., J. Miyakoshi, R.S. Day III and M.C. Paterson. Northern blotting: Efficient RNA staining and transfer. Focus 10:5-6.

Fournier, T., V. Fadok and P.M. Henson. 1997. Tumor necrosis factor-α inversely regulates prostaglandin D_2 and prostaglandin E_2 production in murine macrophages. Journal of Biological Chemistry 272:31065-31072.

Freytag, S.O., D.L. Paielli and J.D. Gilbert. 1994. Ectopic expression of the CCAAT/enhancer -binding protein a promotes the adipogenic program in a variety of fibroblastic cell lines. Genes and Development 8:1654-1663.

Fulceri, R., A. Gamberucci, G. Bellomo, R. Giunti and A. Benedett. 1993. CoA and fatty acyl-CoA derivatives mobilize calcium from a liver reticular pool. Biochemical Journal 295:663-669.

Gaillard, D., R. Negrel, M. Lagarde and G. Ailhaud. 1989. Requirement and role of arachidonic acid in the

differentiation of pre-adipose cells. Biochemical Journal 257:389-397.

Garrity, M.J., E.P. Brass and R.P. Robertson. 1987. PGE binding in isolated hepatocytes: regulation during fasting. Advances in Prostaglandin, Thromboxane, and Leukotriene Research 17:686-690.

Garrity, M.J., M.M. Reed and E.P. Brass. 1989. Coupling of hepatic prostaglandin receptors to adenylate cyclase through a pertussis toxin sensitive guanine nucleotide regulatory protein. Journal of Pharmacology and Experimental Therapeutics 248:979-983.

Garrity, M.J., K.R. Westcott, T.L Eggerman, N.H. Andersen, D.R. Stormm and R.P. Robertson. 1983. Interrelationships between PGE_1 and PGI_2 binding and stimulation of adenylate cyclase. American Journal of Physiology 244:E367-E372.

Gomez-Munoz, A., P. hales and D.N. Brindley. 1991. Unsaturated fatty acids activate glycogen phosphorylase in cultured rat hepatocytes. Biochemical Journal 276:209-215.

Gonzalez, F.J. 1997. The role of peroxisome proliferator activated receptor a in peroxisome proliferation, physiological homeostasis, and chemical carcinogenesis. Advances in Experimental Medicine and Biology 422:109-125.

Gossett, R.E., A.A. Frolov, J.B. Roths, W.D. Behnke, A.B. Kier and F. Schroeder. 1996. Acyl-CoA binding proteins: Multiplicity and Function. Lipids 31:895-918.

Gottlicher, M., E. Widmark, Q. Li and J.-A. Gustafsson. 1992. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proceedings of the National Academy of Sciences 89:4653-4657.

Graber, M.N., A. Alfonso and D.L. Gill. 1997. Recovery of Ca^{2+} pools and growth in Ca2+ pool-depleted cells is mediated by specific epoxyeicosatrienoic acids derived from arachidonic acid. Journal of Biological Chemistry 272:29546-29553.

Graves, R.A., P. Tontonoz, S.R. Ross and B.M. Spiegelman. 1991. Identification of a potent adipocyte-specific enhancer:involvement of an NF-1-like factor. Genes and Development 5:428-437.

Graves, R.A., P. Tontonoz and B.M. Spiegelman. 1992. Analysis of tissue-specific enhancer:ARF6 regulates adipogenic gene expression. Molecular and Cellular Biology 12:1202-1208.

Grimaldi, P.A., S.M. Knobel, R.R. Whitesell and N.A. Abumrad. 1992. Induction of aP2 gene expression by nonmetabolized long-chain fatty acids. Proceedings of the National Academy of Sciences 89:10930-10934.

Grunfeld, C., S. Adi, M. Soued, A. Moser, W. Feirs and K.R. Feingold. 1990. Search for mediators of the lipogenic effects of tumor necrosis factor: potential role of interleukin 6. Cancer Research 50:4233-4238.

Hallakou, S., L. Doare, F. Foufelle, M. Kergoat, M. Guerre-Millo, M.F. Berthault, I. Dugail, J. Morin, J. Auwerx and P. Ferre. 1997. Pioglitazone induces in vivo adipocyte differentiation in the obese Zucker *fa/fa* rat. Diabetes 46:1393-1399.

Hamon, M., J. Berthaut, A. Wisner, F. Dray, B. Descomps and H.T. Dao. 1993. Modulation of human myometrial PGE_2 receptor by GTP characterization of receptor subtype. Prostaglandins 46:251-268.

Hannun, Y.A. 1997. Sphingolipid second messengers: tumor suppressor lipids. Proceedings of Experimental Medicine and Biology 400A:305-312.

Hannun, Y.A and L.M. Obeid. 1995. Ceramide: an intracellular signal for apoptosis. Trends in Biological Sciences 20:73-77.

Harbrecht, B.G., Y.M. Kim, E.M. Wirant, R.A. Shapiro and T.R. Billiar. 1996. PGE_2 and LTB_4 inhibit cytokine-stimulated nitric oxide synthase type 2 expression in isolated rat hepatocytes. Prostaglandins 52:103-116.

Harris, P.K. and R.F. Kletzien. 1994. Localization of a pioglitazone response element in the adipocyte fatty acid-binding protein gene. Molecular Pharmacology 45:439-445.

Hashimoto, N., T. Watanabe, Y. Ikeda, H. Yamada, S. Taniguchi, H. Mitsui and K. Kurokawa. 1997. Prostaglandins induce proliferation of rat hepatocytes through a prostaglandin E_2 receptor EP_3 subtype. American Journal of Physiology 272:G597-604.

Hauner, H., Th. Petruschke, M. Russ, K. Rohrig and J. Eckel. 1995. Effects of tumour necrosis factor alpha (TNFα) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. Diabetologia 38:764-771.

Herrara, R., H.-S. Ro, G.S. Robinson, K.G. Xanthopoulos and B.M. Spiegelman. 1989. A direct role for C/EBP and the AP-1-binding site in gene expression linked to adipocyte differentiation. Molecular and Cellular Biology 9:5331-5339.

Hertz, R., I. Berman, D. Keppler and J. Bar-Tana. 1996. Activation of gene transcription by prostacyclin analogues is mediated by the peroxisome-proliferators activated receptor (PPAR). European Journal Biochemistry 235:242-247.

Hespeling, U., K. Jungemann and G.P. Puschel. 1995a. Feedback-inhibition of glucagon-stimulated glycogenolysis in hepatocyte/Kupffer cell cocultures by glucagon-elicited prostaglandin production in Kupffer cells. Hepatology 22:1577-1583.

Hespeling, U., G.P. Puschel, K. Jungemann O. Gotz and J. Zwirner. 1995b. Stimulation of glycogen phosphorylase in rat hepatocytes via prostanoid release from Kupffer cells by recombinant rat anaphylatoxin C5a but not by native human C5a in hepatocyte/Kupffer cell co-cultures. FEBS Letters 372:108-112.

Hollenberg, A.N., V.S. Susulic, J.P. Madura, B. Zhang, D.E. Moller, P. Tontonoz, P. Sarraf, B.M. Spiegelman and B.B. Lowell. 1997. Functional antagonism between CCAAT/Enhancer binding protein-α and peroxisome proliferator-activated receptor-γ on the leptin promoter. Journal of Biological Chemistry 272:5283-5290.

Hotamisligil, G.S, P. Arner, J.F. Caro, R.L. Atkinson and B.M. Spiegelman. 1995. Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. Journal of Clinical Investigations 95:2409-2415.

Hotamisligil, G.S., P. Peraldi, A. Budavari, R. Ellis, M.F. White and B.M. Spiegelman. 1996b. IRS-1 mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity-induced insulin resistance. Science 271:665-668.

Hu, E., J.B. Kim, P. Sarraf and B.M. Spiegelman. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. Science 274:2100-2103.

Hu, E., P. Tontonoz and B.M. Spiegelman. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proceedings of the National Academy of Sciences 92:9856-9860.

Hughes, B.P., J.N. Crofts, A.M. Auld, L.C. Read and G.J. Barrit. 1987. Evidence that a pertussis-toxin-sensitive substrate is involved in the stimulation by epidermal growth factor and vasopressin of plasma-membrane Ca^{2+} inflow in hepatocytes. Biochemical Journal 248:911-918.

Hunter, J., A. Kassam, C.J. Winrow, R.A. Rachubinski and J.P. Capone. 1996. Crosstalk between the thyroid hormone and peroxisome proliferator-activated receptors in regulation peroxisome proliferator-responsive genes. Molecular and Cellular Endocrinology 116:213-221.

Hwang, D.H., M. Boudreau, P. Chanmugam. 1988. Dietary linolenic acid and longer chain n-3 fatty acids: comparison of effects on arachidonic acid metabolism in rats. Journal of Nutrition 118:427-437.

Hyman, B.T., L.L. Stoll and A.A. Spector. 1982. Prostaglandin production by 3T3-L1 cells in culture. Biochimica et Biophysica Acta 713:375-385.

Im, D.S., T. Fujioka, T. Katada, Y. Kondo, M. Ui and F. Okajima. 1997. Characterization of sphingosine 1-phosphate-induced actions and its signaling pathways in rat hepatocytes. American Journal of Physiology 35:g1091-1099.

Irvine, R.F. 1982. How is the level of free arachidonic acid controlled in mammalian cells? Biochemical Journal 204:3-16.
Review, AA

Izawa, T., T. Mochizuki, T. Komabayashi, K. Suda and M. Tsuboi. 1994. Increase in cytosolic free Ca^{2+} in corticotropin-stimulated white adipocytes. American Journal of Physiology 266:E418-E426.

Jayadev, S., C.M. Linardic and Y.A. Hannun. 1994. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor α. Journal of Biological Chemistry 269:5757-5763.

Jiang, M.S., Q. Tang, J. McLenithan, D. Geiman, W. Shillinglaw, W.J. Henzel and M.D. Lane. 1998. Derepression of the C/EBPα gene during adipogenesis: identification of AP-2α as a repressor. Proceedings of the National Academy of Sciences 95:3467-3471.

Jones, B.H., J.H. Kim, M.B. Zemel, R.P. Woychik, E.J. Michaud, W.O. Wilkison and N. Moustaid. 1996. Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. American Journal of Physiology 270:E192-E196.

Jump, D.B. 1989. Rapid induction of rat liver S14 gene transcription by thyroid hormone. Journal of Biological Chemistry 264:4689-4703.

Jump, D.B., M. Badin and A. Thelen. 1997b. The CCAAT box binding factor, NF-Y, is required for thyroid hormone regulation of rat liver S14 gene transcription. Journal of Biological Chemistry 272:27778-27786.

Jump, D.B., A. Bell and V. Santiago. 1990. Thyroid hormone and dietary carbohydrate interact to regulate rat liver S14 gene transcription and chromatin structure. Journal of Biological Chemistry 265:3474-3478.

Jump, D.B., S.D. Clarke, O. MacDougald and A. Thelen. 1993. Polyunsaturated fatty acids inhibit S14 gene transcription in rat liver and cultured hepatocytes. Proceedings of the National Academy of Sciences 90:8454-8458.

Jump, D.B., S.D. Clarke, A. Thelen and M. Liimatta. 1994. Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids. Journal of Lipid Research 35:1076-1084.

Jump, D.B., S.D. Clarke, A. Thelen, M. Liimatta, B. Ren and M. Badin. 1997a. Dietary fat, genes and human health. Dietary Fat and Cancer, Plenum Press, NY, pp. 167-176.

Jump, D.B., S.D. Clarke, A. Thelen, M. Liimatta, B. Ren and M. Badin. 1996. Dietary polyunsaturated fatty acid regulation of gene transcription. Progress in Lipid Research 35:227-241.

Jump, D.B. and O.A. MacDougald. 1993. Hormonal regulation of gene expression in cultured adipocytes. Journal of Animal Science 71(Suppl):56-64.

Jump, D.B., P. Narayan, H. Towle and J.H. Oppenheimer. 1984. Rapid effects of triiodothyronine on hepatic gene expression. Journal of Biological Chemistry 259:2789-2797.

Jump, D.B. and J.H. Oppenheimer. 1985. High basal expression and 3,5,3-triiodothyronine regulation of messenger ribonucleic acid S14 in lipogenic tissues. Endocrinology 117:2259-2266.

Jump, D.B., B. Ren, S. Clarke and A. Thelen. 1995. Effects of fatty acids on hepatic gene expression. Prostaglandins Leukotrienes and Essential Fatty Acids 52:107-111.

Jump, D.B., A. Veit, V. Santiago, G. Lepar and L. Herberholz. 1988. Transcriptional activation of the rat liver S14 gene during postnatal development. Journal of Biological Chemistry 263:7254-7260.

Jungermann, K. and T. Kietzmann. 1996. Zonation of parenchymal and nonparenchymal metabolism in liver. Annual Reviews of Nutrition 16:179-203.

Kaestner, K.H., R.J. Christy and M.D. Lane. 1990. Mouse insulin-responsive glucose transporter gene: Characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. Proceedings of the National Academy of Sciences 87:251-255.

Kallen, C.B. and M.A. Lazar. 1996. Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. Proceedings of the National Academy of Sciences 93:5793-5796.

Kanemaki, T., H. Kitade, M. Kaibori, K. Sakitani, Y. Hiramatsu, Y. Kamiyama, S. Ito and T. Okumura. 1998. Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. Hepatology 27:1296-1303.

Kanemaki, T., H. Kitade, Y. Hiramatsu, Y. Kamiyama and T. Okumura. 1993. Stimulation of glycogen degradation by prostaglandin E_2 in primary cultured rat hepatocytes. Prostaglandins 45:459-474.

Kass, G.E.N., J. Llopis, S.C. Chow, S.K. Duddy and S. Orrenius. 1990. Receptor-operated calcium influx in rat hepatocytes. Journal of Biological Chemistry 265:17486-17492.

Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato and W. Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proceedings of the National Academy of Sciences 90:2160-2164.

Khan, S.H. and S. Sorof. 1990. Preferential binding of growth inhibitory prostaglandins by the target protein of a carcinogen. Proceedings of the National Academy of Sciences 87:9401-9405.

Kim, J.B. and B.M. Spiegelman. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes and Development 10:1096-1107.

Kim, J.B., G.D. Spotts, Y.-D. Halvorsen, H.-M. Shih, T. Ellenberger, H.C. Towle and B.M. Spiegelman. 1995. Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. Molecular and Cellular Biology 15:2582-2588.

Kim, T.-S. and H.C. Freake. 1996. High carbohydrate diet and starvation regulate lipogenic mRNA in rats in a tissue-specific manner. Journal of Nutrition 126:611-617.

Kinlaw, W.B., P. Tron and L.A. Witters. 1993. Endocrinology 133:645-650.

Kinlaw, W.B., J.L. Church, J. Harmon and C.N. Mariash. 1995. Direct evidence for the role of "Spot 14" protein in the regulation of lipid synthesis. Journal of Biological Chemistry 270:16615-16618.

Kinsella, J.E., K.S. Broughton and J.W. Whelan. 1990. Dietary unsaturated fatty acids: interactions and possible needs in relation to eicosanoid synthesis. Journal of Nutritional Biochemistry 1:123-141.

Kliewer, S.A., J.M. Lenhar, T.M. Willson, I. Patel, D.C. Morris and J.M. Lehmann. 1995. A prostaglandin J2 metabolite binds peroxisome proliferator activated receptor γ and promotes adipocyte differentiation. Cell 83:813-819.

Kliewer, S.A., S.S. Sundsett, S.A. Jones, P.J. Brown, G.B. Wisely, C.S. Koble, P. Devchand, W. Wahli, T.M. Willson, J.M. Lenhard and J.M. Lehmann. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated

receptors α and γ. Proceedings of the National Academy of Sciences 94:4318-4323.

Lamphere, L., C.L. Carpenter, Z.F. Sheng, R.G. Kallen and G.E. Lienhard. 1994. Activation of PI 3 kinase in 3T3-L1 adipocytes by association with insulin receptor substrate-1. American Journal of Physiology 266:E486-E494.

Landshulz, K.T., D.B. Jump, O.A. MacDougald and M.D. Lane. 1994. Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty acids. Biochemical and Biophysical Research Communications 200:763-768.

Lane, M.D., F.-T. Lin, O.A. MacDougald and M. Vasseur-Cognet. 1996. Control of adipocyte differentiation by CCAAT/enhancer binding protein α (C/EBPα). International Journal of Obesity 20:S91-S96.

Laneuville, O., D.K. Breur, N. Xu, Z.H. Huang, D.A. Gage, J.T. Watson, M. Lagarde, D.L. Dewitt and W.L. Smith. 1995. Fatty acid substrate specificities of human prostaglandin-endoperoxide H sythase-1 and -2. Journal of Biological Chemistry 270:19330-19336.

Lehmann, J.M., J.M. Lenhard, B.B. Oliver, G.M. Ringold and S.A. Kliewer. 1997. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. Journal of Biological Chemistry 272:3406-3410.

Lehmann, J.M., L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson and S.A. Kliewer. 1995. An Antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). Journal of Biological Chemistry 270:12953-12956.

Lepak, N.M. and G. Serrero. 1995. Prostaglandin F2α stimulates transforming growth factor-α expression in adipocyte precursors. Endocrinology 136:3222-32229.

Lepar, G.J. and D.B. Jump. 1989. Hormonal regulation of the S14 gene in 3T3-F442A cells. Molecular Endocrinology 3:1207-1214.

Lepar, G.J. and D.B. Jump. 1992. Retinoic acid and dexamethasone interact to regulate S14 gene transcription in 3T3-F442A adipocytes. Molecular and Cellular Endocrinology 84:65-72.

Leung, L.K. and H.P. Glauert. 1996. Reduction of the concentrations of prostaglandins E2 and F2α, and thromboxane B2 in cultured rat hepatocytes treated with the peroxisome proliferator ciprofibrate. Toxicology Letters 85:143-149.

Liimatta, M., H.C. Towle, S. Clarke and D.B. Jump. 1994. Dietary polyunsaturated fatty acids interfere with the insulin/glucose activation of L-type pyruvate kinase gene transcription. Molecular Endocrinology 8:1147-1153.

Lin, F.-T. and M.D. Lane. 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinated gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. Genes and Development 6:533-544.

Lin, F.-T. and M.D. Lane. 1994. CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proceedings of the National Academy of Sciences 91:8757-8761.

Long, S. and P.H Pekala. 1996a. Lipid mediators of insulin resistance:ceramide signaling down-regulates GLUT4 gene transcription in 3T3-L1 adipocytes. Biochemical Journal 319:179-184.

Long, S. and P.H Pekala. 1996b. Regulation of GLUT4 gene expression by arachidonic acid. Journal of Biological Chemistry 271:1138-1144.

MacDougald, O.A., C.-S. Hwang, H. Fan and M.D. Lane. 1995. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. Proceedings of the National Academy of Sciences 92:9034-9037.

MacDougald, O.A. and D.B. Jump. 1992. Localization of an adipocyte-specific retinoic acid response domain controlling S14 gene transcription. Biochemical and Biophysical Research Communications 188:470-476.

Mackall, J.C., A.K. Student, S.E. Polakis and M.D. Lane. 1976. Induction of lipogenesis during differentiation in a preadipoctye cell line. Journal of Biological Chemistry 251:6462-6464.

Makita, K., J.R. Falck and J.H. Capdevila. 1996. Cytochrome P450, the arachidonic acid cascade, and hypertension:new vistas for an old enzyme system. FASEB Journal 10: 1456-1463.

Mandrup, S. and M.D. Lane. 1997. Regulating adipogenesis. Journal of Biological Chemistry 272:5367-5370.

McKay, L.I. and J.A. Cidlowski. 1998. Cross-talk between nuclear factor-κB and the steroid hormone receptors: mechanisms of mutual antagonism. Molecular Endocrinology 12:45-56.

Melien, O., R. Winsnes, M. Refsnes, I.P. Gladhaug and T. Christoffersen. 1988. Pertussis toxin abolishes the inhibitory effects of prostaglandins E_1, E_2, I_2 and $F_{2\alpha}$ on hormone-induced cAMP accumulation in cultured hepatocytes. European Journal of Biochemistry 172:293-297.

Mellgren, G., T. Bruland, A.P. Doskeland, T. Flatmark, O.K. Vintermyr and S.O. Doskeland. 1997. Synergistic antiproliferative actions of cyclic adenosine 3′,5′-monophosphate, interleukin-1β, and activators of Ca^{2+}/calmodulin-dependent protein kinase in primary hepatocytes. Endocrinology 138:4373-4383.

Miller, C.W., D.A. Casimir and J.M. Ntambi. 1996a. The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin $F_{2\alpha}$. Endocrinology 137:5641-5650.

Miller, C.W. and J.M. Ntambi. 1996. Peroxisome proliferators induce mouse liver stearoyl CoA desaturase 1 gene expression. Proceedings of the National Academy of Sciences 93:9443-9448.

Miller, S.G., P.D. Vos, M. Guerro-Millo, K. Wong, T. Hermann, B. Staels, M.R. Briggs and J. Auwerx. 1996b. The adipocyte specific transcription factor C/EBPα modulates *ob* gene expression. Proceedings of the National Academy of Sciences 93:5507-5511.

Mine, T., I. Kojima and E. Ogata. 1990. Mechanism of prostaglandin E_2-induced glucose production in rat hepatocytes. Endocrinology 126:2831-2836.

Mion, F., R. Jasuja and D.E. Johnston. 1995. The contribution of hepatocytes to prostaglandin synthesis in rat liver. Prostaglandins Leukotrienes and Essential Fatty Acids 53:109-115.

Moncur, J.T., J.P. Park, V.A. Memoli, T.K. Mohandas and W.B. Kinlaw. 1998. The "Spot 14" gene resides on the telomeric

end of the 11q13 amplicon and is expressed in lipogenic breast cancers: implications for control of tumor metabolism. Proceedings of the National Academy of Sciences 95:6989-6944.

Moustaid, N., R.S. Beyer and H.S. Sul. 1994. Identification of an insulin response element in the fatty acid synthase promoter. Journal of Biological Chemistry 269:5629-5634.

Moustaid, N., K. Sakamoto, S. Clarke, R.S. Beyer and H.S. Sul. 1993. Regulation of fatty acid synthase gene transcription. Sequences that confer a positive insulin effect and differentiation-dependent expression in 3T3-L1 preadipocytes are present in the 332 bp promoter. Biochemical Journal 292:767-772.

Moustaid, N. and H.S. Sul. 1991. Regulation of expression of the fatty acid synthase gene in 3T3-L1 cells by differentiation and triiodothyronine. Journal of Biological Chemistry 266:18550-18554.

Nagai, M., K. Tuchiya and H. Kojima. 1996. Prostaglandin E2 increases the calcium concentration in rat brown adipocytes and their consumption of oxygen. Prostaglandins 51:377-386.

Neat, C.E., M.S. Thomassen and H. Osmunden. 1980. Induction of peroxisomal β-oxidation of rat liver by high fat diets. Biochemical Journal 186:369-371.

Negishi, M., Y. Sugimoto and A. Ichikawa. 1995. Prostaglandin receptors. Journal of Lipid Mediators and Cell Signaling 12:379-391.

Negrel, R., D. Gaillard and G. Ailhaud. 1989. Prostacylin as a potent effector of adipose-cell differentiation. Biochemical Journal 257:399-405.

Nikolova-Karakashian, M., E.T. Morgan, C. Alexander, D.C. Liotta and A.H. Merrill, Jr. 1997. Bimodal regulation of ceramidase by interleukin-1β. Journal of Biological Chemistry 272:18718-18724.

Ninomiya-Tsuji, J. F.M. Torti and G.M. Ringold. 1993. Tumor necrosis factor-induced c-myc expression in the absence of mitogenesis is associated with inhibition of adipocyte differentiation. Proceedings of the National Academy of Sciences 90:9611-9615.

Ntambi, J.M. and T. Takova. 1996. Role of Ca^{2+} in the early stages of murine adipocyte differentiation as evidenced by calcium mobilizing agents.

Oliw, E.H. 1994. Oxygenation of polyunsaturated fatty acids by cytochrome P450 monooxygenases. Progress in Lipid Research 33:329-354.

Ota, Y., A. Mariash, J.L. Wagner and C.N. Mariash. 1997. Cloning, expression and regulation of human S14 gene. Molecular and Cellular Endocrinology 126:75-81.

Parker, J., J. Lane and L. Axelrod. 1989. Cooperation of adipocytes and endothelial cells required for catecholamine stimulation of PGI_2 production by rat adipose tissue. Diabetes 38:1123-1132.

Paulauskis, J.D. and H.S. Sul. 1988. Cloning and expression of mouse fatty acid synthase and other specific mRNAs. Development and hormonal regulation in 3T3-L1 cells. Journal of Biological Chemistry 263:7049-7057.

Porras, A. and E. Santos. 1996. The insulin/RAS pathway of adipocytic differentiation of 3t3 L1 cells:dissociation between Raf-1 kinase and the MAPK/RSK cascade. International Journal of Obesity 20(Suppl 3):S43-S51.

Puschel, G.P. and B. Christ. 1994. Inhibition by PGE_2 of glucagon-induced increase in phosphoenolpyruvate carboxykinase mRNA and acceleration of mRNA degradation in cultured rat hepatocytes. FEBS Letters 351:353-356.

Raza, H., J.R. Pongubala and S. Sorof. 1989. Specific high affinity binding of lipoxygenase metabolites of arachidonic acid by liver fatty acid binding protein. Biochemical and Biophysical Research Communications 161:448-455.

Reddy, J.K. and G.P. Mannaerts. 1994. Peroxisomal lipid metabolism. Annual Reviews of Nutrition 14:343-370.

Refsnes, M. O.F. Dajani, D. Sandnes, G.H. Thoresen, J. Rottingen, J.-G. Iversen and T. Christoffersen. 1995. On the mechanisms of the growth-promoting effect of prostaglandins in hepatocytes:The relationship between stimulation of DNA synthesis and signaling mediated by adenylyl cyclase and phosphoinositide-specific phospholipase C. Journal of Cellular Physiology 164:465-473.

Reginato, M.J., S.L. Krakow, S.T. Bailey and M.A. Lazar. 1998. Prostaglandins promote and block adipogenesis through

opposing effects on peroxisome proliferator-activated receptor γ. Journal of Biological Chemistry 272:1855-1858.

Ren, B., A.P. Thelen, J.M. Peters, F.J. Gonzalez and D.B. Jump. 1997. Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. Journal of Biological Chemistry 272:26827-26832.

Ren, B., A. Thelen and D.B. Jump. 1996. Peroxisome proliferator-activated receptor α inhibits hepatic S14 gene transcription. Journal of Biological Chemistry 271:17167-17173.

Richelsen, B. 1987. Factors regulation the production of prostaglandin E_2 and prostacyclin (prostaglandin I_2) in rat and human adipocytes. Biochemical Journal 247:389-394.

Richelsen, B., J.D. Borglum and S.S. Sorensen. 1992. Biosynthetic capacity and regulatory aspects of prostaglandin E2 formation in adipocytes. Molecular and Cellular Endocrinology 85:73-81.

Ritz, E., C.C. Heuck and R. Boland. 1980. Phosphate, calcium and lipid metabolism. Advances in Experimental and Medical Biology 128:197-208.

Robertson, R.P., K.R. Westcott, D.R. Storm and M.G. Rice. 1980. Down regulation in vivo of PGE receptors and adenylate cyclase stimulation. American Journal of Physiology 239:e75-E85.

Rocchini, A.P. 1995. Insulin Resistance, Obesity and Hypertension. Journal of Nutrition 125:1718S-1724S.

Rodriguez de Turco, E.B. and J.A. Spitzer. 1990. Eicosanoid production in nonparenchymal liver cells isolated from rats infused with *E. coli* endotoxin. Journal of Leukocyte Biology 48:488-494.

Sadowski, H.B., T.T. Wheeler and D.A. Young. 1992. Gene expression during 3T3-L1 adipocyte differentiation. Journal of Biological Chemistry 266:4722-4731.

Schoonjans, K., J. Peinado-Onsurbe, A. Lefebvre, R.A. Heyman, M. Briggs, S. Deeb, B. Staels and J. Auwerx. 1996a. PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO Journal 15:5336-5348.

Schoonjans, K., B. Staels and J. Auwerx. 1996b. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochimica et Biophysica Acta 1302:93-109.

Schoonjans, K., B. Staels and J. Auwerx. 1996c. Role of peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. Journal of Lipid Research 37:907-925.

Schoonjans, K., M. Watanabe, H. Suzuki, A. Mahfoudi, G. Krey, W. Wahli, P. Grimaldi, B. Staels, T. Yamamoto and J. Auwerx. 1995. Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. Journal of Biological Chemistry 270:19269-19276.

Schwarz, E.J., M.J. Reginato, D. Shao, S.L. Krakow and M.A. Lazar. 1997. Retinoic acid blocks adipogenesis by inhibiting C/EBPβ-mediated transcription. Molecular and Cellular Biology 17:1552-1561.

Sellmayer, A., U. Danesch and P.C. Weber. 1996. Effects of different polyunsaturated fatty acids on growth-related early gene expression and cell growth. Lipids 31:S37-S40.

Serrero, G., N.M. Lepak and S.P. Goodrich. 1992. Paracrine regulation of adipose differentiation by arachidonate metabolites: prostaglandin F2α inhibits early and late markers of differentiation in the adipogenic cell line 1246. Endocrinology 131:2545-2551.

Sessler, A.M., N. Kaur, J.P. Palta and J.M. Ntambi. 1996. Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes. Journal of Biological Chemistry 271:29854-29858.

Sessler, A.M. and J.M. Ntambi. 1998. Polyunsaturated fatty acid regulation of gene expression. Journal of Nutrition 128:923-926.

Shillabeer, G., Z.-H. Li, G. Hatch, V. Kumar and D.C.W. Lau. 1996. A novel method for studying preadipocyte differentiation *in vitro*. International Journal of Obesity 20(Suppl3):S77-S83.

Shillabeer, G. and D.C.W. Lau. 1994. Regulation of new fat cell formation in rats:the role of dietary fats. Journal of Lipid Research 35:592-600.

Skouteris, G.G. and M.R. Kaser. 1991. Prostaglandin E_2 and $F_2\alpha$ mediate the increase in c-myc expression induced by EGF in primary rat hepatocyte cultures. Biochemical and Biophysical Research Communications 178:1240-1246.

Skouteris, G.G. and M. McMenamin. 1992. Transforming growth factor-α-induced DNA synthesis and c-*myc* expression in primary rat hepatocyte cultures is modulated by indomethacin. Biochemical Journal 281:729-733.

Smas, C.M. and H.S. Sul. 1995. Control of adipocyte differentiation. Biochemical Journal 309:697-710.

Smith, W.L. 1989. The eicosanoids and their biochemical mechanisms of action. Biochemical Journal 259:315-324.

Smith, W.L. 1992. Prostanoid biosynthesis and mechanisms of action. American Journal of Physiology 263:F181-191.

Smith, W.L., R.M. Garavito and D.L. Dewitt. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. Journal of Biological Chemistry 271:33157-33160.

Smith, L.K., K.M. Rice and C.W. Garner. 1996. The insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes is mediated by a calcium-dependent thiol protease. Molecular and Cellular Endocrinology 122:81-92.

Soncini, M., S.-F. Yet, Y. Moon, J.-Y. Chun and H. S. Sul. 1995. Hormonal and nutritional control of the fatty acid synthase promoter in transgenic mice. Journal of Biological Chemistry 270:30339-30343.

Spiegelman, B.M. and J.S. Flier. 1996. Adipogenesis and Obesity:Rounding out the big picture. Cell 87:377-389.

Stephens, J.M., R.F. Morrison and P.F. Pilch. 1996. The expression and regulation on STATs during 3T3-l1 adipocytes differentiation. Journal of Biological Chemistry 271:10441-10444.

Stephens, J.M. and P.H. Pekala. 1992. Transcriptional repression of the C/EPB-α and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-α. Journal of Biological Chemistry 267:13580-13584.

Strong, P., R.A. Coleman and P.P.A. Humphrey. 1992. Prostanoid-induced inhibition of lipolysis in rat isolated

adipocytes:probable involvement of EP3 receptors. Prostaglandins 43:559-566.

Stuhlmeier, K.M., and J.J. Kao and F.H. Bach. 1997. Arachidonic acid influences proinflammatory gene induction by stabilizing the inhibitor-κBα/nuclear factor-κB (NF-κB) complex thus suppressing the nuclear translocation of NF-κB.

Stryer, L, editor. 1988. Biochemistry, 3rd ed., W.H. Freeman and Company, New York.

Sudo, Y. and C.N. Mariash. 1996. Lowering glucose depletes a thapsigargin-sensitive calcium pool and inhibits transcription of the S14 gene. Endocrinology 137:4677-4684.

Sudo, Y. and C.N. Mariash. 1994. Two glucose-signaling pathways in S14 gene transcription in primary hepatocytes: a common role of protein phosphorylation. Endocrinology 134:2532-2540.

Sugimoto, Y., T. Namba, A. Honda, Y. Hayashi, M. Negishi, A. Ichikawa and S. Narumiya. 1992. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_3 subtype. Journal of Biological Chemistry 267:6463-6466.

Sul, H.S., N. Moustaid, K. Sakamoto, C.M. Smas, N. Gekakis and A. Jerkins. 1993a. Nutritional and hormonal regulation of genes encoding enzymes involved in fat synthesis. Nutrition and Gene Expression, CRC Press, pp. 207-226.

Sul, H.S., C.M. Smas and N. Moustaid. 1993b. Positive and negative regulators of adipocyte differentiation. Journal of Nutritional Biochemistry 4:554-562.

Tebbey, P.W. and T.M. Buttke. 1992. Arachidonic acid regulates unsaturated fatty acid synthesis in lymphocytes by inhibiting stearoyl-CoA desaturase gene expression. Biochimica et Biophysica Acta 1171:27-34.

Tebbey, P.W., K. M. McGowan, J.M. Stephens, T.M. Buttke and P.H. Pekala. 1994. Arachidonic acid down-regulates the insulin-dependent glucose transporter gene (GLUT4) in 3T3-L1 adipocytes by inhibiting transcription and enhancing mRNA turnover. Journal of Biological Chemistry 269:639-644.

Teboul, L., D. Gaillard, L. Staccini, H. Inaders, E.-Z. Amri and P.A. Grimaldi. 1995. Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. Journal of Biological Chemistry 270:28183-28187.

Thode, J., H.A. Perhadsingh, J.H. Ladenson, R. Hardy and J.M. McDonald. 1989. Palmitic acid stimulates glucose incorporation in the adipocyte by a mechanism likely involving intracellular calcium. Journal of Lipid Research 30:1299-1305.

Tocci, M.J. and J.A. Schmidt. 1997. Interleukin-1: Structure and function. Cytokines in Health and Disease, Marcell Dekker, Inc., New York, NY, pp. 1-23.

Tontonoz, P., R.A. Graves, A.I. Budavari, H. Erdjument-Bromage, M. Lui, E. Hu, P. Tempst and B.M. Spiegelman. 1994a. Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPARγ and RXRα. Nucleic Acids Research 22:5628-5634.

Tontonoz, P., E. Hu, J. Devine, E.G. Beale and B.M. Spiegelman. 1995. PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Molecular and Cellular Biology 15:351-357.

Tontonoz, P., E. Hu, R.A. Graves, A.I. Budavari and B.M. Spiegelman. 1994b. mPPARγ2:tissue-specific regulator of an adipocyte enhancer. Genes and Development 8:1224-1234.

Tontonoz, P., E. Hu and B.M. Spiegelman. 1994c. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147-1156.

Tontonoz, P., J.B. Kim, R.A. Graves and B.M. Spiegelman. 1993. ADD1:a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. Molecular and Cellular Biology 13:4753-4759.

Toussant, M.J., M.D. Wilson and S.D. Clarke. 1981. Coordinate suppression of liver acetyl CoA carboxylase and fatty acid synthase by polyunsaturated fat. Journal of Nutrition 11:146-153.

Towle, H.C., E.N. Kaytor and H.-M. Shih. 1996. Metabolic regulation of hepatic gene expression. Biochemical Society Transactions 24:364-368.

Tracey, K.J. 1997. Tumor Necrosis Factor. Cytokines in Health and Disease, Marcell Dekker, Inc., New York, NY, pp. 223-236.

Uehara, T., S. Hoshino, M. Ui, Y. Tokumitsu and Y. Nomura. 1994. Possible involvement of phosphatidylinositol-specific

phospholipase C related to pertussis toxin-sensitive GTP-binding proteins during adipocytes differentiation of 3T3-L1 fibroblast:negative regulation of protein kinase C. Biochimica et Biophysica Acta 1124:302-310.

Valera, A., G. Solanes and F. Bosch. 1993. Calcium-mobilizing effectors inhibit P-enolypyruvate carboxykinase gene expression in cultured rat hepatocytes. FEBS 333:319-324.

Vance, D.E. and J.E. Vance, eds. 1985. Biochemistry of Lipids and Membranes, The Benjamin/Cummings Publishing Company, Inc.

Varanasi, U., R. Chu, Q. Huang, R. Castellon, A.V. Yeldandi and J.K. Reddy. 1996. Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene. Journal of Biological Chemistry 271:2147-2155.

Vassaux, G., D. gaillard, C. Darimont, G. Ailhaud and R. Negrel. 1992. Differential response of preadipocytes and adipocytes to prostacyclin and prostaglandin E2:physiological implications. Endocrinology 131:2393-2398.

Volpe, J.J. and P.R. Vagelos. 1976. Mechanism and regulation of biosynthesis of saturated fatty acids. Physiological Reviews 56:339-417.

Vu-Dac, N., K. Schoonjans, V. Kosykh, J. Dallongeville, J.-C. Fruchart, B. Staels and J. Auwerx. 1995. Fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor. Journal of Clinical Investigations 96:741-750.

Wagner, M.J., J.A. Sharp and W.C. Summers. 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Proceedings of the National Academy of Sciences 78:1441-1445.

Wang, H., M.S. Goligorski and C.C. Malbon. 1997. Temporal activation of Ca2+ calmodulin-sensitive protein kinase type II is obligate for adipogenesis. Journal of Biological Chemistry 272:1817-1821.

Wang, J.-C., P.E. Stromstedt, R.M. O'Brien and D.K. Granner. 1996. Hepatic nuclear factor 3 is an accessory factor required for the stimulation of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. Molecular Endocrinology 10:794-800.

Wang, P., S.M. Tait, Z.F. Ba and I.H. Chaudry. 1995. Tumor necrosis factor-α administration increases Kupffer cell cyclic adenosine monophosphate levels. Shock 4:351-355.

Watanabe, T., K. Umegaki and W.L. Smith. 1986. Association of a solubilized prostaglandin E_2 receptor from renal medulla with a pertussis toxin-reactive guanine nucleotide regulatory protein. Journal of Biological Chemistry 261:13430-13439.

Wolf, B.A., J. Turk, W.R. Sherman and M.L. McDaniel. 1986. Intracellular Ca^{2+} mobilization by arachidonic acid. Journal of Biological Chemistry 261:3501-3511.

Wright, K.L, T.L. Moore, B.J. Vilen, A.M. Brown and J.P.Y. Ting. 1995. Major histocompatibility complex class II-associated invariant chain gene expression is up-regulated by cooperative interactions of Sp1 and NF-Y. Journal of Biological Chemistry 270:20978-20986.

Wu, Z., N.L.R. Bucher and S.R. Farmer. 1996. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ and glucocorticoids. Molecular and Cellular Biology 16:4128-4136.

Yang, L., G. Baffy, S.G. Rhee, D.R. Manning, C.A. Hansen and J.R. Williamson. 1991. Pertussis toxin-sensitive G_i protein involvement in epidermal growth factor-induced activation of phospholipase C-γ in rat hepatocytes. Journal of Biological Chemistry 266:22451-22458.

Yang, L., A.M. Camoratto, G. Baffy, S. Raj, D.R. Manning and J.R. Williamson. 1993. Epidermal growth factor-mediated signaling of G_i-protein to activation of phospholipases in rat-cultured hepatocytes. Journal of Biological Chemistry 268:3739-3746.

Yenush, L. and M.F. White. 1997. The IRS-signaling system during insulin and cytokine action. BioEssays 19:491-500.

Yu, K., W. Bayona, C.B. Kallen, H.P. Harding, C.P. Ravera, G. McMahon, M. Brown and M.A. Lazar. 1995. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. Journal of Biological Chemistry 270:23975-23983.

Zemel, M.B. 1995. Insulin resistance, obesity and hypertension: An overview. Journal of Nutrition 125:1715S-1717S.

Zhang, B., J. Berger, G. Zhou, A. Elbrecht, S. Biswas, S. White-Carrington, D. Szalkowski and D.E. Moller. 1996. Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome and proliferator-activated receptor γ. Journal of Biological Chemistry 271:31771-31774.