THE ROLE OF FATTY ACID ELONGASES IN GENE EXPRESSION AND LIPID METABOLISM

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ABSTRACT

ROLE OF FATTY ACID ELONGASES ON HEPATIC GENE EXPRESSION AND LIPID METABOLISM

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Fatty acid elongation and desaturation represent key metabolic steps that determine the fatty acyl composition of complex lipids in cells. Of these two metabolic pathways, factors controlling fatty acid elongase expression in mammalian tissues remain poorly defined. My studies established that of the 7 elongase subtypes expressed in mammals, adult rat liver expresses mRNA encoding 4 elongase (Elovl) subtypes; Elovl5 > Elovl1 = Elovl2 = Elovl6.

Once the hepatic profile for elongase subtype expression was established, I examined the nutritional and developmental regulation of hepatic fatty acid elongase as well as the role of key transcription factors in the control of hepatic elongase and desaturase expression in rat primary hepatocytes and in liver *in vivo*. These studies established that hepatic elongase expression is controlled by several transcription factors; including PPARα, SREBP-1, LXR, ChREBP and MLX. The *in vivo* studies show that changes in hepatic lipid composition induced by diabetes or obesity correlated with changes in elongase and desaturase expression.

Even though individual elongase subtypes have different substrate preferences, tissue specific expression, and are regulated differently, the significance of this diversity

is unknown. In an effort to better understand the impact of altered elongase expression on hepatic physiology, I over expressed Elovl2 and Elovl5 in primary hepatocytes using recombinant adenovirus technology. Overexpression of both Elovl2 (Ad-Elovl2) and Elovl5 (Ad-Elovl5) enhanced 20:5,n-3 conversion to 22:5,n-3, but only Elovl2 stimulated the formation of 24:5,n-3. The enhanced rate of 20:5,n-3 to elongated fatty acids attenuated 20:5-mediated induction on PPARα regulated transcripts. Over expressed Elovl2, but not over expressed Elovl5, also enhanced 20:5-mediated suppression of SREBP-1 nuclear content.

To gain a better understanding of the function of these enzymes in vivo, I used the recombinant adenovirus to over express Elovl5 in livers of C57BL/6 male mice. When compared to a control virus, overexpression of Ad-Elovl5 suppressed hepatic glycogen content and lowered blood glucose. I attributed this response to suppressed expression of several genes involved in glucose metabolism, i.e., Glut2, L-PK, PepCK. As in primary hepatocytes, overexpressed Elovl5 also suppressed several PPARα target genes. Over expressed Elovl5 also promoted changes in both the hepatic and plasma lipid profile consistent with Elovl5 overexpression. Over expressed Elovl5 also significantly suppressed blood triglyceride during refeeding. Taken together, these studies indicate that changes in hepatic Elovl5 activity, alone, are sufficient to impact both hepatic and plasma glucose and lipid composition.

In conclusion, these studies establish a role for specific transcriptional regulatory networks in the control of hepatic elongase gene expression and hepatic lipid composition. Based on my overexpression studies, fatty acid elongases play an important role in hepatic and whole body glucose and lipid metabolism.

To my parents
and
my husband

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Images in this dissertation are presented in color

LIST OF ABBREVIATIONS

AA: arachidonic acid

ACC: acetyl CoA Carboxylase

ACS: acyl-CoA synthetase

ACAT: acyl-CoA cholesterol acyltransferase

Ad: Adenovirus

ALA: Alpha-linolenic acid

AOX: Acyl CoA oxidase

bHLH/ZIP: basic helix-loop-helix/leucine zipper

ChoRE: carbohydrate regulatory elements

ChREBP: Carbohydrate regulatory element-binding protein

Cig 30: Cold induced glycoprotein with molecular weight about 30kDa

CNS: central nerve system

CPT: carnitine palmitoyltransferase

CVD: cardiovascular disease

CYP4A: cytochrome P450

DGAT: diacylglycerol acyltransferase

DHA: docosahexaenoic acid

Δ5D: fatty acid desaturase 5

Δ6D: fatty acid desaturase 6

Δ9D: fatty acid desaturase 9

EFAD: essential fatty acid deficiency

Elovl-1: fatty acid elongase family member 1

Elovl-2: fatty acid elongase family member 2

Elovl-3: fatty acid elongase family member 3

Elovl-4: fatty acid elongase family member 4

Elovl-5: fatty acid elongase family member 5

Elovl-6: fatty acid elongase family member 6

Elovl-7: fatty acid elongase family member 7

EPA: eicosapentaenoic acid

ER: endoplasmic reticulum

FAS: fatty acid synthase

GK: glucokinase

GLUT2: glucose transporter 2

HNF-4 α: hepatic nuclear factor-4α

H&E: hepatoxylin and eosin

HMGCS2: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2

LA: linoleic acid

LBD: ligand binding domain

LPK: L-pyruvate kinase

LTB_4: leukotriene B_4

LUC: luciferase

LXR: liver X receptor

MLX: MAX-like factor

MTE-I: mitochondial thioesterase I

MUFA: monounsaturated fatty acid

NEFA: non-esterified fatty acid

NFκB: nuclear factor κB

ORO: oil red O

PAS: Periodic acid shift

PEPCK: phosphoenolpyruvate carboxykinase

PPAR: peroxisome proliferator-activated receptor

PGE_2: prostaglandin E_2

PUFA: polyunsaturated fatty acids

RXRα: retinoid X receptor α

SCAP: SREBP-cleavage activating protein

SCD: Stearoyl-CoA desaturase

SREBP: sterol regulatory element-binding protein

SREs: sterol –regulatory elements

S1P: Site 1 protease

S2P: Site 2 protease

VLCFA: very long chain fatty acid

INTRODUCTION

Fatty acids affect many structural, metabolic, and regulatory components of cells. It has become increasingly apparent that fatty acids are capable of regulating, either directly or indirectly, the expression of numerous genes, both positively and negatively [1-4]. The impact of fatty acids on regulatory systems is determined by the type and quantity of fatty acid. The enzymes involved in the modification of fatty acid structure include fatty acyl CoA synthetases and thioesterases, fatty acid elongases and desaturases, peroxisomal β oxidation (chain shortening) and phospholipid and triglyceride lipases. Our lab has an interest in how the fatty acid elongases lead to structural changes in fatty acids that control transcription via the control of fatty acid-regulated transcription factors, peroxisomal proliferate activator α (PPARα) and sterol regulatory element binding protein-1 (SREBP-1) and carbohydrate regulatory element binding protein (ChREBP).

Four fatty acid elongases (Elovl1, 2, 5 and 6) and 3 desaturases [Δ5 desaturase (Δ5D), Δ6 desaturase (Δ6D) and Δ9 desaturase (Δ9D)] are expressed in rat, mouse and human liver. Among these enzymes, the 3 desaturases are well studied, especially Δ9D. These enzymes are regulated by hormones, development and dietary lipids [5-10]. Δ9D knockout mice are resistant to dietary fat-induced hepatic steatosis and have improved insulin sensitivity in muscle [11-13]. As such, this enzyme has been recognized as a drug target for obesity treatment recently. Compared to the desaturases, however, we are still in the early stages of understanding how fatty acid elongases are regulated and what their roles are in physiology. Moreover, fatty acid structure plays a key role in the regulation

of transcriptional factors [14]. As such, enzymes that modify fatty acid structure are also likely to impact gene expression. The goal of my research has been to gain information about the regulation of the fatty acid elongase family. In addition, I wanted to determine if there was any evidence in support or against the notion that fatty acid elongases play a role in the control of hepatic gene transcription and lipid metabolism in vitro and in vivo.

Chapter 1

Literature Review

1.1. Significance of fatty acid metabolism

1.1.1. Fatty acid metabolism

1.1.1.1. General features of fatty acid

1.1.1.1.1. Classification of fatty acids

Fatty acids are biochemical molecules that are composed of a hydrocarbon chain and a carboxyl group. According to the number of double bonds in the polycarbon chain, fatty acids can be classified to saturated fatty acid and unsaturated fatty acid. Saturated fatty acid, such as palmitic acid (16:0), contains no double bonds in the carbon chain. Unsaturated fatty acids, like oleic acid (18:1,n-9) contain one double bond while linoleic (18:2,n-6) and arachidonic acid (20:4,n-6) contain more than 1 double bonds. Therefore, unsaturated fatty acids are further classified into monounsaturated fatty acid (MUFA), such as oleic acid, and polyunsaturated fatty acid (PUFA) (Fig. 1.1). Depending on the position of the first double bond relative to the methyl end of fatty acid, PUFA can be classified into two major groups, n-3 and n-6. Sometimes the symbol ω is subtituted for the lowercase letter n, making it ω-3 and ω-6. These two groups of PUFA are not convertible and have different biological functions. Alpha(α)-linolenic (ALA, 18:3,n-3),

docosahexaenoic (DHA, 22:6, n-3) and eicosapentaenoic (EPA, 20:5, n-3) acids are representative n-3 fatty acids. Linoleic acid (LA, 18:2, n-6) and arachidonic acid (AA, 20:4, n-6) are n-6 fatty acids (Fig. 1.1).

1.1.1.1.2. Essential fatty acids

The human body can produce all but two of the fatty acids it needs. These two essential fatty acids are 18:2,n-6 and 18:3,n-3; both are widely distributed in plant and fish oils. Since these fatty acids can not be made in the body from other fatty acid substrates and must be supplied by the diet, they are called essential fatty acids. Essential fatty acids are parent compounds of the n-3 and n-6 fatty acid series, respectively. In the body, essential fatty acids are assimilated into membranes as complex phospholipids; they are also used to produce hormone-like substances that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, the immune response, and the inflammation response [15].

1.1.1.2. Dietary fatty acid metabolism

Dietary lipids are absorbed from the small intestines and transported in the blood to various tissues. The transport vehicles for these lipids are large lipoprotein complexes called chylomicrons. The liver removes chylomicron remnants from the circulation; cholesterol and triglycerides are repackaged into very low-density lipoproteins (VLDLs) and secreted from the liver for delivery to various tissues.

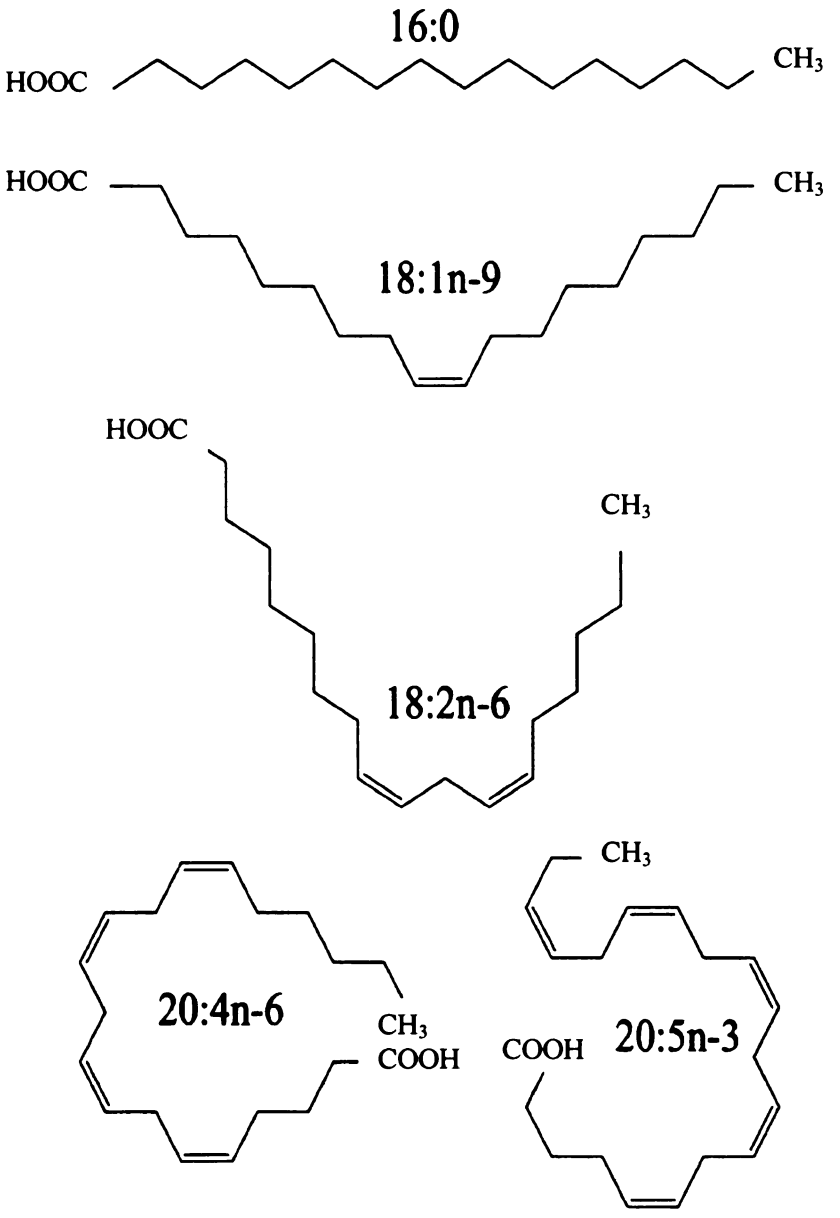


Figure 1.1. Structure of fatty acids. According to the number of double bond in the carbon chain, fatty acid can be classified as a saturated fatty acid (SFA) [Palmitic acid,16:0], monounsaturated fatty acid (MUFA) [Oleic acid, 18:1,n-9], or polyunsaturated fatty acid (PUFA), such as linoleic acid (18:2,n-6), arachidonic acid (20:4,n-6) and eicosapentaenoic acid (20:5,n-3).

Plasma lipids can be derived from the diet (chylomicrons), liver (VLDL) or mobilized from storage depots (adipose tissue) as non-esterified fatty acids (NEFA). Fatty acids in complex lipids, like triglycerides, are excised by the action of lipoprotein lipase. Plasma NEFAs are transported in the blood, loosely bound to albumin; NEFA are taken up by multiple tissues, including the liver, adipose tissue and muscle. Fatty acid can also be synthesized de novo from glucose [16].

When plasma fatty acids reach cell membrane, they enter cells via diffussion or by fatty acid transport proteins, e.g., FATP or CD36 [17]. Once inside the cell, fatty acids are rapidly converted to fatty acyl CoA by the action of fatty acyl CoA synthetases (ACS); some members of the FATP family also can convert NEFA to Fa-CoA during the transport process [18]. The formation of fatty acyl CoA is the rate-limiting step for nearly all subsequent metabolic transformations of fatty acid, such as elongation, desaturation, β-oxidation (mitochondrial or peroxisomal), or assimilation into complex lipids. Microsomal mono-oxidation and eicosanoid synthesis, however, utilizes the NEFA form of the fatty acid as substrate [14](Fig. 1.2).

1.1.1.3. De novo synthesis of fatty acids

Fatty acid synthesis occurs primarily in the cytoplasm of liver, adipose tissue, central nervous system and lactating mammary gland [19]. Among these tissues, liver has the highest rate for fatty acid synthesis. During caloric excess, the liver converts glucose to pyruvate. Pyruvate is converted to citrate in the mitochondrial (tricarboxylic acid cycle). Excessive citrate (not used in aerobic respiration) is transported out of the mitochondria to the cytosol where ATP citrate lyase converts citrate to acetyl-CoA.

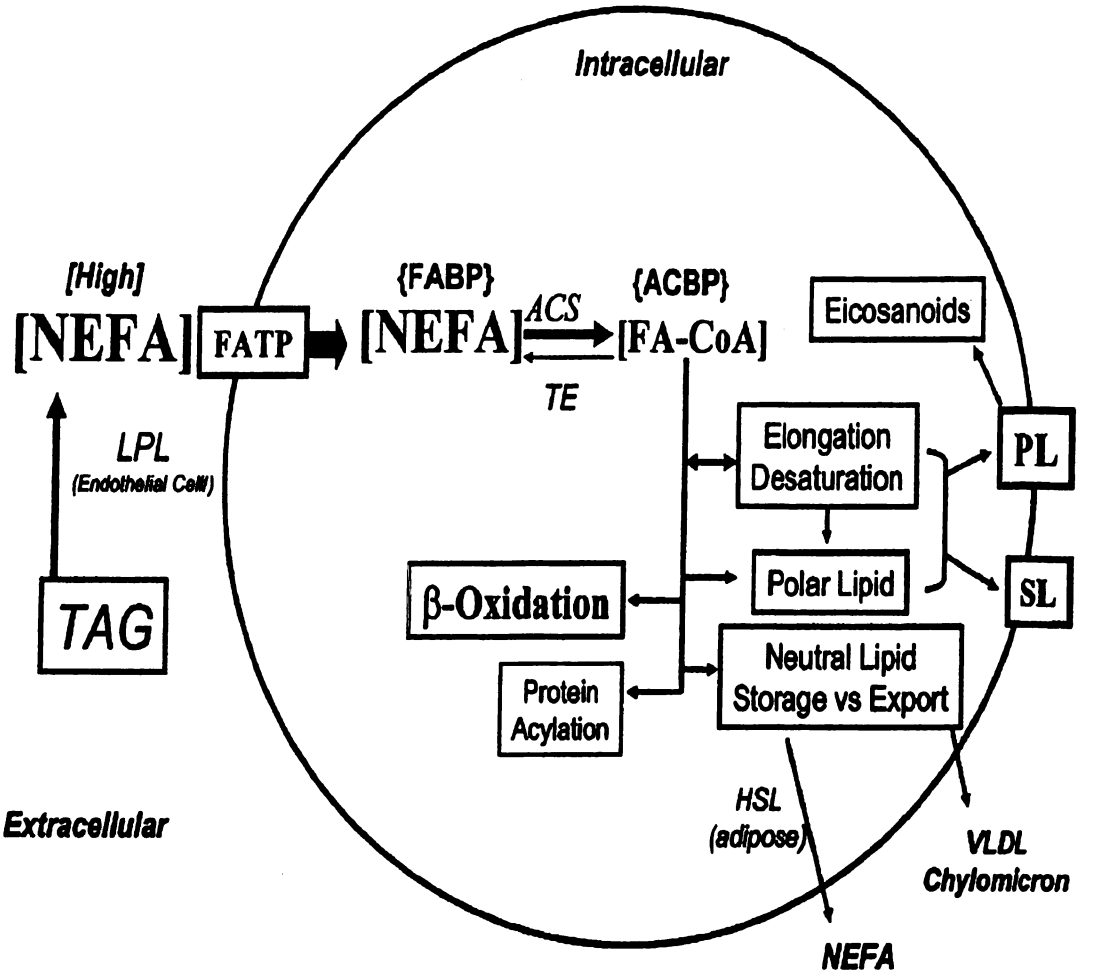


Figure 1. 2. Overview of cellular fatty acid metabolism. When the plasma fatty acid reach the cell membrane, they are either via diffussion or by fatty acid transprot proteins (FATP or CD36) to enter into the cell. Inside, fatty acid must be activated before proceeding through metabolism. Activation consists of conversion of the NEFA to its CoA derivative under the control of acyl CoA synthetases (ACS). The formation of fatty acyl CoA is the rate-limiting step for fatty acid further modification, including elongation/desaturation, β-oxidation (mitochondrial or peroxisomal), or assimilation into complex lipids.

Acetyl-CoA is a substrate for acetyl CoA carboxylase 1 (ACC1) which converts acetyl-CoA to malonyl CoA. Malonyl CoA is the rate-limiting substrate for fatty acid synthesis. A multi-enzyme complex, called fatty acid synthase (FAS), then uses malonyl-CoA, acetyl-CoA, and NADPH to elongate fatty acids in two-carbon increments in the cytosol [20]. The usual end product for this reaction in rodents is palmitate (16:0) [21]. Insulin, triiodothyronine (T3), glucocorticoids and glucose induce, while ≥C18 PUFA, glucagon, and epinephrine suppress de novo fatty acid synthesis [14].

1.1.1.4. Modification of dietary and endogenous fatty acids: elongation and desaturation

The liver plays a central role in whole body glucose, fatty acid and cholesterol metabolism. Once fatty acids enter hepatocytes, they are converted to their active form, acyl CoA. They enter pathways that lead to their elongation, desaturation, β-oxidation or assimilation into complex lipids (phospholipids, triglycerides and cholesterol esters). Fatty acid elongation, desaturation and β-oxidation are three chemical modifications that alter fatty acid structure. Each of these reactions occurs in most tissues. Our focus in the following discussion is on hepatic fatty acid elongation and desaturation.

Fatty acid elongation occurs in both the endoplasmic reticulum (ER) and the mitochondria. The elongation in ER represents the predominant pathway and malonyl CoA is the source of the added carbons. The process resembles the elongation steps of de novo fatty acid synthesis, but involves separate enzymes. These reactions consists of four sequential and independent steps: 1) a condensation between fatty acyl CoA and malonyl CoA to form 3-ketoacyl-CoA mediated by a fatty acid elongase (Elovl) [also known as β-

acylketo CoA synthase] ; 2) a reduction of the 3-ketoacyl-CoA to form 3-hydroxyacyl-CoA using NADPH, mediated by β-acylketo CoA reductase; 3) a dehydration of 3-hydroxyacyl-CoA to *trans*-2,3-enoyl-CoA mediated by (β-hydroxyacyl CoA dehydrase); 4) a reduction of *trans*-2,3-enoyl-CoA to acyl CoA, mediated by trans-2-enol CoA reductase [22]. The overall reactions are shown schematically in Fig. 1.3 [23]. The condensation step catalyzed by the fatty acid elongase (Elovl) is the regulated and rate-limiting step for fatty acid elongation [24]. Unlike de novo fatty acid synthesis, these reactions are separable and are not catalyzed by a multifunctional enzyme.

Mitochondrial elongation is a minor pathway and acetyl CoA is the source of the added carbons. In the mitochondria, fatty acid elongation occurs by a reversal of β-oxidation, except that one NADPH and one NADH are required (β-oxidation yields two NADH). Mitochondrial fatty acid elongation acts primarily on fatty acyl CoA substrates shorter than 16 carbons [25]. Desaturation of fatty acids introduces a double bond into the fatty acid; this reaction also occurs in ER of many tissues. This reaction requires an electron transport system involving: cytochrome b_5, desaturase and NADPH- cytochrome b_5 reductase [9].

In mammals, there are 3 distinct fatty acid desaturases (Δ5, Δ6 and Δ9 desaturase). Each has a different substrate requirement and generates a double bond at specific carbon bonds relative to the carboxyl end. For example, Δ5-desaturase inserts a double bond between the 5^{th} and 6^{th} carbon bond from the carboxyl end. A minimum chain length of 16-18 carbons is required for the desaturation, mammals can not synthesize n-6 and n-3 fatty acids de novo. Desaturation and 2-carbon elongation function together in metabolic

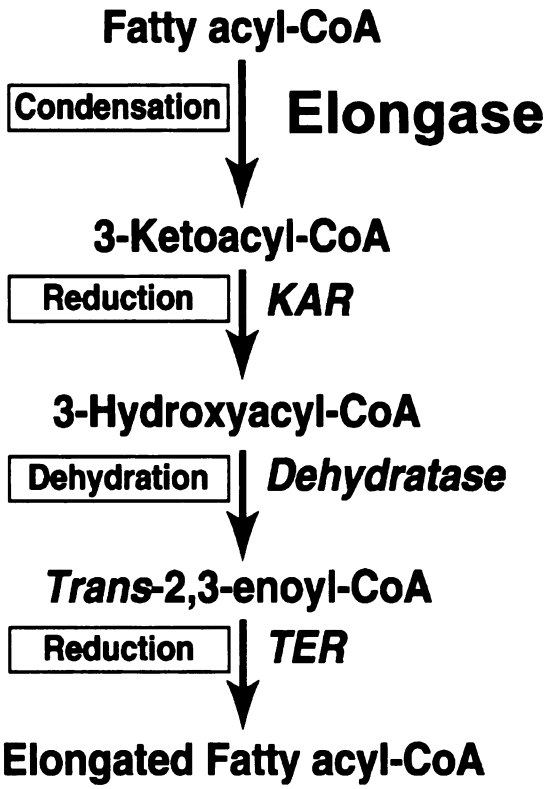


Figure 1. 3. Microsomal fatty acyl elongation. The elongation in ER represents the predominant pathway and malonyl CoA is the source of the added carbons. The process consists of four sequential but independent reactions: 1) a condensation between fatty acyl CoA and malonyl CoA to form 3-ketoacyl-CoA ; 2) a reduction of the 3-ketoacyl-CoA to form 3-hydroxyacyl-CoA using NADPH; 3) a dehydration of 3-hydroxyacyl-CoA to *trans*-2,3-enoyl-CoA; 4) a reduction of *trans*-2,3-enoyl-CoA to acyl CoA KAR, 3-ketoacyl-CoA reductase. TER, trans-2,3-enoyl-CoA reductase.

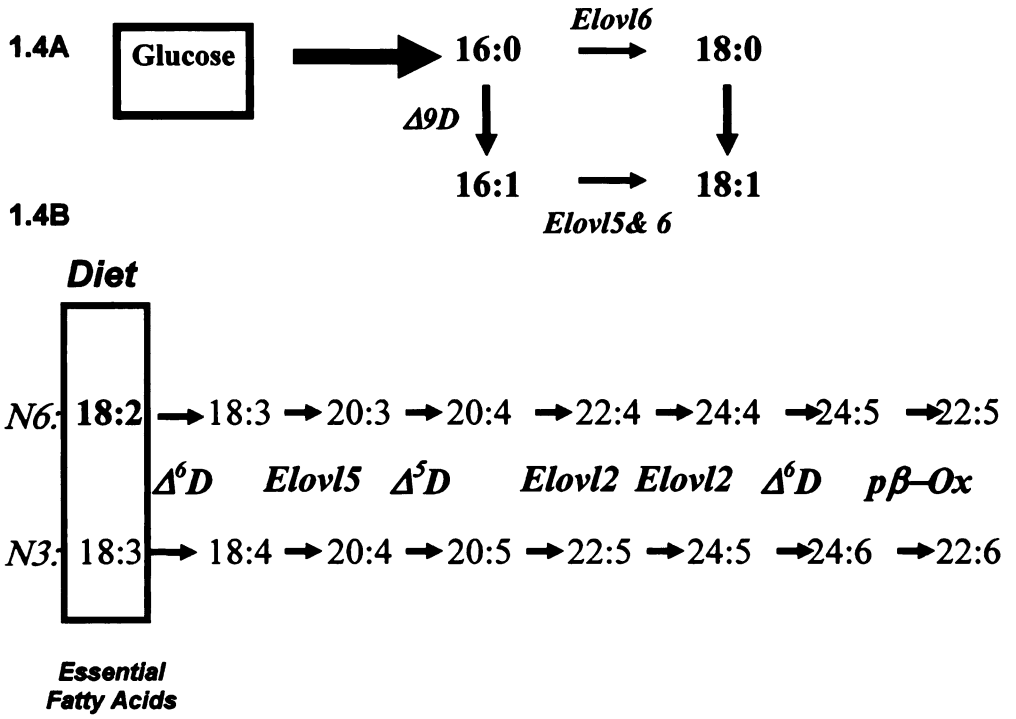


Figure 1. 4. Scheme of endogenous fatty acid synthesis pathway. 4A described the de novo fatty acid synthesis from glucose. Elovl5, Elovl6 and Δ9-desaturase (Δ9D) are involved in this pathway. The end products of this pathway (16:0, 16:1 and 18:1) accumulate in tissues. 4B illustrates the polyunsaturated fatty acid synthesis pathway from essential fatty acids. 18:2n6, and 18:3n3 are converted to long chain unsaturated fatty acids *in vivo* by a series of elongation (Elovl2 & -5) and desaturation (Δ^5-desaturase [Δ^5D] and Δ^6-desaturase [Δ^6D] reactions that take place in the endoplasmic reticulum and the peroxisome via β-oxidation (pβ-Ox)

pathways to generate polyunsaturated fatty acids. As illustrated in Fig. 1.4, these two chemical modifications often alternate in the synthesis of long chain PUFA.

The predominant saturated and monounsaturated fatty acids synthesized in mammals are long chain fatty acids (Fig. 4A). The major products of this pathway are palmitate (16:0), palmitoleate (16:1,n-7), oleate (18:1,n-9) and vaccinate (18:1,n-7). Mead acid (20:3,n-9) can arise from this pathway when essential fatty acids (18:2,n-6 and 18:3,n-3) are deficient or when animals are fed olive oil and treated with peroxisome proliferators, like WY14,643 [26].

Stearoyl-CoA desaturase (SCD), also known as Δ9 desaturase, is the rate-limiting enzyme catalyzing the synthesis of 18:1 and 16:1. 18:1 is the preferred substrate for acyl-CoA:cholesterol acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT), the enzymes responsible for cholesteryl ester and triglyceride synthesis, respectively [27]. In addition, 18:1 is the major MUFA in human adipose tissue and in the membrane phospholipid of most non-neuronal cells. The ratio of 18:0 to 18:1 has been implicated in the regulation of cell growth and differentiation through effects on membrane fluidity and signal transduction [10]. MUFAs also influence apoptosis and may have some role in mutagenesis of some tumors [28-30]. Overall, these fatty acids are important components of phospholipids; they also represent the largest energy storage reservoir in the form of triglycerides, and are the preferred fatty acids used for the esterification of cholesterol.

A second pathway is for synthesis of unsaturated fatty acids utilizes the essential fatty acid precursors (LA, 18:2,n-6 and ALA, 18:3,n-3) to form the long-chain derivatives

arachidonic acid (AA, 20:4,n-6) and docosahexaenoic acid (DHA, 22:6,n-3), respectively (Fig. 1.4B). The first part of this metabolic pathway takes place in the endoplasmic reticulum and consists of sequential alternating elongation and desaturation steps catalyzed by fatty acid elongase-2 (Elovl2) and -5 (Elovl5) and the desaturases, Δ^5-desaturase (Δ5D) and Δ^6-desaturase (Δ6D). The Δ^6-desaturase is the rate-limiting step of the pathway. A part of the pathway that applies only to the synthesis of DHA (22:6,n-3) involves the elongation of eicosapentaenoic acid (EPA, 20:5,n-3) to 22:5,n-3 and 24:5,n-3 by Elovl2, followed by desaturation of 24:5,n-3 by Δ6D to form 24:6,n-3. 24:6,n-3 is then chain shortened in the peroxisome, i.e., peroxisomal β-oxidation, to form 22:6,n-3. These pathways, defined by Sprecher and his colleagues [31], are the only metabolic routes to generate AA (20:4, n6) and DHA (22:6, n3) from dietary precursors, 18:2,n-6 and 18:3,n-3 (Fig. 1.4 B). 18:2,n-6, 20:4,n-6 and 22:6,n-3 are the major PUFA accumulating in membranes of all cells.

Of the two essential fatty acids (18:2,n-6 and 18:3,n-3), linoleic acid (18:2,n-6) is the predominant essential fatty acid in the diet. Dietary abundance, coupled with the fact that much of dietary 18:3,n-3 is β-oxidized limits the availability of 18:3,n-3 for PUFA synthesis [32]. As a result, little 18:3,n-3 accumulates in tissues, appears in the blood or is converted to long-chain PUFA.

The products of these reactions provide substrates for complex lipid synthesis; such as: 1) incorporation into phosphoglycero- and phospho-ether lipids in membranes (AA and DHA), neural tissue is enriched in DHA. Little AA and DHA appear in sphingolipids; 2) substrates for eicosanoid (prostaglandins, thromboxanes, leukotrienes &

prostacyclins are derived from 20:4,n-6) and docosanoid synthesis (resolvins & protectins are derived from 22:6,n-3); 3) β-oxidation for energy metabolism; 4) Stored as neutral lipids, triglycerides and cholesterol esters. AA, but little DHA, appears in cholesterol esters (unpublished observation). Cunnane's laboratory used isotope tracer and other methods to show that membrane lipids in liver and brain preferentially use exogenous long chain PUFAs rather than those produced endogenously [33]. AA that is not incorporated into membranes may be used by eicosanoid metabolism which requires a readily available supply at all times. AA is the major precursor for the synthesis of eicosanoids, which are powerful cellular regulatory substances and mediators of inflammation, platelet aggregation, T-cell proliferation, lymphocyte migration, vasoconstriction and dilation, and the production of several immune and inflammatory substances [34]. EPA (20:5,n-3) is a poor substrate for cyclooxygenase-1 and -2. Eicosanoids derived from 20:5,n-3 typically have weak activity when compared to the AA-derived eicosanoids [17,35,36]. Thus, EPA can moderate the production and activity of AA-derived eicosanoids. It is believed that eicosanoids derived from n-6 fatty acids contribute to the development and deterioration of several chronic diseases [37-40]. DHA, which is the end product of the n-3 PUFA pathway, is the most abundant n-3 PUFA in all tissues. It is critical for the development of central neural system and retina [41-43]. Deficiency of DHA causes a similar phenotype to essential fatty acid deficiency, including severe macular dystrophy and impaired cognitive function.

1.1.2. Fatty acid regulation of gene transcription

Fatty acids regulate the expression of numerous genes, both positively and negatively [14,44-46]. Fatty acids directly bind to and regulate several nuclear receptors, including members of the peroxisome proliferator-activated receptor (PPAR) family (PPARα, β/δ, γ1 & γ2), liver X receptor (LXRα, but not LXRβ), retinoid X receptor α (RXRα) , hepatic nuclear factor-4α (HNF-4 α) [47-55]. In this case, fatty acids act like hydrophobic hormones to control the activity of these key transcriptional factors. Of these, however, the PPAR family are generally considered bona fide in vivo targets of fatty acids [14].

In contrast, fatty acids also regulate other transcriptional factors through indirect mechanism by changing their nuclear abundance or activity. These transcription factors include sterol regulatory element binding protein-1 (SREBP-1, a & c) [56], MAX-like factor-X (MLX), carbohydrate regulatory element-binding protein (ChREBP) complex [55,57] and nuclear factor κB (NFκB) [14]. In vivo studies with rats and mice have established that hepatic PPARα, SREBP-1, ChREBP and MLX are target for control by dietary fatty acids [58-61].

PPARα induces fatty acid oxidation, whereas SREBP-1 induces fatty acid synthesis. ChREBP/MLX induce glucose utilization for fatty acid synthesis [55,57,62]. These mechanisms control, not only hepatic lipid metabolism, but also whole body lipid metabolism by affecting VLDL composition and secretion. As such, these regulatory pathways may contribute to the onset and progression of chronic disease, such as

atherosclerosis, diabetes and obesity [44,63]. This review will mainly focus on the three transcriptional factors, PPARα, SREBP-1 and the ChREBP/MLX heterodimer.

1.1.2.1 PPARα

PPARα is the predominant PPAR subtype expressed in liver. It can be activated by PUFA and their oxidized derivatives as well as by hypolipomic drugs of the fibrate family, including fenofibrate or gemfibrozil [51]. PPARα plays a role in the regulation of an extensive network of genes involved in glucose and lipid metabolism [58,64-67]. PPARα, as well as other PPARs, bind DNA as a heterodimer with RXR at direct repeat in the 5' flanking regions of many genes, such as carnitine palmitoyltransferase (CPT), acyl-CoA oxidase (AOX), and fatty acyl-CoA synthetase-1 (ACS1) [68-70]. Like other nuclear receptors, ligand binding recruits co-regulators to the promoter and changes gene transcription [71].

PPARα was the first transcription factor identified as a prospective fatty acid receptor [51]. PUFAs are recognized as the PPARα activators [53]. This finding parallels the metabolic findings that PUFA induce fatty acid oxidation [72]. In cells, non-esterified fatty acids (NEFA) serve as PPARα ligands. Since NEFA bind PPARα with different affinities, the composition of intracellular NEFA is an important determinant in the control of PPAR activity. Intracellular NEFA composition is affected by the concentration of exogenous fatty acids entering cells, their rate of removal via acyl CoA-synthetase-dependent and -independent mechanisms, *e.g.* microsomal mono-oxygenation, and the return of NEFA or oxidized lipids to the NEFA pool as a result of lipid metabolism [14]. The relative abundance of several putative fatty acid ligands for PPARα in rat primary

hepatocytes is 20:4,n-6 = 18:2,n-6 = 18:1,n-9 > 22:6,n-3 > 18:3,n-3 or n-6 = 20:5,n-3 [53]. Challenging cells with 18:1,n-9, 18:2,n-6 or 20:4,n-6 has little impact on the mass of these fatty acids in the NEFA fraction of cells. In contrast, the addition of 20:5,n-3, 22:5,n-3 and 22:6,n-3 to hepatocytes significantly enriches the NEFA fraction of cells with these fatty acids. Of the 3 n-3 PUFA, 20:5,n-3 significantly induced PPARα activity and PPARα target genes, like AOX and CYP4A [53]. Structural analysis of PPARβ indicated that the carboxyl end of fatty acid inserts into the hydrophobic pocket of ligand binding domain (LBD) [73]. 20:5,n-3 and 22:6,n-3 have a similar structure near the carboxyl end of carbon chain. However, elongation of 20:5,n-3 to 22:5,n-3 increases the rigidity of the fatty acid, which may influence its binding to PPARα [14]. This indicates that major changes in intracellular NEFA composition and mass alone may not be sufficient to trigger a PPARα response. Fatty acid structure may be a key factor in the control of PPARα activity.

Although PUFA are weak agonists of PPAR compared with pharmacological agonists (e.g., fibrates and thiazolidinediones), PUFA have significant effects on cell physiology. PUFA-mediated induction of PPARα-regulated genes shifts hepatic metabolism away from lipid synthesis and storage toward lipid oxidation [74,75]. It also affects insulin sensitivity in various tissues, particularly skeletal muscle [76]. As such, PUFA composition of cells will likely impact gene expression and sensitivity to key hormonal regulatory systems.

1.1.2.2. SREBP-1

SREBPs are basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factors involved in cholesterol, fatty acid and complex lipid synthesis [77]. SREBPs bind DNA cis-regulatory elements called sterol-regulatory elements (SREs) as dimers. There are two separate genes encoding the SREBP-1 and SREBP-2. SREBP-1 regulates the expression of genes involved in fatty acid and triglyceride synthesis, while SREBP-2 regulates the expression of genes involved in cholesterol synthesis [78]. Two isoforms of SREBP-1 have been described. These isoforms, SREBP-1a and SREBP-1c, arise from differential promoter and exon usage. SREBP-1c is the predominant SREBP-1 isoform expressed in rodent and human liver. Despite its greater abundance, SREBP-1c is reported to be the weaker of the two transcription factors in activating gene transcription. This is because SREBP-1c lacks a 29 acidic rich amino acid region present in SREBP-1a.

When SREBPs are first translated as large precursor proteins (~125 kd). These proteins are tethered to the endoplasmic reticulum (ER). SREBP binds a second membrane protein SREBP-cleavage activating protein (SCAP) at its C-terminal end. A third family of proteins, INSIGs, interacts with SCAP in the endoplasmic reticulum. Depletion of sterol from cells leads to the movement of the SCAP-SREBP complex from the endoplasmic reticulum to the Golgi. SCAP plays an escort role for this movement, while INSIG interacts with SCAP and prevent this movement when intracellular sterols are high [79,80]. Two proteases, called Site 1 protease (S1P) and Site 2 protease (S2P) are present in the Golgi. These proteases cleave SREBP precursors to release the mature nuclear form of SREBP (nSREBP, ~65 kd) from the Golgi. SREBPs dimerized and bind

to importin-β for transport to the nucleus. Once in nuclei, SREBP bind as dimers at SRE in promoters of target genes. SREBP binding to SREs promotes recruitment of co-activators, e.g., CBP, RNA polymerase II and initiates gene transcription. There are no reports of SREBP functioning as receptors for either cholesterol or fatty acids. As such, genes regulated by SREBPs are controlled by factors affecting the nuclear abundance of SREBP.

In the liver, SREBP-2 is more sensitive to the cholesterol mediated inhibition than SREBP-1. In contrast to SREBP-2, the nuclear content of SREBP-1 is suppressed by unsaturated fatty acids. This phenomenon is observed in livers of animals fed PUFA-containing diets as well as in primary hepatocytes and some, but not all, SREBP-1c-expressing cells lines treated with PUFA [74,81]. The effects of PUFA on SREBP-1 levels are very complex. It has been attributed to inhibition of transcription of the SREBP-1c gene as well as enhanced turnover of the mRNA encoding SREBP-1, interference with the SREBP-1 processing, and enhanced proteasomal degradation [61,82]. The effect of 20-22 carbon PUFA on SREBP-1 mRNA and gene transcription is similar. 22:6,n-3, however, has an added impact on controlling SREBP-1 nuclear content. 22:6,n-3enhances 26S proteasomal degradation of nuclear SREBP-1, but not the SREBP-1 precursor or nuclear SREBP-2 [83]. As such, 22:6,n-3 is the most potent fatty acid suppressor of SREBP-1 nuclear abundance and SREBP1-mediated gene expression.

1.1.2.3. ChREBP/MLX

ChREBP and MLX are bHLH-LZ transcription factors. They bind as heterodimers to carbohydrate regulatory elements (ChoRE) located in promoters of

glucose-responsive genes, like L-type pyruvate kinase (LPK), ACC, FAS, S14, SCD-1 and GLUT2 [62,84-86]. Both our group [55] and the Postic group [57] have reported that components in the ChREBP/MLX heterodimer are regulated by PUFA in liver and primary hepatocytes. However, the results from these two groups are not identical. Postic's group indicated that PUFA suppressed ChREBP activity by enhancing ChREBP mRNA decay as well as inhibiting its nuclear translocation. Our group reported that n-3 PUFA have no effect on ChREBP nuclear abundance, but suppressed MLX nuclear abundance. This controversy may come from the different animal species as well as the experiment design. Whatever the case, finding that PUFA control ChREBP/MLX nuclear abundance provides an explanation for those fatty acid-regulated hepatic genes that were independent of PPARα and SREBP-1 [55].

1.1.2.4. Other transcription factors regulated by fatty acids

1.1.2.4.1 Liver X receptor (LXRα)

LXRs were first identified as fatty acid regulated transcriptional factors by Brown and Goldstein group [87]. LXRs, like PPARs, belong to the steroid-thyroid receptor superfamily. There are two subtypes, LXRα and LXR β in this family. LXRα is the predominant one expressed in liver. LXRs bind oxysterols and regulate the expression of genes involved in hepatic bile acid synthesis, reverse cholesterol synthesis, lipogenesis and glucose uptake [81,88,89]. PUFA antagonize oxysterol activation of LXRα in HEK 293 and hepatoma cell lines by interfering with oxysterol binding [54,87]. However, a recent study from our group showed that LXR-regulated transcripts, such as CYP7A, ABCA1, ABCG5 and ABCG8, were not sensitive to PUFA suppression in rat primary

hepatocytes and in liver [52]. The condition used to access PUFA control of transcriptional factors in this study was sufficient to suppress SREBP-1c and induce PPARα targeted genes. These observations raised doubt as to whether LXRα were *bona fide* targets for PUFA control in vivo.

1.1.2.4.2 Hepatic nuclear factor-4 (HNF4)

HNF4 is an orphan nuclear receptor regulating an extensive network of genes controlling glycolysis and lipogenesis in liver and other tissues [14]. Few of these genes are regulated by dietary PUFA in vivo except L-PK, glucose 6 phosphatases, apolipoproteins A1 and CIII. Our laboratory first reported that HNF-4 recognition sequence is a component of the PUFA-response region of L-PK gene [90]. Later, the Bar-tana group showed evidence that HNF4α bound fatty acyl CoAs. They suggested that fatty acyl CoA might account for the effect of fatty acids on transferring and Apo CIII promoter activity [47]. However, structural studies on HNF-4 protein indicated that fatty acids and not fatty acyl CoA co-crystallized with HNF-4 ligand binding domain. These findings triggered controversy over HNF-4 ligands. Recent studies from our laboratory showed that 20:5,n-3 treatment in rat primary hepatocytes promotes transient change HNF-4α–LPK promoter interaction, but no effect on HNF4 transactivation on LPK promoter [55]. 20:5, n-3 is a minor fatty acid in the pool of NEFA and esterified lipid fraction of liver and hepatocytes [83]. Greater than 99% of intracellular 20:5,n-3 was esterified after hepatocytes were treated. Intracellular non-esterified 20:5,n-3 is highest at 1.5 and lowest at 24 hours after challenging cells with 20:5,n-3 [55]. This suggest that non-esterified 20:5,n-3 can regulate HNF-4 binding activity to LPK promoter. The

mechanism for this control, however, is unknown. In the view of the Jump lab, this control does not involve direct binding of 20:5,n-3 by HNF-4.

This discussion of fatty acid regulated transcription factors highlights those factors that are well-established targets for PUFA control, i.e., PPARα and SREBP-1, and those that are either recently identified (ChREBP/MLX) or there is some controversy (LXRα and HNF-4α). An underlying fact is that the type and quantity of intracellular fatty acid controls these transcription factors and their regulatory networks. This thesis focuses on a metabolic pathway (fatty acid elongation) that alters the type of fatty acid in the cells. I am predicting that changes in fatty acid elongation will impact certain transcriptional regulatory networks.

1.1.3. Fatty acid and chronic disease

1.1.3.1. Cardiovascular disease

Numerous studies have revealed the benefits of n-3 PUFA on cardiovascular disease (CVD) [91-93]. N-3 PUFA can be obtained from two dietary sources: seafood and certain nut and plant oils. Fish or fish oil contains EPA and DHA, whereas canola and walnut oil contain ALA. ALA is less potent than EPA and DHA. Mechanisms by which n-3 fatty acids reduce CVD risk are under active investigation. Research to date, however, suggests there are several mechanisms involved in the protective effect of PUFA on cardiovascular health.

Evidence from epidemiological studies and clinical trials suggest that n-3 PUFA have an important anti-arrhythmic effect in patients with CVD [94,95]. However, the details of the anti-arrhythmic action of n-3 PUFA remain to be elucidated. In addition, n-3 PUFA is reported to decrease risk for thrombosis due to decreases in platelet aggregation. A decrease in thromboxane (TXA_2) coupled with increases in prostaglandin (PGI2 and PGI3) production, decrease in whole blood viscosity and an increase in bleeding time [96]. As described above, eicosanoid metabolic products from AA (n-6 PUFA) are more biologically active than those derived from EPA (n-3 PUFA) [14]. Supplementation of n-3 PUFA will replace n-6 PUFA as the precursor of eicosanoids, while increasing the amount of n-6 PUFA consumption contributes to the development of thrombi and athomas. PUFA is also reported to decrease triglyceride and remnant lipoprotein levels by replacing saturated fat in diet. Additional benefit is shown by n-3 PUFA which consistently lowers blood triacylglycerol levels in hypertriglyceridemic patients. In contrast, n-6 fatty acids either have no effect or increase blood triglycerides [97]. In addition, PUFA can decrease rate of growth of the atherosclerotic plaque by improving endothelial function and reducing inflammatory responses [98].

1.1.3.2 Diabetes

Type 2 diabetes (non-insulin dependent diabetes melittus, NIDDM) is the most prevalent form of diabetes in western societies. NIDDM is a metabolic disorder characterized by hyperglycemia in the presence of insulin resistance and hypertriglyceridemia. Skeletal muscle is the principal site of insulin-mediated glucose disposal. Campbell and colleagues showed that insulin resistance and hyperinsulinemia

are inversely associated with the amount of C20 and C22 fatty acids in the phospholipids of muscle cell membranes in patients with coronary heart disease and normal volunteers [99]. Increasing the amount of C20 and C22 PUFA in membrane leads to an increase in membrane fluidity, insulin receptor and insulin action [100]. High dietary intake of saturated and trans fat interferes with the elongation and desaturation of essential fatty acids to AA, EPA and DHA. These fatty acids also contribute to the development of insulin resistance [100,101]. Skeletal muscle triglyceride levels are also inversely related to insulin action [102]. Clinical trials of dietary supplemention of n-3 PUFA to type 2 diabetic patients lowers triacylglycerol concentrations with no adverse effects on glucose control [103].

Leptin is a hormone that suppresses appetite and enhances basal metabolic rate. As such, it has a remarkable effect on the regulation of body fat [104]. It is reported that serum leptin levels are influenced in patients with type 1 diabetes by the type of fat in the diet [105]. In particular, n-3 PUFA has been found to decrease leptin gene expression both in vivo and in vitro [106].

1.1.3.3 Inflammation

Inflammation is characterized symptomatically by pain, redness, and swelling. The disordered or excessive inflammation also contributes to the loss of function. This results from the release of inflammatory mediators, predominantly from activated leukocytes that migrate into the target area. Among the key inflammatory mediators are prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), which are derived from the n-6 PUFA, AA [107]. Eicosanoids derived from AA and EPA have very similar molecular

structures, but very different biologic effects. Those derived from EPA are generally much less potent in inflammation than those derived from AA. As such, a majority of n-6 fatty acids will result in a proinflammatory status by producing prostaglandins of the 2 series and leukotrienes of the 4 series. As the ratio of n-3 to n-6 increases, more prostaglandins of the 3 series and leukotrienes of the 5 series are produced. As such, n-3 fatty acids exert anti-inflammatory effects via competitive inhibition of the n-6-derived proinflammatory eicosanoids [108].

The immune response also plays a role in the development of inflammation by producing immunologic mediators such as cytokines. PUFA have been shown to have effects on immune response [109]. Dietary supplementation of n-3 fatty acids influences the cytokine production and lymphocyte proliferation. This effect is more dramatic on older versus younger women.

1.1.3.4 Cancer

Cancer is a disease characterized by abnormal cell division without any control. Many cancers have a high mortality rate. Epidemiological studies indicate that women in countries with high-fat diets have a higher risk (5-fold) of breast cancer than women in countries with low-fat consumption. Such observations strongly suggest that a high intake of dietary fat could increase breast cancer risk [110,111]. This also provides evidence that dietary or exogenously derived fatty acids may play an important role in the carcinogenesis, evolution and/or progression of breast cancer. Several studies also showed that diets containing corn oil, with high levels of n-6 PUFA, enhance breast and colon tumorgenesis in rodents. In contrast, diets enriched in n-3 PUFA, e.g., fish oil,

reduces carcinogenesis [112]. Saturated fatty acids have no discernible effect on mammary carcinogenesis or progression. Several mechanisms were proposed to explain the mechanism by which the intake of n-3 PUFA might lower the risk of cancer. Inhibition of eicosanoid biosynthesis from AA is the most important one among these mechanisms. High intake of n-6 PUFA experimentally induces various physiological and metabolic effects [112,113]. It can increase ornithine decarboxylase activity in colon mucosa which leads to the enhanced epithelial polyamine levels and increase colon crypt cell proliferation. It can also up-regulate activities of protein kinases, such as protein kinase C in rodent mammary gland and increases number of estrogen receptor binding sites. Prostaglandins, thromboxanes, leukotrienes and hydroxy and hydroxyperoxy fatty acids are involved in tumor initiation and promotion, cell proliferation, tissue invasion and metastatis. Tumor cells produce more eicosanoids than their normal cell counterparts; oxidized lipids ultimately derived from linoleic acid have been linked to increased cell growth and tissue metastasis. The finding that oleic acid and n-3 PUFA, specifically EPA, inhibits the desaturation reaction, the first step from linoleic acid to arachidonic acid and eventually to eicosanoid production, may partially explain their inhibitory effects on tumorigenesis.

Clearly, the impact of dietary fat on chronic disease is significant. What is less clear is how chronic disease might impact enzymes involved in fat metabolism. Equally unclear is how changes in these enzymes impact cellular fat composition and impact the onset and progression of these diseases. This thesis will begin to address these important medical issues.

1.2. Fatty acid elongases

The focus of this thesis is on fatty acid elongases. This section will review our current understanding to the role these enzymes play in lipid metabolism.

Fatty acid elongases (Elovls) are the condensing enzymes that interact with 3-keto acyl-CoA reductase, a dehydratase and trans-2,3-enoyl-CoA reductase to elongate fatty acids (Fig. 1.3). The rate of fatty acid elongation is determined by the activity of elongase (condensing enzyme). There are 7 distinct elongase subtypes (Elovl-1, -2, -3, -4, -5, -6 & -7) present in the mouse, rat and human genomes. Each has a specific substrate preference in terms of chain length and the degree of fatty acid unsaturation. The members of this family are predicted to have 5 or 6 transmembrane spanning domain that traverse the endoplasmic reticulum. Since the third transmembrane stretch is very long, it is not clear whether 5 or 6 transmembrane domains in this family protein [114]. Also, this protein family share a common structure of a single histidine-box motif (HXXHH), and an ER retention signal (KKXX-like) [115].

Elovl3 was the first elongase to be identified by Jacobsson group in 1997 [116]. It was originally named cig30 (Cold induced glycoprotein with molecular weight about 30kDa) because the gene can be induced significantly in brown adipose tissue when the mice are exposed to cold environment. In silica search identified two homologues of cig30, which were first termed Ssc1 and Ssc2 (sequence similarity to cig30) [114]. The official names for these two enzymes are Elovl1 and Elovl2, respectively. Shortly after that, a murine long chain fatty acid elongase was identified by gene array analysis from

SREBP1 transgenic mice liver [117]. This enzyme was called Elovl6. Elovl5 was the first human elongase identified [118]. Later, the rat Elovl5 was reported to have similarity to human Elovl5 [119]. Mutation in the human Elovl4 gene were described as being responsible for an autosomal dominant macular degeneration [120] (Table 1.1). Because of their separate isolations and cloning over the years, fatty acid elongases have been given many names, e.g, Cig30, SSC1, etc. With the discovery of multiple subtypes in human and rodent genomes, the nomenclature was revised to the following. Elovl, elongase of very long fatty acid. (Table 1.2).

Table 1. 1. Alignment of mouse fatty acid elongases

```
Accession# Elovl#
nm_019422      1        -----------------MEAVVNLYHELMKHADPRIQSYPLMGSPLLITSILLTYVYFI
bab31310       7        ------------MAFSDLTSRTVRFYDNWIKDADPRVEDYLLMSSPLPQTIILGLYVYFV
af170908       2        -----------MEQLKAFDNEVNAFLDNMFGPRDSRVRGWFLLDSYLPTFILTITYLLSI
nm_134255      5        --------------MEHFDASLSTYFKAFLGPRDTRVKGWFLLDNYIPTFVCSVIYLLIV
nm_148941      4        MGLLDSEPGSVLNAMSTAFNDTVEFYRWIWTIADKRVADWPLMQSPWPTISISTLYLLFV
bc016468       3        --------MDTSMNFSRGLKMDLMQPYDFETFQDLR---PFLEEYWVSSFLIVVVYLLLI
ay053453       6        ------------MNMS----VLTLQEYEFEKQFNENEAIQWMQENWKKSFLFSALYAAFI
                                                          : .     :             *   :

nm_019422      1        LSLGPRIMANRKPFQLRGFMIVYNFSLVILSLY-IVYEFLMSGWLSTYTWRCDPIDFSNS
bab31310       7        TSLGPKLMENRKPFELKKAMITYNFFIVLFSVY-MCYEFVMSGWGTGYSFRCDIVDYSQS
af170908       2        W-LGNKYMKNRPALSLRGILTLYNLAITLLSAY-MLVELILSSWEGGYNLQCQNLDSAGE
nm_134255      5        W-LGPKYMKNRQPFSCRGILQLYNLGLTLLSLY-MFYELVTGVWEGKYNFFCQGTRSAGE
nm_148941      4        W-LGPKWMKDREPFQMRLVLIIYNFGMVLLNLF-IFRELFMGSYNAGYSYICQSVDYSND
bc016468       3        V-VGQTYMRTRKSFSLQRPLILWSFFLAIFSILGTLRMWKFMATVMFTVGLKQTVCFAIY
ay053453       6        F-GGRHLMNKRAKFELRKPLVLWSLTLAVFSIFGALRTGAYMLYILMTKGLKQSVCDQSF
                           *   *  *  :. :  :  :.: :.::.                    :

                                                                  His-Box
nm_019422      1        PEALRMVRVAWLFMLSKVIELMDTVIFILRKKDGQVTFLHVFHHSVLPWSWWWGIKIAPG
bab31310       7        PRAMRMVHTCWLYYFSKFIELLDTIFFVLRKKNSQVTFLHVFHHTIMPWTWWFGVKFAAG
af170908       2        G-DVRVAKVLWWYYFSKLVEFLDTIFFVLRKKTNQITFLHVYHHASMFNIWWCVLNWIPC
nm_134255      5        S-DMKIIRVLWWYYFSKLIEFMDTFFFILRKNNHQITVLHVYHHATMLNIWWFVMNWVPC
nm_148941      4        VNEVRIAAALWWYFVSKGVEYLDTVFFILRKKNNQVSFLHVYHHCTMFTLWWIGIKWVAG
bc016468       3        TDDAVVRFWSFLFLLSKVVELGDTAFIILRKR--PLIFVHWYHHSTVLLFTSFGYKNKVP
ay053453       6        YNGPVSKFWAYAFVLSKAPELGDTIFIILRKQ--KLIFLHWYHHITVLLYSWYSYKDMVA
                              : : .**  *  ** :::***.   : .:* :**  :        :

nm_019422      1        GMGSFHAMINSSVHVVMYLYYGLSALGPVAQPYLWWKKHMTAIQLIQFVLVSLHISQYYF
bab31310       7        GLGTFHAFLNTAVHVVMYSYYGLCAMGPAYQKYLWWKKHLTSLQLVQFVLVTIHIGQIFF
af170908       2        GQSFFGPTLNSFIHILMYSYYGLSVF-PSMHKYLWWKKYLTQAQLVQFVLTITHTLSAVV
nm_134255      5        GHSYFGATLNSFIHVLMYSYYGLSSI-PSMRPYLWWKKYITQGQLVQFVLTIIQTTCGVF
nm_148941      4        GQAFFGAQMNSFIHVIMYSYYGLTAFGPWIQKYLWWKRYLTMLQLVQFHVTIGHTALSLY
bc016468       3        SGGWF-MTMNFGVHSVMYTYYTMKAA--KLKHPNLLPMVITSLQILQMVLGTIFGILNYI
ay053453       6        GGGWF-MTMNYGVHAVMYSYYALRAA--GFRVSRKFAMFITLSQITQMLMGCVINYLVFN
                         . . *   :*  :* :** ** :       :        :*  *: *: :

nm_019422       1       MPS-----CNYQYPIIIHLIWMYGTIFFILFSNFWYHSYTKG-----KRLPRAVQQ----
bab31310        7       MED-----CNYQYPVFLYIIMSYGCIFLLLFLHFWYRAYTKG-----QRLPKTLE-----
af170908        2       KP------CGFPFGCLIFQSS-YMMTLVILFLNFYIQTYRKK---PVKKELQEKE-----
nm_134255       5       WP------CSFPLGWLFFQIG-YMISLIALFTNFYIQTYNKKGASRRKDHLKGHQNGSVA
nm_148941       4       TD------CPFPKWMHWALIA-YAISFIFLFLNFYTRTYNEP-----KQSKTGKTATNGI
bc016468        3       WRQEKG--CHTTTEHFFWSFMLYGT-YFILFAHFFHRAYLRP-----KGKVASKSQ----
ay053453        6       WMQHDNDQCYSHFQNIFWSSLMYLS-YLVLFCHFFFEAYIG------KVKKATKAE----
                                *               *    . ** :*: .:*        :

nm_019422       1       --NGAPATTKVKAN------------
bab31310        7       --NGN---CKSKRH------------
af170908        2       VKNGFPKAHLIVANGMTDKKAQ----
nm_134255       5       AVNGHTNSFPSLENSVKPRKQRKD--
nm_148941       4       SSNGVNKSEKALENGKPQKNGKPKGE
bc016468        3       -------------------------
ay053453        6       ---------------------------
```

Table 1. 2. Chromosomal Location of Elongase Subtypes in Mouse, Rat and Human.

Elongase Subtype (Elovl)		Mouse	Rat	Human
1	Genbank #	NM_019422		AAL71993
	Alias	SSC1		CGI-88, SSC1
	Chromosome #	4	1	1
	Location, mb	117.3	138.9	43.2
2	Genbank #	AF170908		AAH60809
	Alias	SSC2		SSC2
	Chromosome#	13	17	6
	Location, mb	41.2	29.5	11.1
3	Genbank#	BC016468		AAH34344
	Alias	CIG30		ELO3
	Chromosome #	19	1	10
	Location mb	46.3	251.6	103.7
4	Genbank #	NM_148941	Q9GZR5	AAH38506
	Alias	ELO4		
	Chromosome #	9	8	6
	Location, mb	86.5	88.9	80.6
5	Genbank #	NM_134255	NM_134382	AAF70631
	Alias	FAE1	RELO1	HELO1
	Chromosome #	9	8	5
	Location, mb	80.5	83	53.2
6	Genbank #	AY053453	NM_134383	NP076995
	Alias	LCE, FACE	Elo2	
	Chromosome #	3	2	4
	Location, mb	133.1	226.9	111.4
7	Genbank #	BAB31310,	AKA050441	
	Alias	9130013K24RIK		Q9NT66
	Chromosome #	13	2	5
	Location, mb	106.8	39.5	60.1

1.2.1 Elovl1

Elovl1 was first discovered by its homology to Elovl-3. Complementation studies with yeast mutants with defective fatty acid elongation with the murine Elovl1 indicated that Elovl-1 was involved in elongation of very long chain fatty acids (up to 26 carbons) and sphingolipid formation [121]. Sphingolipids are essential for cell proliferation. Abnormal sphingolipid synthesis hinders cell growth [122]. Numerous studies show that sphingolipids, as well as very long chain fatty acid synthase (VLCFAS) are important for the formation of functional lipid microdomains (lipid rafts) in plasma membrane [123]. Disruption of the yeast Elovl1 homologue, Elo3, leads to a broad range of defects including altered expression of the plasma membrane ATPase and lower glucose uptake [124]. The overlapping functions of yeast Elo3 and murine Elovl1 indicates that mammalian enzymes are also important in membrane related functions associated with the lipid rafts.

Elovl1 is ubiquitously expressed in mouse tissues. This means most mammalian tissues are endowed with the capacity to synthesize VLCFA up to C26. Interestingly, the mRNA level of Elovl1 is particularly high in stomach, lung, kidney, skin and intestine. The common feature of these tissues is that they have separate compartments with widely different water content. Therefore, it is conceivable that higher Elovl1 levels are needed for VLCFA synthesis in order to reduce water permeability of the epithelial barrier [125]. Quaking and Jimpy are two mouse mutant strains that have dramatically decreased Elovl1 mRNA level. Both strains are deficient in nerve myelination throughout the central nerve system (CNS), which results in severe motor problems and early death.

Moreover, both strains show decreased elongation activity towards 20:0-CoA and 22:0-CoA and lower levels of VLCFA in the brain [126]. The severity of elongation defect is proportional to Elovl1 mRNA expression.

1.2.2 Elovl2

When Elovl2 (SSC2) gene was first described in 2000, there was no functional characterization for this enzyme. Investigators hypothesized that it may play a role in PUFA synthesis based on the complementation studies from yeast system [114]. Later on, both mouse Elovl2 and human Elovl2 were shown to use arachidonic acid (20:4,n-6), eicosapentaeonic acid (20:5,n-3), docosatetraenoic acid (22:4,n-6) and docosapentaenoic acid (22:5,n-3) in yeast and mammalian HEK 293 cells when they are transfected with the vector expressing Elovl2 gene [117,127]. In contrast, Elovl2 did not elongate saturated and monounsaturated fatty acids. Northern blot analysis revealed that the highest level of expression of Elovl2 was found in mouse liver and testis, followed by brain and kidney [127].

1.2.3 Elovl3

Compared to other mammalian fatty acid elongases, Elovl3 is the one most studied. Elovl3 was first cloned from brown adipose tissue (BAT) of mice exposed to cold temperature [116]. When mice were exposed to cold temperature for 3 days, the microsomal fraction from BAT of Elovl3 ablated mice has decreased ability to elongate saturated C16-C22 substrates. There was, however, little difference in elongation activity after mice were exposed to low temperatures for 3 weeks [128,129]. Further analysis of

fatty acid profile in these mice revealed a transient alteration of several different fatty acid species in the mice after 3 days of cold exposure but not in the mice exposed to cold for 3 weeks cold [129]. Moreover, there are almost no lipid droplets in BAT of Elovl3 ablated mice when they are under thermoneutral conditions (30^0C). Such studies support a role for short term, but not long term, adaptive changes in BAT lipid metabolism following cold exposure.

Jacobsson's group showed that cold exposure induced Elovl3 gene expression required PPARα regulation [130]. In cultured brown adipocytes, a mixture of norepinephrine, dexamethasone, and the PPARα ligand Wy14,643, which rendered the adipocytes a high oxidative state, was required for substantial induction of Elovl3 expression. In contrast, this treatment suppressed Elovl1 mRNA levels. Interestingly, the LXR agonist, T0901317, repressed Elovl-3 expression [130].

A most prominent phenotypic feature in Elovl3 -/- mice was found in the skin. Skin functions as a water barrier and very long chain fatty acids are important for this physiological function. Elovl3-/- mice showed an impaired water barrier function which was attributed to a change of VLCFA synthesis [128]. Although most of identified fatty acid elongases are expressed in murine skin [114,127], nothing was known about the specific roles of the individual enzymes in skin. In situ hybridization indicated that Elovl3 has a distinct expression in the skin that was restricted to the sebaceous glands and the epithelial cells of the hair follicles [128].

Recent studies from the Baler group revealed circadian regulation of Elovl3 in liver that involved RevErbα and SREBP-1. This regulation was sex- and tissue-specific

[131]. The reason for circadian regulation of Elovl3 can be explained by the needs for the liver to synthesize long-chain fatty acids. The main fraction of intracellular VLCFAs in liver is esterified in various lipids, predominantly in sphingolipids. As such, circadian expression of Elovl3 as well as other lipogenic enzymes may contribute to maintenance membrane fluidity, which consequently regulates a rhythm of the membrane-linked signaling pathways [131].

1.2.4 Elovl4

Tissue expression of Elovl4 is one of the most restricted amongst the elongases; it is expressed predominantly in the retina and to a less extent in brain, and testis [120]. The high expression of Elovl4 in the retina suggest an involvement in the synthesis of docosahexaenoic acid, 22:6,n-3. Nearly 50% of all acyl chains in phospholipids are DHA, the highest content of all mammalian tissues.

Mutation of Elovl-4 was the first mutation of a fatty acid elongase linked to human disease. Mutation of the Elovl4 locus is found in patients with the genetic autosomal dominant disease, Stargardts-like macular dystrophy [120]. Stargardts-macular dystrophy is one of several forms of macular dystrophy. This disease is characterized by decreased visual acuity, macular atrophy and extensive fundus flecks. Genetic mapping by Zhang's group identified a 5-bp deletion in the Elovl4 gene that may be responsible for this for of inherited macular degeneration [120]. About the same time, Allikmets, et al [132] found another mutation in Elovl4 gene associated with this disease. This mutation results in a frame shift and the truncation of the ELOVL4 protein, similar to the effect of 5-bp deletion.

In vitro studies show that the mutant Elovl4 protein exerts a dominant-negative effect by recruiting wild-type protein into perinuclear cytoplasmic inclusions [133]. The interaction between the wild-type and mutant forms of Elovl4 and the resultant alteration in the trafficking of the wild-type ELOVL4 protein suggest a mechanism for the pathology associated with in patients harboring the autosomal dominant Stargardts-like macular dystrophy genotype.

1.2.5 Elovl5

The Elovl5 gene encodes a protein of 299 amino acids, which shares 56.4% amino acid sequence identity with Elovl2. Elovl5 is involved in the synthesis of mono and polyunsaturated fatty acids of C16-C22, but not polyunsaturated fatty acids beyond C22 [127]. Elovl5 is widely expressed with high levels in testis, adrenal gland, lung and brain [115]. This is consistent with the fact that these tissues contain very high levels of docosapentanoic acid, 22:5,n-3 and adrenic acid, 22:4, n-6.

1.2.6. Elovl6

The identification of Elovl-6 was described by Moon et al. in 2001. It was discovered in livers of transgenic mice overexpressing SREBP-1 [117]. This enzyme is ubiquitously expressed. High fat-containing tissues such as white and brown adipose tissue, liver, and brain, displayed high levels of Elovl6 transcripts, whereas the low fat-containing tissues like spleen, skeletal muscle, and heart exhibit low levels of the Elovl6 transcript. In vitro microsomal fatty acid elongation assays demonstrated that Elovl6 possessed elongase activity specific for C_{12}-C_{16} saturated and monounsaturated fatty

acids. The enzyme is induced by SREBP1. Its expression is also induced in the liver and adipose tissue in the refed state after fasting, but suppressed by the presence of dietary PUFAs [117,134]. It encodes 267 amino acid residues and has 96% identity with the human homologue of 265 amino acids [115].

1.2.7 Elovl7

In silica analysis of the human and mouse genomes revealed the existence of the seventh member in elongase family. This gene is located on chromosome #13 in mouse, and #2 in rat. It encodes 281 amino acids residues and contains a histidine-rich motif (HXXHH), and ER retension signal (KKXX-like), both typical for members of fatty acid elongase family. The histidines in the HXXHH motif may act with aspartate and glutamate to coordinate an Fe-O-Fe cofactor to receive electrons from cytochrome b5 or a cytochrome b5-like domain in an NAD(P)H-dependent manner [114,117]. The similarities in sequence organization amongst the 7 proteins suggest Elovl7, i.e., BAB31310, mouse; Q9NT66, human, is a fatty acid elongase. Unfortunately, there is no experimental data on Elovl-7 substrate specificity or its regulation.

1.3. Statement of the Problem and Hypothesis.

Multiple mammalian fatty acid elongases and fatty acid desaturases play a major role in determining cellular lipid content. Of these enzymes, the most extensively studied is Δ9D. Multiple factors regulate Δ9D expression. Studies with the D9D -/- mouse implicate this enzyme in the management of hepatic and whole body lipid metabolism,

energy expenditure and tissue sensitivity to insulin [12,135]. These studies have targeted Δ9D as a candidate enzyme for the development of inhibitors of its activity to manage of whole body lipid metabolism.

In contrast to desaturases like D9D, we are in the early stage of understanding the role fatty acid elongases play in cell function and composition. We have little information on their regulation; we are unclear of their substrates and products. We do not know how they are regulated in chronic disease or which transcriptional regulatory networks control their expression. Studies in our laboratory have established a strong link between fatty acid structure and the control of key transcriptional regulatory networks controlling glucose and lipid metabolism [83,136]. Elongase activity alters fatty acid structure. As such, these enzymes have the potential to impact cellular lipid composition and affect key transcriptional regulatory networks. If so, then, elongases might join with Δ9D as targets for drug development in the management of fatty acid-linked chronic diseases.

The goal of this study is to gain information about the regulation of the elongase family of enzymes. A second goal is to gain evidence in support or against the notion that fatty acid elongases play a dual role in hepatic physiology: to control hepatic lipid composition and to impact hepatic gene transcription both in cells (in vitro) and in the mouse (in vivo).

I hypothesized *that fatty acid elongases play a dual role in hepatic physiology: a) to regulate cellular levels of long chain unsaturated fatty acids, and b) to regulate cellular levels of ligands controlling hepatic gene expression.* The aims are:

Aim 1: To identify substrates of elongases in rat primary hepatocytes.

To identify regulatory factors controlling fatty acid elongase family expression.

Aim 2: To elucidate the role of Elovl-2 & Elovl5 on hepatic gene expression in rat primary hepatocytes.

Aim 3: To determine how over expression of specific fatty acid elongases affect hepatic function and blood lipid composition.

The outcome of these studies will provided new information on the role fatty acid elongases play in hepatic physiology.

Chapter 2

Tissue-Specific, Nutritional and Developmental Regulation of Rat Fatty Acid Elongases.

2.1. Introduction

Most cells have the capacity to synthesize fatty acids from glucose, *de novo*. This pathway utilizes products from glycolysis and along with the two enzymes, acetyl CoA carboxylase and fatty acid synthase, generates palmitate (16:0). Insulin, T_3, glucocorticoids and glucose induce, while C_{20} PUFA, glucagon and epinephrine suppress *de novo* lipogenesis [14,46,137]. Many cells also have the capacity to modify fatty acid structure through metabolic pathways that include desaturation, elongation, mono-oxidation and peroxisomal β-oxidation (chain shortening). Such modifications occur to fatty acids generated *de novo* as well as fatty acids derived from the diet. These metabolic pathways play an important role in the maintenance of membrane lipid composition, the generation of precursors for certain signaling molecules, like eicosanoids. These pathways may also contribute to the control of cellular fatty acids impacting specific nuclear receptors, e.g., PPARα [14,53,54].

Of the various pathways known to affect fatty acid structure, physiological control of fatty acid elongation remains poorly defined. The predominant pathway for fatty acid elongation occurs in the endoplasmic reticulum and uses malonyl-CoA and fatty acyl-CoA as substrates for C_2 additions to fatty acids. Elongases are condensing enzymes that interact with 3-keto acyl-CoA reductase, a dehydratase and trans 2,3-enoyl CoA

reductase to elongate fatty acids [24,115,138]. The rate of fatty acid elongation is determined by the activity of the elongase (condensing enzyme) and not the reductases or the dehydratase. Six distinct fatty acid elongase subtypes (Elovl1 through Elovl6) are present in the mouse, rat and human genomes. Elovl1 (Ssc1) and Elovl6 (LCE, FACE, rElo2) elongate saturated and monounsaturated fatty acids. Disorders of sphingolipid metabolism, like the Quaking and Jimpy phenotypes [114] have been associated with impaired Elovl1 activity. Elovl6 is induced in transgenic mice over expressing SREBP-1 [117,134,139]. Elovl2 (Ssc2) substrates include, C_{20-22} PUFA, while Elovl5 (FAE1, Relo1, Helo1) may utilize a broad substrate array, C_{16-22} [117,119]. Elovl2 and Elovl5 likely play a role in endogenous PUFA synthesis; i.e., the conversion of essential fatty acid precursors, linoleic acid (18:2n6) and α-linolenic acid (18:3n3) to arachidonic acid (20:4n6) and docosahexaenoic acid (22:6n3) [139]. Elovl3 (Cig30, Elo3) and Elovl4 (Elo4) are expressed in the skin [140] and retina [141], respectively. Both Elovl3 and Elovl4 elongate a broad array of fatty acids $\leq C_{26}$. Elovl3 is induced in brown adipose tissue following exposure of animals to the cold [142]. Stargardt-like macular dystrophy and autosomal dominant macular dystrophy is associated with defective Elovl4 expression [120].

Many studies have examined the regulation of mammalian elongases at the level of enzyme activity [25,116,117,119,127,134,138,143-156]. Overlapping substrate/product profiles for these enzymes has made it difficult to ascertain which enzyme subtypes are regulated under specific physiological conditions. More recent studies have examined nutritional and tissue-specific regulation of elongase gene expression [116,117,134,140,153,155]. We are unaware of any study that has examined

the expression of all elongase subtypes in specific tissues. We have cloned 5 of the 7 fatty acid elongases known to be expressed in mammals and examined their expression in rat liver. Elovl4 was not examined because of its retina-specific expression. Elovl7 was not studies because it is poorly expressed in liver (unpublished observation). Our studies included an analysis of the tissue-specific expression of elongases as well as the effect of starvation, fish oil and PPARα agonist feeding on hepatic elongase expression and elongase enzymatic activity. Developmental regulation of hepatic elongase expression and activity is also included. Of the 4 fatty acid elongases expressed in rat liver, Elovl5 emerges as the predominant elongase expressed, based on mRNA abundance. Elovl5 may account for much of the developmental and nutritional regulation of rat hepatic fatty acid elongation.

2.2. Methods and Materials

Cloning of mouse and rat fatty acid elongases and desaturases. cDNAs for fatty acid elongases and desaturases were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) methods. Primers corresponding to the open reading frame of each elongase and desaturase (Table 2.1) were designed based on sequences obtained from Genebank and the location of the open reading frame as determined using DNA-Star. Mouse liver was used as template to clone all desaturases and elongases, except Elovl5; Elovl5 was cloned from rat liver. The RT-PCR products were inserted into TOPO-cloning-plasmids (Invitrogen, Carlsbad, CA) and screened by blue-white selection. Positive clones were selected and sequenced at the Genomic Technology Support Facility at Michigan State University. Sequences were verified by alignment to sequences in

Genebank (Blast, http://www.ncbi.nlm.nih.gov/blast/ bl2seq/bl2.html). Rat and mouse elongases are ≥85% homologous at the protein sequence level.

Animals. All procedures for the use and care of animals for laboratory research have been approved by the All University Committee for Animal Use and Care at Michigan State University. Rats are maintained on Harlan-Teklad laboratory chow (#8640) and water *ad lib*. Tissues for the analysis of elongase expression were derived from 60 day old Spraque-Dawley male rats maintained on a chow diet *ad lib*. Nutritional regulation of elongases used a meal-feeding protocol [53,157,158]. Briefly, rats were acclimated to meal-feeding of a high carbohydrate (glucose) diet (HiCHO) [ICN Biochemicals, Aurora, OH] supplemented with olive (Pompiean, Baltimore, MD) at 10% w/w for 7 days. The meal began at 8 AM and ended at noon. After the acclimation period, rats were either maintained on the olive oil diet or switched to a HiCHO diet supplemented with fish oil (Dyets, Inc., Bethlehem, PA) at 10% w/w or a HiCHO diet supplemented with WY14,643 at 0.1% w/w [158]. Animals were maintained on the olive oil, fish oil or WY14, 643 diets for 7 days. Fasted rats were meal fed the HiCHO-olive oil diet 7 days, but were fasted for 24 hrs before euthanasia. Two hours after completion of the final meal animals were euthanized for tissue and blood collection. The developmental studies used timed pregnant female Spraque-Dawley rats from Charles River Laboratories (Kalamazoo, MI). Fetal livers were obtained at 18-19 days post coitum and pooled for analysis. Male suckling pups (1-20 days of age) and weaned male animals (30 days of age) were used as a source of liver. Rats were weaned onto Harlan-Teklad laboratory chow at 21 days postpartum.

Primary hepatocytes. Rat primary hepatocytes from Teklad chow-fed (ad lib) male Spraque-Dawley rats were prepared and treated with fatty acids or WY14,643 as previously described [52,136].

In vitro fatty acid elongation assay. Rat liver microsomes were isolated by differential centrifugation [159]. Elongation reactions were carried out with modifications to the procedure described by Moon et al [117]. Briefly, reaction mixtures contained 50 μg microsomal proteins in a total reaction volume of 100 μl. The elongation assay used fatty acyl CoA as substrates. The reaction constituents are: 50 mM potassium phosphate buffer- pH 6.5, 5 μM rotenone (Sigma), 40 μM fatty acyl CoA (Avanti Polar Lipids, Alibaster, Al and Sigma, St. Louis, MO), 60 μM malonyl CoA (Sigma) [6.5 dpm/pmol] [2-^{14}C]-malonyl CoA (Perkin Elmer), 1 mM NADPH (Sigma) 20 μM BSA (fatty acid free). Reactions (at 37°C) were initiated with the addition of NADPH. Reactions were terminated after 20 minutes with the addition of 100 μl 5 N KOH + 10% methanol; lipids were saponified for 1 hr at 65°C. The saponification reaction was acidified with 100 μl 5 N HCL; 100 μl ethanol was added to aid hexane extraction of fatty acids. Elongated fatty acids were collected by 2 independent extractions with hexane (800 μl). Hexane extracts were pooled and ^{14}C-radioactivity was quantified by β -scintillation counting. Results are expressed as Elongase Activity Units, nmoles ^{14}C-malonyl CoA incorporated/mg protein/20 mins. Formation of reaction products was dependent on the presence of NADPH and the fatty acid CoA. Fatty acid elongation products were verified by reverse-phase-HPLC chromatography using a flow-through β-scintillation counter [136].

RNA extraction, northern analysis and RT-PCR. RNA was extracted and separated in denaturing (formaldehyde) agarose gels, transferred to nitrocellulose and hybridized with

^{32}P-cDNAs. The cDNAs for the various transcripts has been described previously [52,136,159]. Hybridization was visualized by phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA). To ensure an accurate assessment of all elongases, reverse transcriptase-polymerase chain reaction (RT-PCR) was used; transcript-specific primers are described in Table 2.1.

Immunoblotting. Hepatic nuclear and microsomal proteins were separated by SDS-PAGE and transferred to nitrocellulose. SREBP-1, SREBP-2 and CYP4A were detected as previously described [159]. PPARα was measured using antibodies obtained from Santa Cruz Biotechnology. Anti-goat antibodies were also obtained from Santa Cruz. The detection system employed the Super Signal West Pico chemiluminescence kit (Pierce).

Quantitation of hepatic and plasma mead acid (20:3,n-9) levels. Total lipid was extracted from liver or plasma in chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene [136]. 7-Nonadecenoic acid (19:1) was added as a recovery standard at the time of extraction. Protein (Bio-Rad) was measured in extracts after the initial homogenization step. Total lipids were saponified, fractionated and quantified by reverse phase HPLC (RP-HPLC) using a YMC J-Sphere (ODS-H80) column and a sigmoidal gradient starting at 86.5% acetonitrile + acetic acid (0.1%) and ending at 100 % acetonitrile + acetic acid (0.1%) over 50 mins with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the MSU Mass Spectrometry facility by GC/MS (www.bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for RP-

HPLC were obtained from Nu-Chek Prep (Elysian, MN). Mead acid (20:3,n-9) was obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Statistical analysis involved Student's t-test; *p* values were calculated using Microsoft-excel t-test for 2 samples with unequal variance.

Table 2.1 Primers used to clone Elovls and desaturases from mouse or rat liver.

Enzyme	**Accession No.**	**Primer Sequence**
Elovl1	BC006735	Sense:ATGGAGGCTGTTGTCAACTTG Antisense: TCAGTTGGCCTTGACCTTGGT
Elovl2	NM_019423	Sense:ATGGGCGGCCGCATGGAGCAGCTGAAGGCCTTT Antisense: TTATTGAGCCTTCTTGTCCGT
Elovl3	AF054504	Sense: ATGGACACATCCATGAATTTC Antisense: TCATTGGCTCTTGGATGCAAC
Elovl5	NM_134382	Sense: ATGGAACATTTCGATGCGTCA Antisense: TCAATCCTTCCGCTGCTTCCT
Elovl6	AY053453	Sense: ATGAACATGTCAGTGTTGACT Antisense: TCTAGACTACTCAGCCTTCGTGGCTTTCTT
Δ5D	NM_146094	Sense: ATGGCTCCCGACCCGGTGCCG Antisense: CTATTGGTGAAGGTAAGCGTC
Δ6D	BC057189	Sense: AGTCGACATGGGGAAGGGAGGTAACCAG Antisense: TCATTTATGGAGGTAAGCATC
Δ9D	NM_009127	Sense: ATGCCGGCCCACATGCTCCAA Antisense: TCAGCTACTCTTGTGACTCCC

2.3. Results

2.3.1. Tissue-specific expression of rat fatty acid elongases.

Northern and RT-PCR approaches were used to determine the profile of fatty acid elongase expression in several rat tissues (Fig. 2.1). Elovl1 was well expressed in lung, brain, kidney and heart, but is barely detectable in liver, brown adipose tissue and skin. Because northern analysis of hepatic Elovl2 indicates this transcript is expressed at low levels (see Fig. 2.2), a RT-PCR approach was used to examine its expression in various rat tissues. Elovl2 is expressed in liver, lung, brain and kidney; Elovl2 expression was not detected other tissues. Elovl3 was only detected in skin. Elovl5 expression was readily detected in all tissues examined. Elovl6 mRNA is also expressed at low levels in rat liver (Fig. 2.2). RT-PCR was used to show that Elovl6 is expressed in all tissues; its highest expression is in brain. The tissue distribution of Elovl1[155], Elovl2 [155], Elovl3 [140,155], Elovl5 [119] and Elovl6 [117,119] is similar to previous reports. Rat liver expresses 4 distinct fatty acid elongases, Elovl1, Elovl2, Elovl5 and Elovl6. The relative abundance of the elongase mRNAs in livers of adult male rats fed Teklad chow diets *ad lib* is: Elovl5 > Elovl1 = Elovl2 = Elovl6 (Fig. 2.1 and 2.2).

2.3.2. Dietary Effects on Hepatic Elongase and Desaturase Gene Expression.

To examine the effects of diet on hepatic elongase expression, adult male rats were meal fed high carbohydrate diets supplemented with olive oil (10% w/w), fish oil (10%w/w) or the PPARα agonist, WY14,643 (0.1% w/w) for 7 days. Because elongases

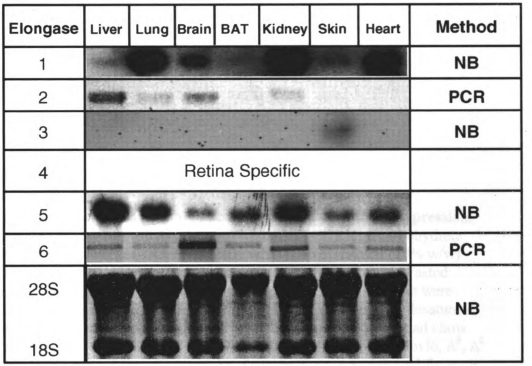
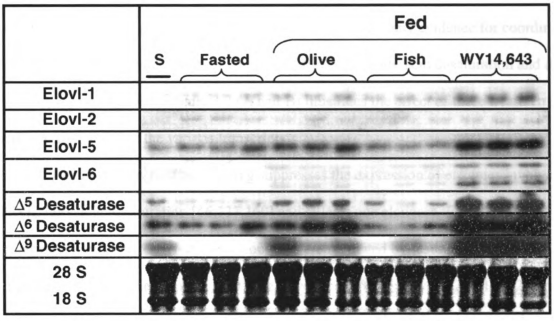


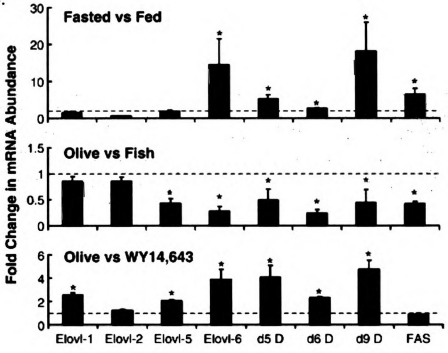
Figure 2. 1. Tissue specific expression of rat fatty acid elongases. RNA was extracted from rat liver, lung, brain, brown adipose tissue (BAT), kidney, skin and heart. These tissues were obtained from a 60 day old male rat maintained on a Teklad-Harlan chow diet, *ad lib*. Elovl1, Elovl3, and Elovl5 mRNAs were detected by northern analysis using cDNAs cloned as described in Materials and Methods. Elovl2 and Elovl6 were detected by RT-PCR (35 cycles).

Figure 2. 2. Effect of diet on rat hepatic elongase and desaturase expression. Male Spraque-Dawley rats (50 days of age) were meal-fed a high carbohydrate diet (HiCHO) supplemented with olive (10% w/w) [Olive], fish oil (10% w/w) [Fish] or olive plus WY14, 643 (0.1% w/w) [WY14, 643] for 7 days. Fasted animals were meal fed the HiCHO-olive oil diet as described above, but were fasted for 24 hrs prior to euthanasia. A: Northern blot of elongase and desaturase mRNAs. A rat liver standard (S) derived from a 90 day old rat fed Teklad chow (8240) was included in all northern blots for Elovl1, Elovl2, Elovl5, Elovl6, Δ^5, Δ^6 and Δ^9 desaturase mRNA measurement. Single transcripts were detected for each mRNA, except Elovl6. Two transcripts have been reported for Elovl6 [117]. In all cases, the size of the mRNA corresponded with the reported size of the transcript. A representative ethidium bromide stain of 18 S and 28 S mRNA documents equal loading of the RNA samples. Elovl3 was not detected in any treatment (not shown). B: Fold changes in fatty acid elongase, fatty acid desaturase and fatty acid synthase (FAS) mRNA abundance following dietary challenge. Top panel: Effect of refeeding fasted animals on mRNA expression. Results are expressed as the Mean $\pm$ SD; N=3. *$p \leq 0.05$; fasted vs fed. Middle panel: Effect of fish oil feeding on mRNA expression. Results are expressed the Mean $\pm$ SD,n=6/group. *$p \leq 0.05$, olive vs fish. Bottom panel: Effect of WY14, 643 feeding on mRNA expression. Results are expressed as the Mean $\pm$ SD,n=6/group. *$p \leq 0.05$; olive vs olive + WY14, 643.

2.2A


Fed
S
Fasted
Olive
Fish
WY14,643
Elovl-1
Elovl-2
Elovl-5
Elovl-6
Δ5 Desaturase
Δ6 Desaturase
Δ9 Desaturase
28 S
18 S

2.2B.


Fasted vs Fed
Olive vs Fish
Olive vs WY14,643
Fold Change in mRNA Abundance
Elovl-1
Elovl-2
Elovl-5
Elovl-6
d5 D
d6 D
d9 D
FAS

act in concert with desaturases for PUFA synthesis, we examined the hepatic expression of 4 elongases (Elovl1, -2, -5 and -6), 3 desaturases (Δ^5, Δ^6 and Δ^9) and fatty acid synthase (FAS). Our goal was to determine whether there is any evidence for coordinate regulation of pathways involved in fatty acid elongation, fatty acid desaturation and *de novo* lipogenesis (DNL). Figure 2.2 (A and B) illustrates the effect of these treatments on mRNA abundance of the various transcripts.

Fasting and refeeding: Fasting suppresses the expression of enzymes involved in DNL, fatty acid desaturation [46,137,160] and at least one elongase, Elovl6 [119,134]. To determine if other hepatic fatty acid elongases are regulated by fasting, rats meal-fed a high carbohydrate diet supplemented with olive oil (10 % w/w) were fasted for 24 hrs and refed the same diet for 4 hrs (Figs. 2.2). mRNAs encoding Elovl1 and Elovl5 were induced marginally (1.5- and 1.9-fold) by refeeding fasted rats. Only Elovl6 mRNA displayed a robust (14-fold) induction in response to refeeding fasted rats. In contrast, all of the desaturase (Δ^5, Δ^6, Δ^9) mRNAs, as well as $mRNA_{FAS}$, were induced 5.2-, 2.6-, 18- and 6.5-fold, respectively. Unlike the desaturases, the elongases do not display a uniform response to fasting and refeeding.

Fish oil Feeding. Feeding rats diets enriched in N3-PUFA, i.e., fish oil, inhibits DNL and the expression of all fatty acid desaturases [2,5,160]. While fish oil suppressed FAS and each desaturase mRNA by $\geq$50%, fish oil feeding had no significant effect on Elovl1 and Elovl2 mRNA abundance. Only $mRNA_{Elovl5}$ and $mRNA_{Elovl6}$ were significantly ($\geq$50%) suppressed by fish oil (Fig. 2.2). Fish oil feeding does not uniformly suppress expression of all hepatic fatty acid elongases.

WY14,643 Feeding. All hepatic fatty acid desaturases are induced by PPARα agonists, like WY14,643 [134,160,161]. To determine if the elongases are regulated by WY14,643, rats were fed WY14,643 (0.1% w/w) in the high carbohydrate-olive oil diet for 7 days (Fig. 2.2). mRNAs encoding all of the desaturases and 3 of the elongases were induced by WY14, 643. Elovl1 and Elovl5 were induced 2- and 2.5-fold; Elovl6 was induced nearly 4-fold. Elovl2 mRNA was not affected by WY14,643 feeding. Δ^5, Δ^6 and Δ^9 desaturase mRNAs were induced 4.1-, 2.3- and 4.8-fold respectively, while $mRNA_{FAS}$ was not significantly affected by WY14,643 feeding. The effect of WY14,643 on both elongase and desaturase expression suggest PPARα agonist may induce long chain polyunsaturated synthesis.

Dietary Effects on Hepatic Elongase Activity. We next determined if changes in hepatic elongase mRNA abundance is consistent with hepatic fatty acid elongase activity. Elongase assays used 7 substrates, i.e., 16:0-CoA, 18:0-CoA, 18:1,n-9-CoA, 20:0-CoA, 20:4,n-6-CoA, 22:0-CoA and 24:0-CoA, in separate assays (Fig. 2.3). Based on reports by others, these fatty acyl-CoAs likely serve as substrates for 1 or more of the hepatic elongases. Specifically, 16:0-CoA is a substrate for Elovl1 and Elovl6, but not Elovl2 [117,134,155]. 18:1-CoA is not a substrate for Elovl6 [134], but is a substrate for other elongases. 20:4,n-6 is a substrate for Elovl2 and Elovl5, but not Elovl6 [117,119,134]. 20:0-CoA, 22:0-CoA and 24:0-CoA are substrates for Elovl1[155].

When compared to the olive oil-fed rats, hepatic microsomes isolated from fasted rats had a significantly lower (~50%) elongase activity when using 16:0-CoA and 20:4-CoA as substrate. Elongation of other fatty acyl CoAs was not significantly affected by fasting and refeeding. This effect correlates with the suppression of Elovl1, Elovl5 and

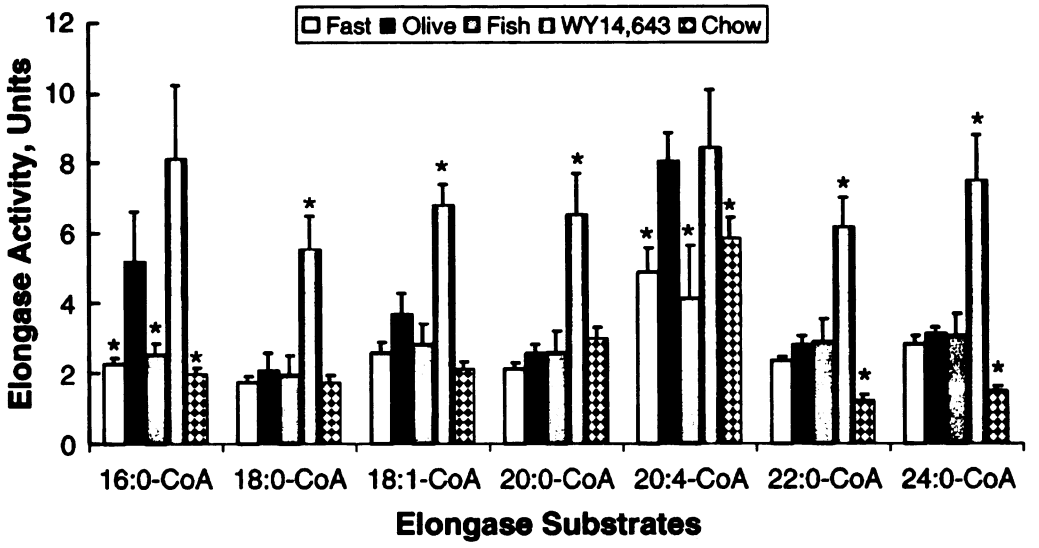


Figure 2. 3. Effect of diet on hepatic fatty acid elongase activity. In vitro assays for elongase activity used microsomes isolated from livers of rats fasted or fed a high carbohydrate diet containing olive oil, fish oil or WY14, 643 as described in the Methods. Separate reactions were run using 16:0-CoA, 18:0-CoA, 18:1n9-CoA, 20:0-CoA, 20:4n6-CoA, 22:0-CoA and 24:0-CoA as substrate. Results are expressed as Elongase Activity, Units (nmoles ^{14}C-Malonyl CoA incorporated/mg protein), Mean $\pm$ SD,n=3/group. *p$\leq$ 0.05, Olive oil fed vs Fasted, Fish oil fed or WY14,643 fed.

Elovl6 mRNA in starved animals (Fig. 2.2). A comparison of the elongase activity in microsomes isolated from olive and fish oil fed animals indicates that fish oil feeding suppressed (~60%) the elongation of both 16:0-CoA and 20:4-CoA. Fish oil suppressed only Elovl6 and Elovl5 mRNA, respectively (Fig. 2.2). Feeding rats WY14,643 increased elongation of all saturated and monounsaturated fatty acyl-CoA substrates, except 16:0-CoA, 2- to 3-fold. Increased elongase activity following WY14,643 feeding correlates with increased hepatic abundance of mRNAs encoding Elovl1, Elovl5 and Elovl6 (Fig. 2.2). Increased Elovl1 expression in livers of WY14,643-fed rats is likely involved in the increased elongation activity toward saturated and monounsaturated fatty acids.

Effect of fish oil and WY14,643 on hepatic and plasma fatty acid composition. We next determined if changes in elongase gene expression and elongase activity had any effect on hepatic or plasma lipid composition (Fig. 2.4). For this analysis, we focused on mead acid (20:3,n-9) levels, because: 1) 20:3,n-9 is not a dietary fat; its presence in cells is due to elongation and desaturation of 18:1,n-7 or 18:1,n-9 [162]; 2) dietary n-3 and n-6 PUFA suppress tissue levels of 20:3,n-9, by competing with 18:1,n-9 for Δ^6 desaturase [163] and suppressing the expression of mRNAs encoding Δ^5 and Δ^6 desaturases. The contribution of elongases or WY14, 643 to 20:3,n-9 synthesis is unknown.

Fish oil and WY14,643 affect tissue and plasma levels of 20:3,n-9 and 20:4,n-6 (Fig. 2.4). In olive oil-fed rats, the mole % of 20:3,n-9 and 20:4,n-6 in the plasma is 0.7 and 6.4. In liver, the mole % of 20:3,n-9 and 20:4,n-6 is 1.8 and 14.2. The ratio of 20:3,n-9/20:4,n-6 in plasma and liver is 0.1 and 0.13. These values are in line with essential fatty acid sufficiency [164]. Rats fed the fish oil diet have a > 95% reduction in plasma and hepatic 20:3,n-9. In contrast, olive oil-WY14,643 fed animal have 4- and 5-fold increased

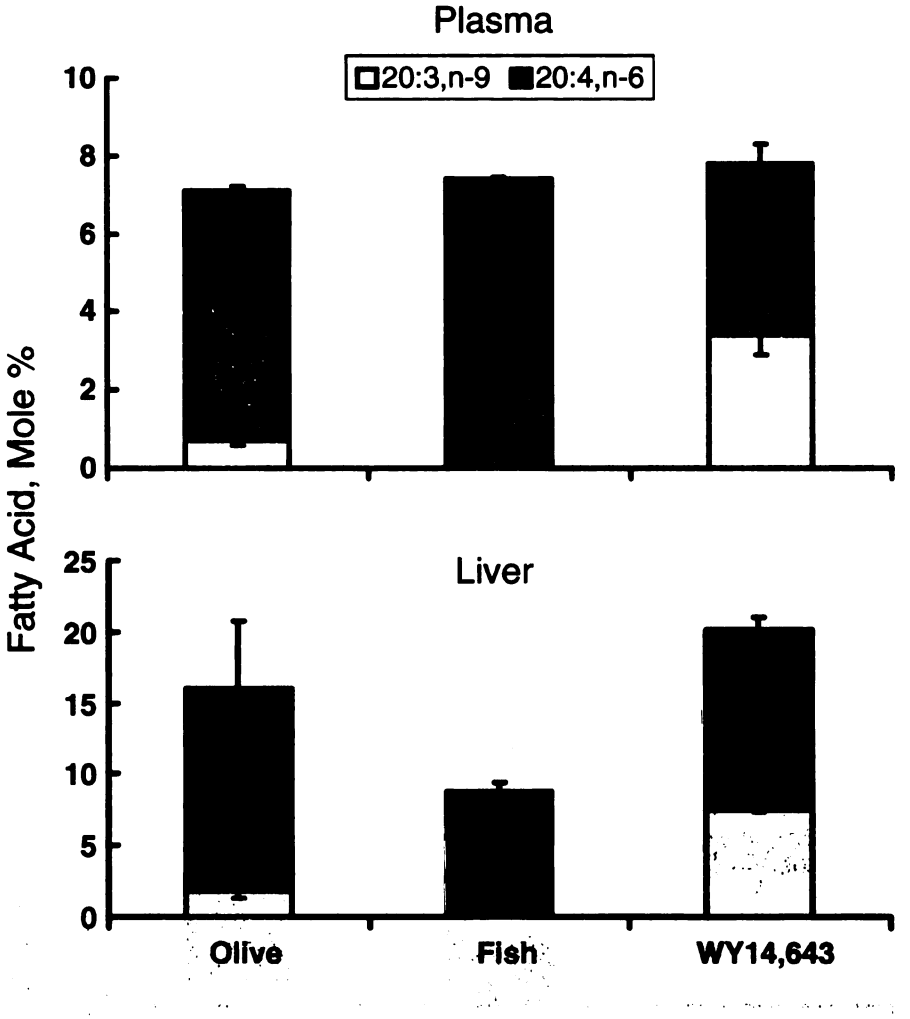


Figure 2.4. Effect of diet on plasma and liver mead acid levels. Total lipids extracted from rat liver and plasma of animals fed olive oil, fish oil or WY14,643 were saponified, separated by RP-HPLC and quantified (Methods and Materials). The identity of all fatty acids was confirmed by gas chromatography-mass spectrometry. Levels of mead acid (20:3,n-9) and arachidonic acid (20:4,n-6) were quantified and the results are presented as Mole % of the total saponified lipid fraction, Mean $\pm$ SD,n = 3. The mole % of 20:3,n-9 in olive oil vs fish oil-fed animals and in olive oil and olive oil + WY14, 643-fed animals was significantly different, *$p<0.01$.

in 20:3,n-9 levels in plasma and liver, respectively. The 20:3,n-9/20:4,n-6 ratio in these animals is 0.8 and 0.6 in plasma and liver, a value found in essential fatty acid deficiency (EFAD). The elevation in 20:3,n-9 levels can be correlated to increased elongation of 18:1-CoA (Fig. 2.3) and elevated Elovl1, Elovl5, Δ^5 and Δ^6 desaturase mRNA abundance (Fig. 2.2) plus the increased dietary intake of 18:1,n-9 in the olive oil diet.

2.3.3. Developmental regulation of hepatic fatty acid elongase and desaturase gene expression.

SREBP-1c, a key transcription factor for controlling hepatic lipid synthesis [78], has been implicated in the regulation of at least one fatty acid elongases (Elovl6) [117,134,139]. In an effort to determine if changes in SREBP-1c nuclear content correlate with elongase expression, we have used the model of early postnatal development. In this model, SREBP-1 is present in fetal hepatic nuclei (Fig. 2.5, upper panel). Shortly after birth, however, nuclear SREBP-1 levels fall and do not rise again until the animals are weaned at 21 days postpartum [159]. We examined the expression of hepatic elongases and desaturases in rat livers derived from fetal animals (18 days post coitum), at 10 days postpartum (suckling) and 30 days postpartum (weaned). Fatty acid synthase (FAS) was included for comparison (Fig. 2.5 lower panel). Expression of all 4 elongases (Elovl1, -2, -5 and -6), all three desaturases (Δ^5, Δ^6, and Δ^9) and FAS were detected in fetal liver. Elovl1 decreased to adult values after birth. Elovl2 remains unchanged from 3 days prepartum to 30 days postpartum. Fetal Elovl 5 expression is <10% of the 30 day value and increased 5-fold by 10 days and 10-fold by 30 days postpartum. Elovl6 mRNA fell after birth to undetectable levels, but increased to adult values after weaning. The developmental regulation of Elovl6, Δ^9 desaturase and FAS

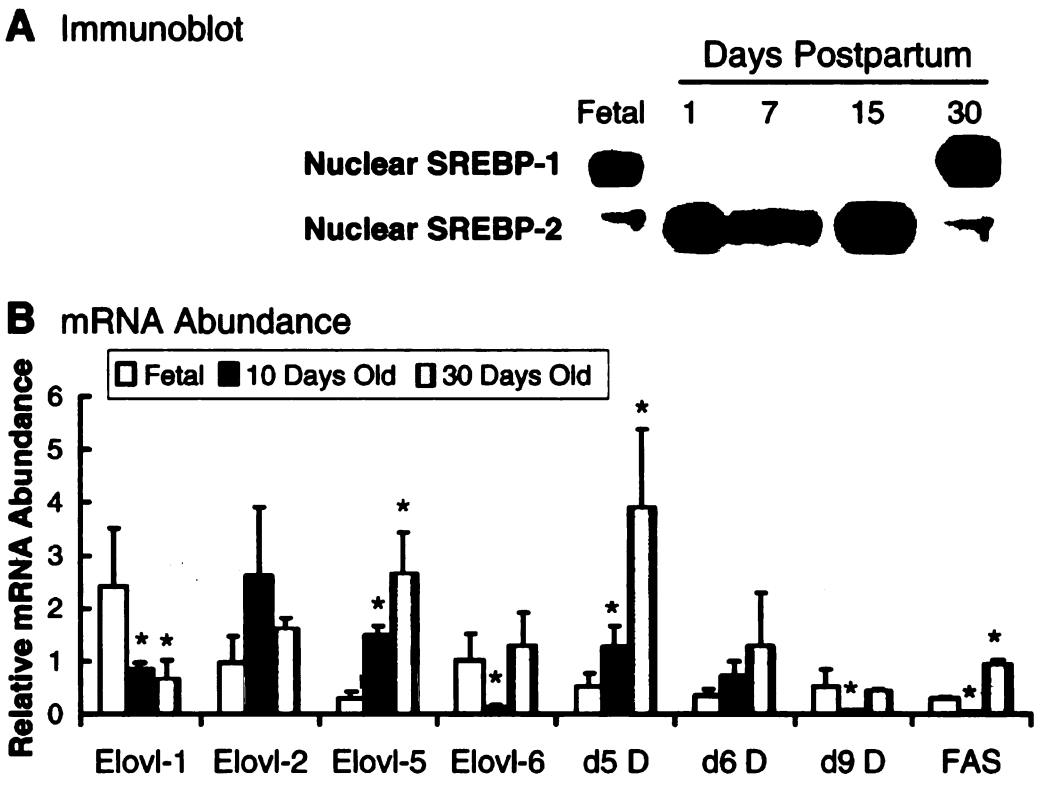


Figure 2. 5. Developmental regulation of rat hepatic SREBP-1 and -2 nuclear content and mRNA abundance of fatty acid elongase, fatty acid desaturase and fatty acid synthase. A. Immunoblot: Nuclear proteins isolated from livers at 18 days postcoitum (Fetal) , 1, 7, 15 and 30 days postpartum were prepared, separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies for SREBP-1 and SREBP-2 [159]. The inclusion of SREBP-2 shows that hepatic SREBP-1 and SREBP-2 nuclear content is regulated differently during development. B. mRNA Abundance: Rat liver mRNA from 18 days postcoitum (Fetal), 10 and 30 days postpartum was used to quantify levels of fatty acid elongases (Elovl1, Elovl2, Elovl5, Elovl6), fatty acid desaturases (Δ^5D, Δ^6D and Δ^9D) and fatty acid synthase (FAS). The results are represented as "Relative RNA Abundance" and are normalized to the level of expression in a 90 day old male rat. Mean $\pm$ SD,number of animals (N)/group = 3. *$p \leq 0.05$, Fetal vs 10 or 30 days.

parallels changes in SREBP-1 nuclear content during early development. However, at no time in this analysis was Elovl6 mRNA found to be an abundant transcript. In contrast, mRNAs encoding Elovl5, Δ^5 and Δ^6 desaturase are very low in fetal liver and increase after parturition. Developmental changes in Elovl5, Δ^5 and Δ^6 desaturase do not parallel changes in SREBP-1 nuclear content.

At parturition, animals ingest a high fat milk diet where 65% of the calories are fat. The ingestion of the high fat milk diet is associated with the activation of PPARα-regulated transcripts [165,166]. Nuclear PPARα content changes little in livers of fetal, neonatal or weaned animals (Fig. 2.6). The PPARα-regulated transcript, CYP4A, is induced (~10-fold) within 1 day of birth; both $mRNA_{CYP4A}$ and microsomal CYP4A protein increase >10-fold. The induction of $mRNA_{CYP4A}$ parallels the induction of $mRNA_{Elovl5}$ after birth. Both Δ^5 and Δ^6 desaturase mRNAs rapidly increase after birth (not shown). This observation, coupled with the fact that $mRNA_{Elovl5}$ is induced by WY14,643 (Fig. 2.2), suggest that PPARα and the high fat milk diets contribute to the postnatal increase in $mRNA_{Elovl5}$.

Developmental regulation of hepatic fatty acid elongase activity. Fatty acid elongation activity was examined in microsomes isolated from fetal liver and livers of 10 and 30 days old male rats (Fig. 2.7). Fatty acid elongation activity for all substrates is low in fetal liver and increased 5- to 20-fold by 10 days postpartum. However, elongation of C_{22-24} saturated fatty acids is ~2 to 3-fold higher than C_{16-18} saturated fatty acids. Elongation activity for each substrate was induced significantly by 10 days post partum. The level of elongase activity at 10 days postpartum was at or near adult (90 days) values.

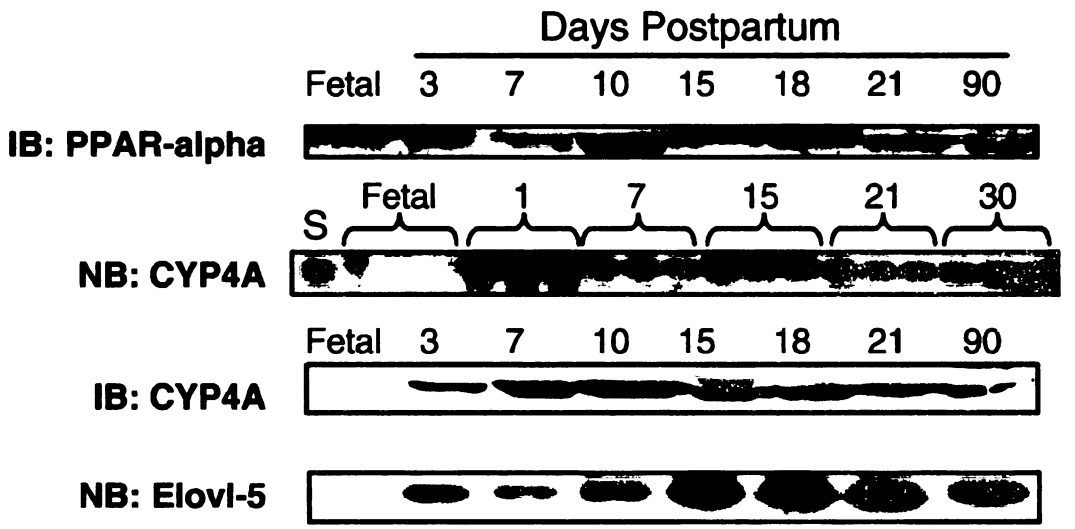


Figure 2. 6. Rapid induction of hepatic Elovl5 and CYP4A mRNA following parturition. NB Elovl5: mRNA was extracted from livers of fetal rats and from male rats 3, 7, 10, 15, 18, 21 and 90 days old. A northern blot illustrating the induction of Elovl5 is shown. IB CYP4A and IB PPARα: nuclear (PPARα) or microsomal (CYP4A) proteins were isolated from livers at 18 days postcoitum (fetal) and 3, 7, 10, 15, 18, 21 and 30 days postpartum. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies for PPARα or CYP4A, Immunoblot (IB) [159]. Total RNA was extracted from rat livers at 18 days post coitum (fetal) and 1, 7, 15, 21 and 30 days postpartum (3 animals/group); S, RNA from 90 day old rat liver. RNA was separated and probed for CYP4A [75].

of the substrates used, 2-3 times more 20:4-CoA was converted to its elongation product (22:4,n-6) than any other fatty acyl CoA tested.

The low elongase activity in fetal livers correlates with the low level of expression of all hepatic elongases. Note that Elovl1, Elovl2 and Elovl6 are expressed at low levels in both fetal and adult livers (Figs. 2.1, 2.2 & 2.5). The postnatal increase in hepatic elongase activity correlates with only one elongase transcript, i.e., Elovl5. $mRNA_{Elovl5}$ was induced (5-fold) by 10 days postpartum (Fig. 2.5 and 2.6). Since no other elongase was significantly induced by 10 days postpartum, our studies suggest that Elovl5 is the likely elongase responsible for a major fraction hepatic fatty acid elongation. If so, then Elovl5 is capable of elongating a broad range of fatty acid substrates, including saturated (16:0, 18:0, 20:0, 22:0 & 24:0), monounsaturated (18:1n9) and polyunsaturated (20:4n6) fatty acids (Fig. 2.7). Elovl5 mRNA is expressed at higher levels than all other elongases. If changes in elongase mRNA parallel changes in elongase protein and elongase activity, then much of the increase in hepatic fatty acid elongase activity that occurs during postnatal development can be attributed to Elovl5.

2.4. Discussion

Liver (Fig. 2.7), primary hepatocytes [53], Hek293 [54] and FTO-2b hepatoma cells display robust elongation activity toward C_{20} PUFA. C_{20-22} PUFA fatty acids regulate the activity of fatty acid-regulated nuclear receptors, like PPARα and LXRα [53,54]. As such, changes in C_{20} PUFA elongation might impact PPARα and LXRα action. In an effort to determine which elongase might be responsible for C_{20} elongation, we examined the expression and activity of rat liver fatty acid elongases. These studies

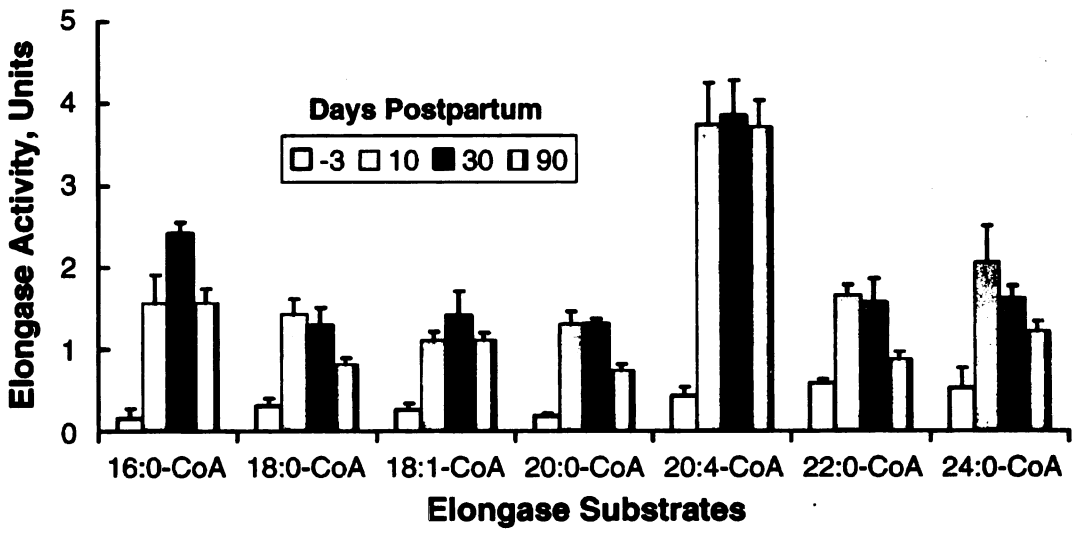


Figure 2. 7. Developmental regulation of hepatic fatty acid elongase activity. *In vitro* assays for elongase activity used microsomes isolated from livers of fetal rats (18 days post coitium), 10, 30 and 90 days postpartum. Separate reactions were run using 16:0-CoA, 18:0-CoA, 18:1,n-9-CoA, 20:0-CoA, 20:4,n-6-CoA, 22:0-CoA and 24:0-CoA as substrate. Results are expressed as Elongase Activity, Units (^{14}C-Malonyl CoA nmoles incorporated/mg protein), Mean $\pm$ SD,n=3/group. Elongase activity in fetal liver was significantly lower than postpartum liver for all substrates examined, *$p \leq 0.05$.

have revealed new information on the regulation of elongase expression and which elongase likely contributes to C_{20} PUFA metabolism in liver.

Of the 4 fatty acid elongases (Elovl1, Elovl2, Elovl5 and Elovl6) expressed in liver (Figs. 2.1 and 2.2), only Elovl1, Elovl2 and Elovl5 use C_{20} fatty acid substrates [117,119,155]. The mRNAs encoding Elovl1 and Elovl2 are expressed at very low levels relative to $mRNA_{Elovl5}$ in adult rat liver. Moreover, Elovl1 and Elovl2 mRNAs do not increase during postnatal development, a time in which the liver substantially increases its capacity for C_{20} PUFA elongation (Fig. 2.7). Elovl5 is the only fatty acid elongase readily detected in primary hepatocytes. Based on this observation, we suggest that Elovl5 is responsible for elongation of C_{20} PUFA to C_{22} PUFA in primary hepatocytes. Finding that Elovl5 mRNA is expressed in broad array of tissues (Fig. 2.1) [119], regulated during postnatal development (Fig. 2.5), by dietary fat and PPARα agonist (WY14,643) (Fig. 2.2), suggest changes in Elovl5 expression and activity may be important for the maintenance of cellular long chain fatty acids. Whether changes in Elovl5 expression and activity affect fatty acid-regulated nuclear receptor function is an issue addressed in a later chapter.

It is unclear if changes in elongase activity during postnatal development are due solely to changes in elongase expression. Elongases are condensing enzymes that interact with 3-keto acyl-CoA reductase, a dehydratase and trans 2,3-enoyl CoA reductase to elongate fatty acids [24,138,153]. The rate of fatty acid elongation is generally viewed as being determined by the activity of the elongase (condensing enzyme) and not the reductases or dehydratase. However, the low elongase activity in fetal liver may be due to

deficient expression of the reductases and dehydratase. Additional studies will be required to examine the expression of these enzymes during postnatal development.

These developmental and nutritional studies have implicated 2 transcription factors in control of elongase expression, i.e., PPARα and SREBP-1. During postnatal development two distinct patterns of fatty acid elongase and desaturase expression are evident. These two patterns of expression can be linked to changes in SREBP-1 nuclear content or the activation of PPARα. Both SREBP-1 and PPARα play major roles in whole body and hepatic lipid metabolism [51,78]. Changes in SREBP-1 nuclear content closely parallel changes in FAS, Δ^9 desaturase and Elovl6 expression (Fig. 2.5). SREBP-1 is expressed in fetal livers at levels near adult values. During the suckling period, however, SREBP-1 is absent from the nucleus. FAS, Δ^9 desaturase and Elovl6 increase after weaning when SREBP-1 nuclear content increases. SREBP-1 nuclear abundance has been linked, directly or indirectly, to the transcriptional control of each of these genes [78,139,167]. The regulation of SREBP-1 nuclear content during postnatal development may be mediated by changes in proteolytic conversion of the SREBP-1c precursor (~125 kd) to the mature form (~65 kd) and its transport to the nucleus [159]. Ingestion of the high fat milk diet (~65% calories as fat) begins at parturition. The ingestion of the high fat milk diet, coupled with low blood insulin levels [168] likely contributes to this regulatory scheme.

The expression of the other elongases, particularly Elovl5, does not parallel changes in SREBP-1 nuclear content during postnatal development (Fig. 2.5). Treatment of primary hepatocytes with T1317, an LXR agonist, significantly elevates SREBP-1 nuclear content as well as several SREBP-1 regulated transcripts [52]. T1317 only

modestly induced $mRNA_{Elovl5}$ (<30%) in primary hepatocytes (not shown). Unlike Elovl6 [117,134], Elovl5 does not appear to be a target for either SREBP-1 or LXRα.

Despite the finding that the postnatal induction of Elovl5, Δ^5 and Δ^6 desaturase closely parallels the induction of PPARα-regulated transcripts, e.g., Cyp4A (Fig. 2.6) [75,158], the role PPARα plays in [144] this regulatory scheme is less clear. PPARα agonist, like WY14,643 (Fig. 2.2) and fibrates induce hepatic elongase activity toward a broad range of fatty acids. Our studies show that WY14,643 induced Elovl1, -5 and -6 mRNA (Fig. 2.2). Studies with primary hepatocytes, however, have indicated that while PPARα target genes, like CYP4A and AOX, are well induced in primary hepatocytes [136,158], transcripts encoding elongases, e.g., Elovl5, are only marginally (<25%) induced (not shown). The lack of a significant WY14,643 effect on Elovl5 (or other elongases) expression in primary hepatocytes suggest these enzymes are not a direct target of PPARα. Clearly, many factors change during postnatal development, e.g., glucocorticoids, leptin, T_3 [169,170], that could impact hepatic elongase expression. These and other factors were evaluated for their contribution to elongase expression in the next chapter.

Our studies reveal two regulatory patterns for elongase and desaturase expression during postnatal development. These different patterns of expression likely have physiological significance. Blood of pregnant female rats fed a Teklad chow diet contains levels of 20:4,n-6 and 22:6,n-3 at 10 and 5 mole %, respectively (not shown). In contrast, rat milk is deficient in 20:4,n-6 and 22:6,n-3, i.e., 0.5 and 0.1 mole % respectively. Analysis of hepatic fatty acid profiles in fetal, 10 and 30 day old liver shows that the mole % of 20:4,n-6 and 22:6,n-3 remains essentially unchanged, at 15-20 mole% (not

shown). Since neonates grow very rapidly, the only mechanism available to sustain the constant level of hepatic 20:4,n-6 and 22:6,n-3 in the absence of an extrahepatic source of C_{20-22} PUFA is to induce PUFA synthesis. The capacity of the neonatal liver to synthesize 20:4,n-6 and 22:6,n-3 from dietary precursors (18:2,n-6 and 18:3,-n3) is achieved, at least in part, by inducing mRNAs encoding Δ^5 and Δ^6 desaturase and Elovl5 (Fig. 2.6 and 2.7).

Fetal liver has high blood levels of 20:4,n-6 and 22:6,n-3 and low elongase activity, and low levels of mRNAs encoding Elovl5, Δ^5 and Δ^6 desaturase. Adult animals fed fish oil (a rich source of 20:5,n-3, 22:5,n-3 and 22:6,n-3), have low 20:4,n-6 and 20:5,n-3 elongation activity (Fig. 2.5) and suppressed expression of mRNAs encoding Elovl5, Δ^5 and Δ^6 desaturase (Fig. 2.2). Thus, changes in Elovl5, Δ^5 and Δ^6 desaturase expression respond to changes in dietary PUFA composition and tissue requirements for C_{20-22} PUFA. Elovl2 has also been implicated in PUFA synthesis [118,127]. Yet $mRNA_{Elovl2}$ remains unresponsive during postnatal development, following fasting/refeeding and fish oil treatment. Based on these findings, Elovl5, and not Elovl2, appears to play the major role, along with Δ^5 and Δ^6 desaturase, in the adaptive response of PUFA synthesis following changes in dietary lipid composition. This response is mediated, at least in part, through the regulation of the cellular abundance of the mRNAs encoding these enzymes. Additional studies will be needed to further define the roles Elovl2 and Elovl5 play in the control of hepatic PUFA synthesis. In this regard, raising rats on n3-PUFA deficient diets leads to changes in blood and tissue lipid profiles as well as diminished cognitive function [171]. Whether similar effects occur in null mutations of specific elongases or desaturases remains to be determined.

Feeding rats fish oil suppresses lipogenic gene expression [52,157,172] and $mRNA_{Elovl5}$ (≥50%) (Fig. 2.2). Primary hepatocytes treated with 20:5,n-3 showed ~50% reduction in Elovl5 mRNA (not shown). Many of the effects of fish oil on hepatic gene expression can be attributed to suppression of nuclear levels of SREBP-1c or activation of PPARα [14]. Our findings indicate that neither factor induces major changes in Elovl5 expression in primary hepatocytes. Hepatic L-pyruvate kinase gene transcription is suppressed by 20:5,n-3 through mechanisms that are independent of PPARα and SREBP-1c [157,158]. The target for 20:5,n-3 action on L-PK is a region that binds a carbohydrate regulatory element binding protein (ChREBP), Mlx and HNF-4 [90,173]. Elovl5 mRNA is unresponsive to changes in glucose treatment of primary hepatocytes suggesting that CHREBP and Mlx are not involved in Elovl5 expression (not shown). The role of HNF-4 in Elovl5 expression is under investigation.

Finally, finding that WY14,643 elevated hepatic and plasma levels of mead acid (20:3,n-9) in olive oil-fed rats was a surprise (Fig. 2.4). Mead acid is a elongation and desaturation (Δ^5 and Δ^6 desaturase) product of vaccenic (18:1,n-7) and oleic (18:1,n-9) acid [162]. The effect of dietary n3 and n6 PUFA on tissue and plasma levels of 20:3,n-9 has been well described [164]. It is generally accepted that much of the PUFA-mediated suppression of 20:3,n-9 content in cells is due to competition of PUFA for Δ^6 desaturase (n3 > n6 > n9) [163]. The elevated tissue content of $C_{20\text{-}22}$ PUFA, coupled with the decline in both elongation and desaturation capacity (Fig. 2.2-2.4), likely accounts for the low levels of 20:3,n-9 in fish oil-fed animals. Contrary to the effects of fish oil, WY14,643 significantly increased hepatic and plasma levels of 20:3,n-9. The level of 18:2,n-6 in the olive oil diet (3.3mole %) is sufficient to prevent EFAD. Ingestion of a

18:1n9-enriched diet, however, coupled with the induction of hepatic Elovl1, Elovl5 and Elovl6 plus Δ^5 and Δ^6 desaturases by WY14,643, is apparently sufficient to increase 20:3,n-9 synthesis and its accumulation in tissues and plasma. The consequence of elevated 20:3,n-9 production is an increase in the hepatic 20:3,n-9/20:4,n-6 to 0.62, a value consistent with essential fatty acid deficiency (EFAD) [164]. Clearly, the elevated expression of key enzymes involved in PUFA synthesis, plus ingestion of a 18:1,n-9 enriched diet, is sufficient to shift the tissue balance of 20:3,n-9 and 20:4,n-6.

Chapter 3

Regulation of Hepatic Fatty Acid Elongase and Desaturase Expression in Diabetes and Obesity

3.1 Introduction

The liver plays a central role in whole body lipid metabolism. Fatty acids are synthesized *de novo* from glucose. This pathway utilizes products from glycolysis and along with the two enzymes, acetyl CoA carboxylase and fatty acid synthase, generates palmitate (16:0). Insulin, triiodothyronine (T_3), glucocorticoids and glucose induce, while C_{20} polyunsaturated fatty acids (PUFA), glucagon and epinephrine suppress *de novo* lipogenesis [14,137,174]. The liver also modifies fatty acid structure through metabolic pathways that include desaturation, elongation, mono-oxidation and peroxisomal β-oxidation (chain shortening). Such modifications occur to fatty acids generated *de novo,* as well as fatty acids derived from the diet. These pathways are particularly critical for the generation of end products of PUFA synthesis. Arachidonic acid (20:4,n-6) and docosahexaenoic acid (22:6,n-3) are the main C20-22 PUFA accumulating in membranes of all tissues [31]. Together, these metabolic pathways play an important role in the maintenance of membrane lipid composition and lipid storage, the generation of precursors for signaling molecules, like eicosanoids, and the control of fatty acid-regulated transcription factors [14,52-54].

Of the various pathways known to affect fatty acid structure, physiological control of fatty acid elongation remains poorly defined. We recently reported on the tissue-specific, nutritional and developmental regulation of fatty acid elongases in the rat [175]. The outcome of those studies suggested that hepatic elongase expression might be controlled by at least two transcription factors, peroxisome proliferator-activated receptor (PPARα and sterol regulatory element binding protein-1 (SREBP-1). This report extends those findings by examining SREBP-1, PPARα, liver X receptor (LXR), carbohydrate regulatory element binding protein (ChREBP) and MAX-like factor X (MLX) control of elongase and desaturase expression. Our analysis also includes *in vivo* studies to determine if changes in hepatic lipid composition induced by diabetes or obesity correlated with changes in elongase and desaturase expression. Overall, these studies establish a role for specific transcriptional regulatory networks in the control of hepatic desaturase and elongase gene expression and hepatic lipid composition.

3.2 Materials and Methods

Animals. All procedures for the use and care of animals for laboratory research have been approved by the All University Committee for Animal Use and Care at Michigan State University.

Streptozotocin-Induced Diabetes: Male Spraque-Dawley rats (200-250 g) (Charles River Laboratories, Kalamazoo, MI) were maintained on Harlan-Teklad laboratory chow (#8640) and water *ad lib*. Rats were injected, intraperitoneally with streptozotocin (7.5 mg/100 g. BW) and 3 ml of 25% glucose [176]. Three weeks later, blood glucose was measured on animals receiving no streptozotocin (control) or streptozotocin (diabetic, blood glucose ≥120 mg/dl). Blood glucose was measured in isoflurane anesthetized rats

using a glucose meter (Freestyle Flash, Thera Sense, Inc, Alameda, CA). Control and diabetic rats were euthanized (isoflurane anesthesia and exsanguinations) for recovery of blood (plasma) and liver.

High Fat Feeding of C57BL/6 Mice: Male C57BL/6 mice (Jackson Laboratories, Bar Harbor Maine), 2 months of age, were fed diets containing 10% lard (D12450B) or 60% lard (D12492), Research Diets, Inc.) *ad lib* for 10 weeks. Four days prior to euthanasia, mice were subjected to a glucose tolerance test. Briefly, mice were injected with glucose (2 g/kg, IP). Blood was withdrawn from the tail vein prior to and after glucose treatment. Blood glucose was measured using a hand-held glucose meter (Freestyle Flash, Thera Sense, Inc, Alameda, CA). Mice were euthanized using isoflurane and exsanguinated; blood and liver was recovered. Livers were used for RNA and lipid extraction.

Lean and Obese Mice. Livers from lean and obese C57BL/6 mice were obtained from Drs. Romsos and Claycomb (Department of Food Science and Human Nutrition, MSU). Male lean C57BL/6J-*Lep*$^{ob/+}$ and obese (C57BL/6J-*Lep*$^{ob/ob}$) mice (B6.V-Lep ob/J , #000632, Jackson Labs) were maintained on a Harlan-Teklad laboratory chow (#8640) diet and water ad lib. Livers were used for RNA, lipid and protein extraction.

Wild type and PPARα (-/-) mice. Homozygous wild type and PPARα-null (-/-) mice on an Sv/129 genetic background [177,178]were fed either a control diet or one containing WY14643 (at 50 or 500 ppm: Bio-Serv, Piscataway, NJ) for 1 week. Mice were euthanized, livers were removed and RNA was isolated for analysis of gene expression.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) treated mice. Female C57BL/6 mice were treated by gavage with 0.1 ml of sesame oil for a nominal dose of 0 (vehicle control) or 30 μg/kg body weight of TCDD. Mice were sacrificed 168 h after dosing. Livers were

removed and RNA was isolated for analysis of gene expression and fatty acid composition.

Primary Rat Hepatocytes: Male Spraque Dawley rats (Charles River Laboratories, Kalamazoo, MI) were maintained on Harlan-Teklad laboratory chow (#8640) and water *ad lib.* Rat primary hepatocytes were prepared from Teklad chow-fed (*ad lib*) male Spraque-Dawley rats, cultured on BioCoat (type 1 collagen) plates (Beckon Dickinson, Belford, MA.[179]. For RNA and protein extraction, cells were plated onto 100 mm type I collagen-coated plates (BD Bioscience, Bedford, MA) at 10^7 cells/plate in Williams E (Gibco/Invitrogen, Carlsbad CA), 10 mM lactate, 10 nM dexamethasone (Sigma, St. Louis), and 10 % fetal bovine serum (Gibco/Invitrogen). For adenoviral infection studies, cells were plated in the same media onto 6 well type 1 collagen-coated plates at 1.5 x 10^6 cells/well. The ratio of culture media to cell number was maintained constant for the different plating conditions. For treatments, hepatocytes were incubated in medium (Williams E + 10 nM dexamethasone without or with 100 nM insulin and/or 25 mM glucose.

RNA extraction, northern analysis and quantitative real time-PCR (qRT-PCR). Total RNA was extracted from primary hepatocytes or liver samples and used for template for qRT-PCR or northern analysis as previously described [175]. Specific primers for each gene (Table 3.1) were designed using Primer Express software (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized using the SuperScript II RNase H-Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific forward and reverse primers and subjected to real-time PCR quantification using the ABI

PRISM 7700 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative C_T method (User Bulletin #2, Applied Biosystems). Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. Primers used for quantitative real time PCR are listed in Table 3.1.

Lipid extraction and quantitation of hepatic fatty acids composition. Total lipid was extracted from liver in chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene [175]. 7-nonadecenoic acid (19:1) was added as a recovery standard at the time of extraction. Protein (Bio-Rad, Hercules, CA) was measured in extracts after the initial homogenization step. Total lipids were saponified, fractionated and quantified by reverse phase HPLC (RP-HPLC) using a YMC J-Sphere (ODS-H80) column and a gradient starting at 77.5% acetonitrile + acetic acid (0.1%) and ending at 100 % acetonitrile + acetic acid (0.1%) over 90 mins with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the MSU Mass Spectrometry facility by GC/MS (www.bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for RP-HPLC were obtained from Nu-Chek Prep (Elysian, MN).

Immunoblotting. Liver microsomal and nuclear extracts were prepared as described previously [159,175]. Proteins (50-100 μg) extracted from microsomal or nuclear fractions were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12% polyacrylamide Bis-Tris, Invitrogen) and transferred to nitrocellulose

Table 3. 1. Primers for quantitative reverse transcriptase-polymerase chain reaction

Gene	Forward	Reverse
Rat		
Elovl1	CCCTACCTTTGGTGGAAGAA	TCCAGATGAGGTGGATGATG
Elovl2	TTTGGCTGTCTCATCTTCCA	GGGAAACCGTTCTTCACTTC
Elovl5	TACCACCATGCCACTATGCT	GACGTGGATGAAGCTGTTGA
Elovl6	CAACGGACCTGTCAGCAA	GTGGTACCAGTGCAGGAAGA
Δ^5D	TGGAGAGCAACTGGTTTGTG	GTTGAAGGCTGACTGGTGAA
Δ^6D	TGTCCACAAGTTTGTCATTGG	ACACGTGCAGGCTCTTTATG
Δ^9D	ACATTCAATCTCGGGAGAACA	CCATGCAGTCGATGAAGAAC
LXRα	TCAGCATCTTCTCTGCAGACCGG	TCATTAGCATCCGTGGGAACA
LXRß	AAGCTGGTGAGCCTGCGC	CGGCAGCTTCTTGTCCTG
SREBP-1c	GATTGCACATTTGAAGACATGCTT	GTCCCAGGAAGGCTTCCAGAGA
L-PK	AGGAGTCTTCCCCTTGCTCT	ACCTGTCACCACAATCACCA
Cyclophilin	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG
ß-Actin	ACTATTGGCAACGAGCGGTT	TGTCAGCAATGCCTGGGTACA
Mouse		
Elovl1	CCCTACCTTTGGTGGAAGAA	ATCCAGATGAGGTGGATGATG
Elovl2	ACGCTGGTCATCCTGTTCTT	GCCACAATTAAGTGGGCTTT
Elovl5	GGTGGCTGTTCTTCCAGATT	CCCTTCAGGTGGTCTTTCC
Elovl6	ACAATGGACCTGTCAGCAAA	GTACCAGTGCAGGAAGATCAGT
Δ^5D	TGTGTGGGTGACACAGATGA	GTTGAAGGCTGATTGGTGAA
Δ^6D	CCACCGACATTTCCAACAC	GGGCAGGTATTTCAGCTTCTT
Δ^9D	TCAACTTCACCACGTTCTTCA	CTCCCGTCTCCAGTTCTCTT
Cyclophilin	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG
ß-Actin	GACGGCCAGGTCATCACTAT	CGGATGTCAACGTCACACTT
Human		
Elovl1	GCTGGCTGAGCACCTATACC	TCAGCTCAATGAACTTGGAGAA
Elovl2	CCCTTCGGTTGTCTCATCTT	CAGGTGGCTCTTGCATATCTT
Elovl5	GTGCACATTCCCTCTTGGTT	TGGTCCTTCAGGTGGTCTTT
Elovl6	CTAAGCAAAGCACCCGAACT	GGCAACCATGTCTTTGTAGGA
Δ^5D	TTGGCCTGGATGATTACCTT	CTGTGTCACCCACACAAACC
Δ^6D	ATCCCTTTCTACGGCATCCT	TAGGCCTCCTGGTCAATCTC
Δ^9D	CACCCAGCTGTCAAAGAGAA	GATGAAGCACATCATCAGCAA
ß-Actin	CTCTTCCAGCCTTCCTTCCT	TGTTGGCGTACAGGTCTTTG

membranes. Membranes were incubated with antibodies for SREBP-1 (IgG-2A4, sc-13551) (Santa Cruz Biotechnology, San Cruz, CA). HNF-4α (C-19), MLX (N-17), anti-goat and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ChREBP antibody was obtained from (Novus Biologicals, Littleton, CO). Anti-mouse and anti-rabbit secondary antibodies were obtained from Bio-Rad (Hercules, CA); anti-goat antibodies were obtained from Santa Cruz Biotechnology. The SuperSignal West Pico chemiluminescence kit (Pierce) detection system was used.

Recombinant adenovirus. Cloning of cDNA for Elovl2, Elovl5 and Elovl6 was described previously [175]. The coding region for each transcript was ligated into Ad-Easy XL adenoviral vector system (Stratagene), recombined in BJ 5183 cells and propagated in XL10 Gold ultra-competent cells. Ad-DNA was packaged into adenoviral particles Ad-293 cells. The resultant adenovirus was amplified in HEK293 cells.

Recombinant adenovirus expressing dominant negative MLX (Ad-dnMLX) and the doxycycline inducible nuclear SREBP-1c (Ad-nSREBP1) were obtained from H. Towle, University of Minnesota, Minneapolis, MN [62,180]. Adenovirus was propagated and amplified Hek293 cells. Viral lysates were titered using the Adeno-X Rapid Titer Kit, Clontech. Confluent primary hepatocytes were infected (5-10 plaque forming units [PFU]/cell). Using Ad-GFP as a control for infection, greater than 80% of primary hepatocytes expressed functional protein at the 5-10 PFU/cell level.

In vitro fatty acid elongation assay. Rat liver microsomes were isolated by differential centrifugation [159]. Elongation reactions were carried out with modifications to the procedure described by Moon et al [117]. Briefly, reaction mixtures contained 50 μg microsomal proteins in a total reaction volume of 100 μl. The reaction constituents are:

50 mM potassium phosphate buffer- pH 6.5, 5 μM rotenone (Sigma), 40 μM fatty acyl CoA (Avanti Polar Lipids, Alibaster, Al and Sigma, St. Louis, MO), 60 μM malonyl CoA (Sigma) [6.5 dpm/pmol] [2-^{14}C]-malonyl CoA (Perkin Elmer), 1 mM NADPH (Sigma) 20 μM BSA (fatty acid free). Reactions (at 37°C) were initiated with the addition of NADPH. When fatty acids were used as substrate, NaOH neutralized fatty acid (40 μM) replaced fatty acyl CoA. CoASH (at 100 μM), MgCl2 (1 mM) and ATP (1 mM) were added to the reaction to generate fatty acyl-CoA. Elongase reactions were terminated after 20 minutes with the addition of 100 μl 5 N KOH + 10% methanol; lipids were saponified for 1 hr at 65°C. The saponification reaction was acidified with 100 μl 5 N HCl; 100 μl ethanol was added to aid hexane extraction of fatty acids. Elongated fatty acids were collected by 2 independent extractions with hexane (800 μl). Hexane extracts were pooled and ^{14}C-radioactivity was quantified by β-scintillation counting. Results are expressed as Elongase Activity Units, nmoles ^{14}C-malonyl CoA incorporated/mg protein/20 mins. Formation of reaction products was dependent on the presence of NADPH and the fatty acid CoA. Fatty acid elongation products were verified by reverse-phase-HPLC chromatography using a flow-through β-scintillation counter [53].

Statistical Analysis. Statistical analysis used Student's t-test and ANOVA plus post hoc Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html).

3.3 Results

3.3.1. Elongase and desaturase expression in rat and mouse liver.

Our first objective was to compare fatty acid elongase and desaturase expression in rat and mouse (Fig. 3.1A). Of the 7 elongases identified in the genomes of these species, qRT-PCR analysis indicated that only 4 elongases are expressed in liver, Elovl1, Elovl2, Elovl5 and Elovl6. Based on relative mRNA abundance, the hierarchy of elongase expression in rat and mouse liver is: Elovl5 > Elovl1 = Elovl2 = Elovl6. The hierarchy of desaturase expression is: Δ^9D (stearoyl CoA desaturase-1, SCD-1) > Δ^5desaturase (Δ^5D) = Δ^6 desaturase (Δ^6D).

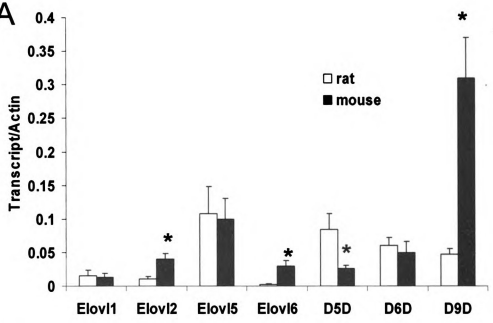
Fatty acid elongase activity was assessed using 3 substrates. 16:0-CoA is a substrate for Elovl1 and Elovl6; 20:4-CoA is a substrate for Elovl2 and Elovl5; and 24:0-CoA is a substrate for Elovl1 [153]. Elongation of 16:0-CoA to 18:0-CoA was highest in mouse liver, while elongation of 24:0-CoA to 26:0-CoA was comparable amongst species. Differences in elongation activity can be attributed to elongase subtype expression amongst species.

3.3.2. Role of PPARα in the control of hepatic elongase and desaturase expression.

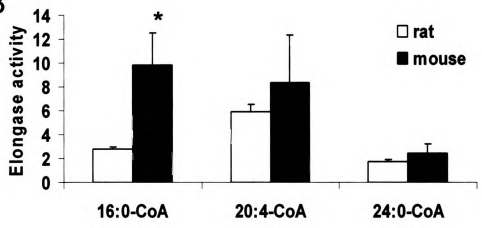
Feeding rats the PPARα agonist, WY14643, induces certain hepatic fatty acid elongases and desaturases and promotes changes in hepatic and plasma lipid composition [26]. Herein, we evaluate further the role PPARα plays in the control of hepatic elongase and desaturase expression. The effect of WY14643 on elongase and desaturase expression in wild type and PPARα null (-/-) mice was examined. Mice were fed a control diet or one containing WY14643 (at 50 or 500 mg WY14643/kg of diet) for 1

Figure 3. 1. Fatty acid elongase and desaturase expression in rat, mouse and human liver. A. Liver from male Spraque-Dawley rats (2-3 months of age) and C57BL/6 mice (2-4 months of age) were used for RNA extraction and measurement of elongase and desaturase expression as described in Materials and Methods. Rats and mice were maintained on chow diets *ad lib.* mRNA abundance for each elongase and desaturase was measured by qRT-PCR. Results are expressed relative to an internal standard, βactin (transcript/βactin) mean ± SD,n=4. B. Rat and mouse were extracted for microsomes to assay elongase activity using three separate substrates, 16:0-CoA, 20:4-CoA and 24:0-CoA. Results are expressed as Elongase Activity (nmoles ^{14}C-malonyl CoA assimilated into fatty acids/mg protein). Mean ± SD,n=4. *$p \leq 0.05$ vs rat liver, *ANOVA*.

3.1A


Transcript/Actin
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
rat
mouse
*
Elovl1
Elovl2
Elovl5
Elovl6
D5D
D6D
D9D

3.1B


Elongase activity
14
12
10
8
6
4
2
0
rat
mouse
*
16:0-CoA
20:4-CoA
24:0-CoA

week (Fig. 3.2). Of the 4 elongases expressed in mouse liver, WY14643 induced only Elovl5 and Elovl6, 14- and 4-fold, respectively. Previous studies established that the PPARα agonist increased desaturase expression [134]. Our results indicate that Δ^6D and Δ^9D transcript levels were induced ~6-fold by WY14643, while Δ^5D was weakly induced. Elovl1 and Elovl2 mRNA abundance was unresponsive to WY14643 treatment. The absence of a WY14643 effect on elongase and desaturase expression in PPARα (-/-) mice indicates that PPARα plays a role in controlling both elongase (Elovl5 and Elovl6) and desaturase (Δ^6D and Δ^9D) expression.

3.3.3. Regulation of elongase and desaturase expression in primary rat hepatocytes.

Insulin, SREBP-1c [134,181], LXR agonist (T0901317, T1317), glucose, ChREBP and MLX [62] control desaturase expression [9]. The following studies determined if these same transcriptional regulatory systems control elongase expression in rat primary hepatocytes.

Regulation of elongases and desaturases by insulin and LXR agonist.

Insulin regulates lipid synthesis, at least in part, by controlling SREBP-1 nuclear abundance [179,182]. LXR agonist stimulate lipogenesis through direct and indirect mechanisms [183]. LXR/RXR heterodimers bind LXRE in promoters of responsive lipogenic genes. LXR agonist also induce lipogenic gene expression through the induction of SREBP-1c gene transcription [182,183]. The effect of insulin and T1317 on hepatocyte elongase and desaturase expression was examined.

In the absence of insulin or T1317, SREBP-1 nuclear abundance in hepatocytes is low (Fig. 3.3 insert). Treatment of rat primary hepatocytes with insulin or T1317 induced

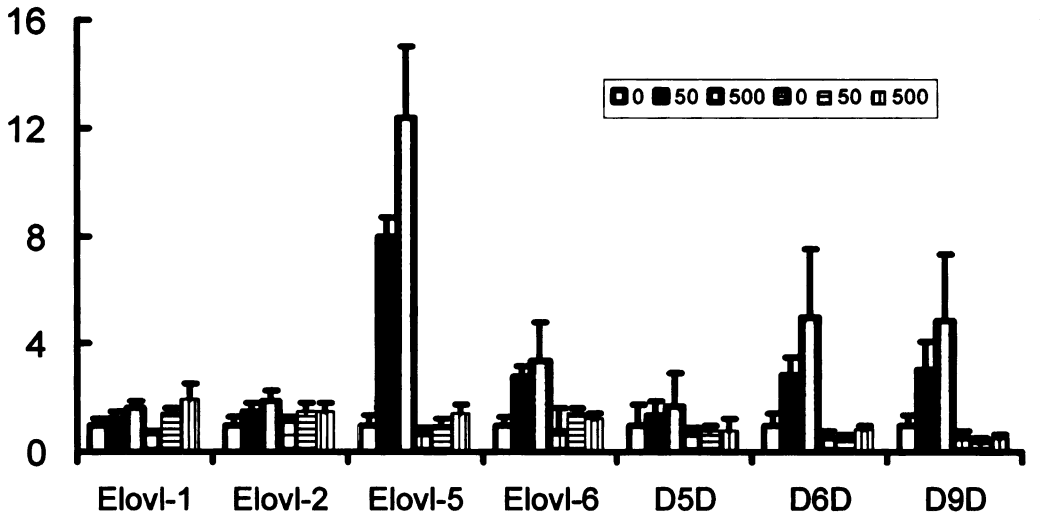


Figure 3. 2. The role of PPARα in the control of hepatic elongase and desaturase expression. Homozygous wild type and PPARα-null (-/-) mice on a Sv/129 genetic background [177,178] were fed either a control diet or one containing WY14643 (at 50 or 500 mg/kg of diet; Bioserv, Piscataway, NJ) for 1 week. Liver RNA was extracted and used as template for qRT-PCR. Results are reported as Fold Change in mRNA abundance (transcript/cyclophilin) for each enzyme, mean ± SD,n=4. *$p \leq 0.05$, *Students T-Test* vs wild type on a control diet.

nuclear SREBP-1 ~4-fold, but had no effect on SREBP-2 nuclear abundance. T1317 (1 μM) had no significant effect on Elovl1, -2 or -5 expression in rat primary hepatocytes and only modestly induced Elovl6, ~1.5-fold (Fig. 3.3). In contrast, all 3 desaturases were induced between 2- and 15-fold; Δ^9D was most responsive. Insulin induced Elovl6 and Δ^5D $\leq$1.5-fold, while Δ^6D and Δ^9D were induced >3-fold. Co-treatment with insulin and T1317 had no additive effect on SREBP-1 nuclear abundance or the expression of any elongase or desaturase.

These studies suggest that the induction of Elovl6, Δ^5D, Δ^6D and Δ^9D by insulin and T1317 likely involves control of SREBP-1 nuclear abundance. Although others have reported that insulin induces LXRα in primary hepatocytes [184], we found no evidence for an insulin effect on either LXRα or LXRα mRNA abundance (not shown). Glucocorticoids, T_3 and leptin had no effect on elongase expression in primary rat hepatocytes. None of the hormones tested induced hepatic Elovl3, Elovl4 or Elovl7 (not shown).

Effect of over expressed nuclear SREBP-1c on elongase and desaturase expression.

To further evaluate the SREBP-1 control of elongase and desaturase expression, primary hepatocytes were infected with a recombinant adenovirus containing a doxycycline-inducible nuclear form of SREBP-1c [Ad-nSREBP-1c] [180]. These cells received no insulin or LXR agonist. Treatment of primary hepatocytes with doxycycline significantly induced the nuclear form of SREBP-1c (not shown) as well as the endogenous SREBP-1 transcript (Fig. 3.4). The endogenous SREBP-1c promoter contains a SRE and is activated by elevated levels of nuclear SREBP [182]. Over

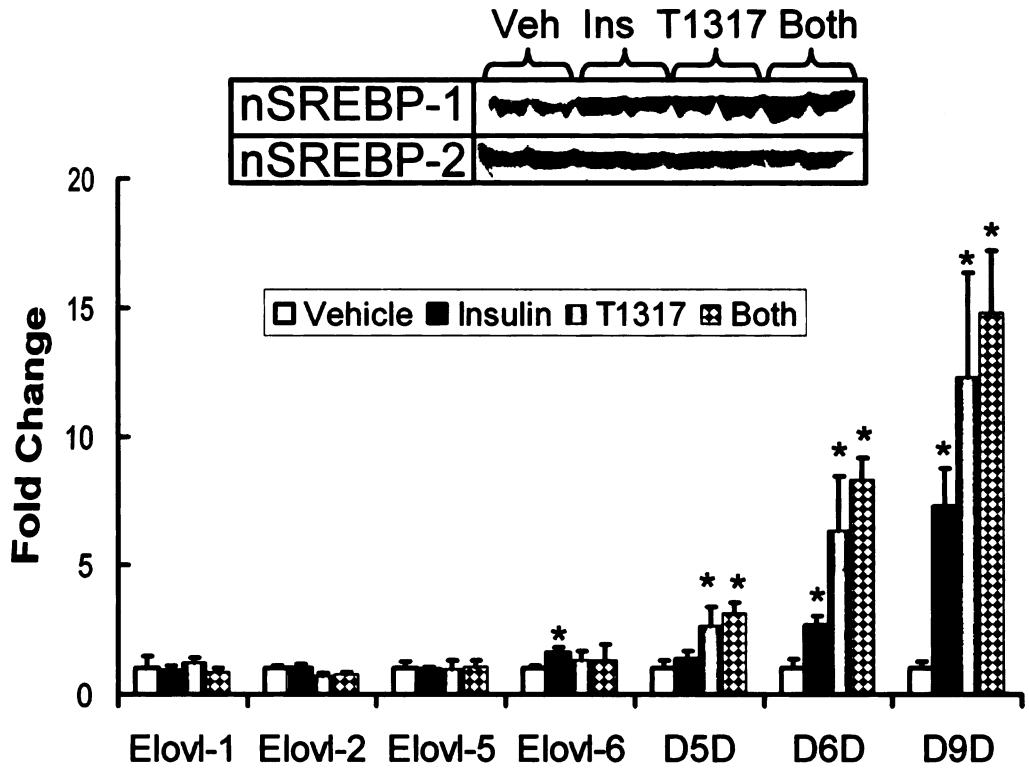


Figure 3. 3. Effect of insulin and LXR agonist (T1317) on fatty acid elongase and desaturase expression in rat primary hepatocytes. Rat primary hepatocytes were maintained in Williams E medium containing 10 mM lactate, 10 nM dexamethasone (DEX) with no insulin overnight. The next day, cells were switched to Williams E medium containing 25 mM glucose, 10 nM DEX (Vehicle) in the presence or absence of insulin (100 nM) or the LXR agonist, T0901317 [T1317, Cayman Chemical Co. Ann Arbor, MI] (1 μM). After 24 hours of treatment, cells were harvested for RNA extraction and the measurement of nuclear SREBP-1 and SREBP-2 by immunoblot (insert, duplicate samples/treatment). Elongase and desaturase mRNA abundance was quantified by qRT-PCR. Results are expressed as the Fold Change in mRNA versus Vehicle (transcript/cyclophilin); mean ± SD,n=4. These results are representative of 2 separate studies. *$p \leq 0.05$ vs vehicle, *ANOVA*.

expressed nSREBP-1c induced transcripts encoding Elovl2, Elovl6, Δ^5D, Δ^6D and Δ^9D ~3-fold at the maximal doxycycline dose.

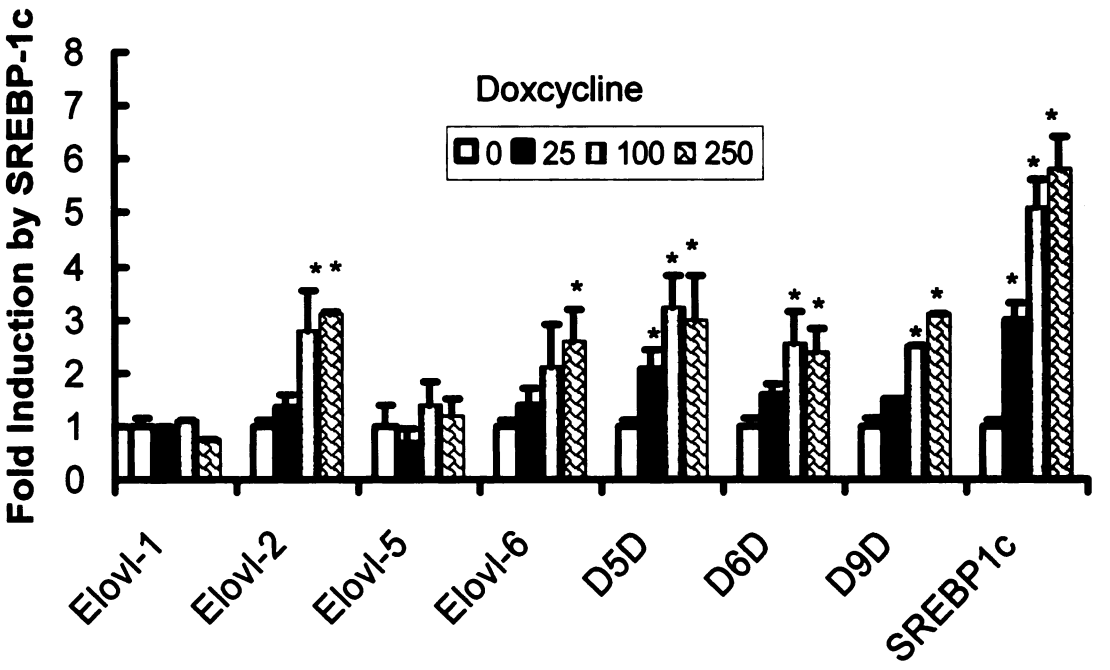
In a second study, Ad-nSREBP-1c infected cells were treated with insulin or T1317 (Fig. 3.4B). The goal of this study was to determine if insulin or T1317 had effects on elongase or desaturase expression independent of SREBP-1c. Over expressed SREBP-1c (Ad-SREBP-1c) induced Elovl2 (2.6-fold) and Elovl6 (6-fold), while Δ^5D, Δ^6D and Δ^9D transcripts were induced 3.6-, 4.8- and 3.1-fold, respectively. Addition of insulin had no additional effect on the expression of any elongase or desaturase. Addition of T1317 induced Elovl1 50%, while Δ^9D was induced 4-fold. The expression of no other elongase or desaturase was affected by T1317. Based on these results, the induction of Elovl6, Δ^5D, Δ^6D by insulin and T1317 (Fig. 3.4A) is due to elevated SREBP-1 nuclear abundance. Only Δ^9D expression is induced by elevated SREBP-1 nuclear abundance and independently by LXR agonist. Elovl1 was modestly responsive to LXR agonist only when SREBP-1 was over expressed. Elovl5 mRNA abundance was unresponsive to changes in insulin, LXR agonist and SREBP-1 nuclear abundance (Fig. 3.4B).

Glucose, ChREBP & MLX regulation of elongase and desaturase expression.

Insulin stimulated glucose metabolism induces the translocation of ChREBP to the nucleus where ChREBP/MLX heterodimers bind to carbohydrate regulatory elements (ChoRE) in promoters of glucose responsive genes involved in glycolysis and lipogenesis [185,186]. The effect of glucose on elongase and desaturase expression in primary hepatocytes was examined. Primary hepatocytes were maintained in medium containing lactate (20 mM) and insulin (100 nM) or switched to medium containing 25 mM glucose

Figure 3. 4. Effect of over expressed nuclear SREBP-1c on elongase and desaturase expression. A. Primary rat hepatocytes in Williams E medium + 10 mM lactate + 10 nM DEX, but no insulin, were infected with a recombinant adenovirus expressing the nuclear form of SREBP-1c under the control of doxycycline [180]. Cells were switched to Williams E medium + 25 mM glucose with no insulin in the absence or presence of doxycycline (25, 200 or 250 ng/ml). Cells were harvested 24 hrs afterward for RNA extraction and measurement of elongase and desaturase mRNA abundance by qRT-PCR (transcript/cyclophilin). Endogenous SREBP-1 expression was quantified by northern analysis. Results are represented as Fold Induction by SREBP-1c, mean $\pm$ SD,n=4. These results are representative of 2 separate studies. *$p<0.05$ vs vehicle, *ANOVA*. B. Primary rat hepatocytes in Williams E medium + 10 mM lactate + 10 nM DEX, but no insulin, were infected with a recombinant adenovirus expressing the nuclear form of SREBP-1c under the control of doxycycline [180]. Cells were switched to Williams E medium + 25 mM glucose with doxycycline at 250 ng/ml, in the absence or presence of insulin (100 nM) or T1317 (1 μM). Cells were harvested 24 hrs afterward for RNA extraction and measurement of elongase and desaturase mRNA abundance by qRT-PCR (transcript/cyclophilin). Results are represented as Fold Induction by insulin or T1317, mean $\pm$ SD,n=3. These results are representative of 1 study. *$p<0.05$ vs vehicle, *ANOVA*.

3.4A.


Fold Induction by SREBP-1c
Doxcycline
0 25 100 250
Elovl-1
Elovl-2
Elovl-5
Elovl-6
D5D
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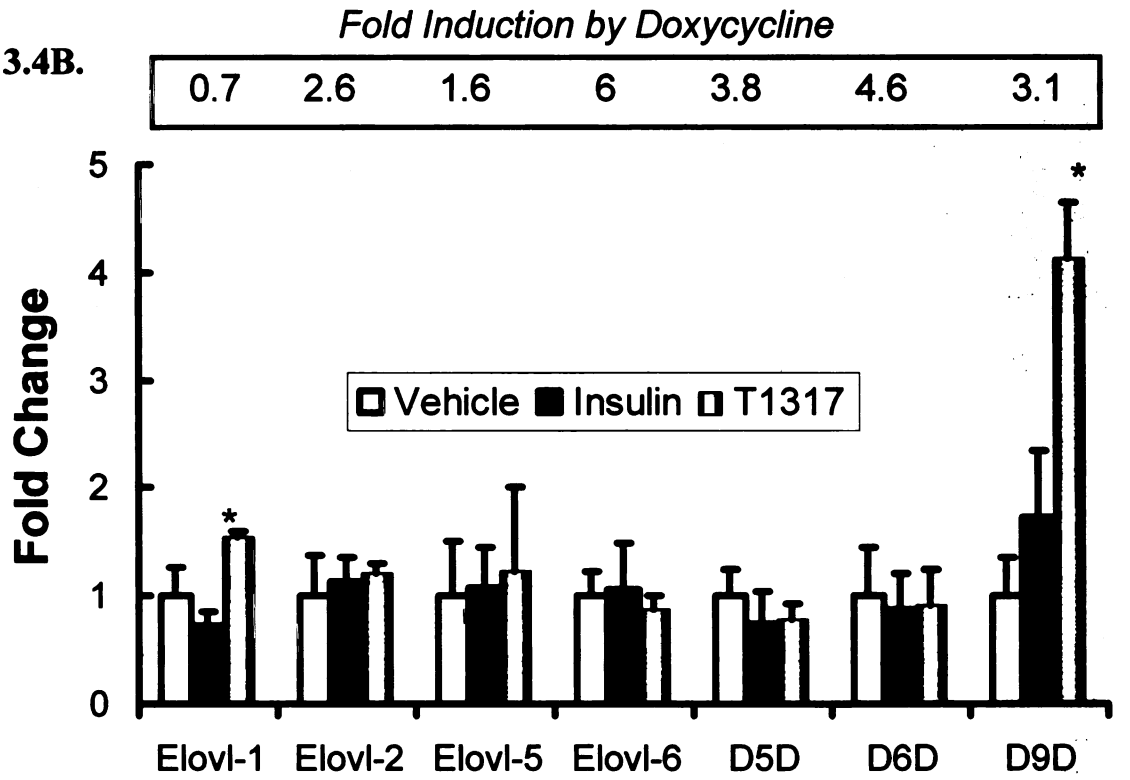
D9D
SREBP1c

3.4B.


Fold Induction by Doxycycline
0.7 2.6 1.6 6 3.8 4.6 3.1
Fold Change
Vehicle Insulin T1317
Elovl-1
Elovl-2
Elovl-5
Elovl-6
D5D
D6D
D9D

plus insulin. This treatment induced the accumulation of ChREBP in hepatocyte nuclei with no effect on MLX nuclear abundance Fig. 3.5 (insert). L-pyruvate kinase (LPK), a glucose-responsive transcript, is controlled at the transcriptional level by binding the ChREBP/MLX heterodimer to its promoter [62]. Switching hepatocytes from lactate to glucose induced $mRNA_{L\text{-}PK}$ ~60-fold (Fig. 3.5). Elovl6 and Δ^9D mRNAs were induced 7.6- and 10-fold, respectively. No other elongase or desaturase was affected by glucose.

To verify the role ChREBP/MLX heterodimers play in this regulatory process, primary hepatocytes in lactate-containing medium were infected with recombinant adenovirus expressing luciferase (Ad-Luc) or a dominant negative version of MLX (Ad-dnMLX). MLX is required for ChREBP to bind ChoRE in glucose-responsive promoters. Overexpressed dn-MLX attenuates the glucose induction of L-PK [173].

Infecting cells with Ad-Luc had no effect on the glucose induction of Elovl6, Δ^9D or L-PK (Fig. 3.5). Infection of cells with Ad-dnMLX completely attenuated the glucose induction of L-PK, Elovl6 and Δ^9D. Ad-Luc or Ad-dnMLX expression in primary hepatocytes had no effect on Elovl1, Elovl2, Elovl5, Δ^5D or Δ^6D. These studies indicate that glucose regulates both Elovl6 and Δ^9D expression by mechanisms that control the nuclear abundance of ChREBP and MLX. ChREBP and MLX play no role in the control of Elovl1, Elovl2, Elovl5, Δ^5D or Δ^6D expression.

3.3.4. Metabolism of fatty acids by fatty acid elongases.

Fatty acid elongases have overlapping substrate specificities [153,187]. Herein, the substrate specificity of 3 fatty acid elongases was examined. Recombinant adenovirus expressing luciferase (Ad-Luc), Elovl2 (Ad-Elovl2), Elovl5 (Ad-Elovl5) and Elovl6 (Ad-Elovl6) were used to over express these enzymes in rat primary hepatocytes. Substrate

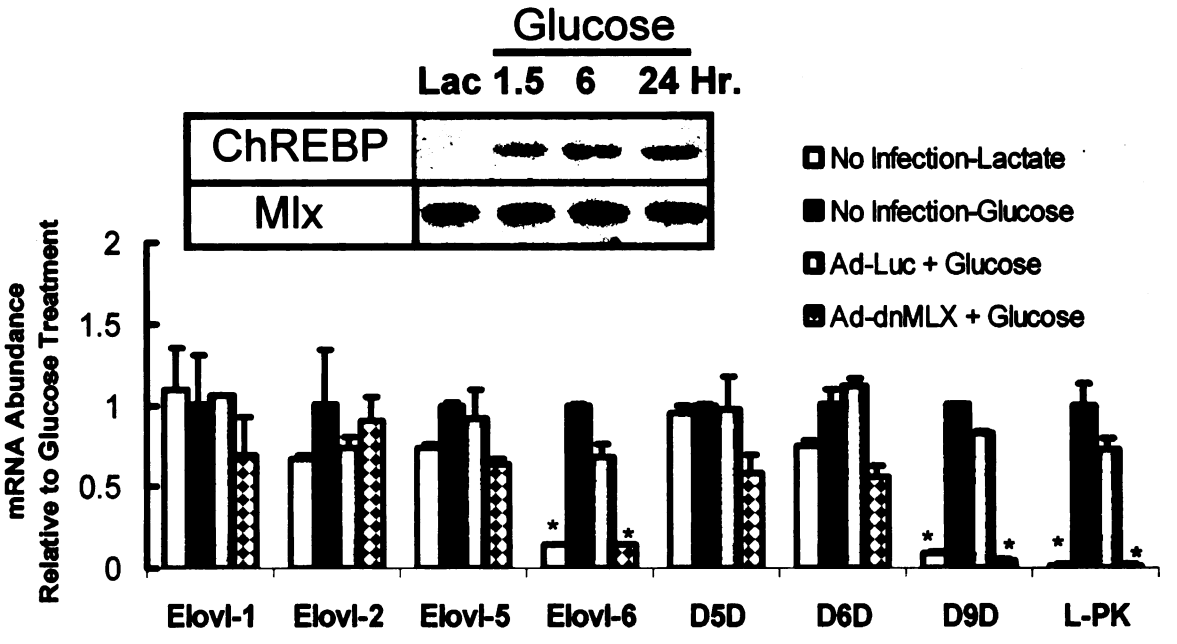


Figure 3. 5. Effect of glucose on elongase and desaturase expression. Primary rat hepatocytes in Williams E medium + 10 mM lactate + 10 nM DEX + 10 nM insulin were switched to Williams E medium + 25 mM glucose + 10 nM DEX + 10 nM insulin. After 24 hrs of treatment cells were harvested for extraction of nuclear proteins and RNA. The nuclear abundance of ChREBP and MLX was measured by immunoblotting at the times indicated [59] (Insert). Elongase, desaturase and L-pyruvate kinase (L-PK) mRNA abundance was quantified by qRT-PCR (transcript/cyclophilin). White bars, cells maintained in lactate; black bars, cells switched to glucose.

In a second experiment, primary hepatocytes in Williams E medium + 10 mM lactate + 10 nM DEX + 10 nM insulin were infected with recombinant adenovirus expressing luciferase (Luc, gray bars) or dominant negative MLX (dn-MLX, checked bars). After 24 hours of treatment, cells were switched to Williams E medium + 25 mM glucose + 10 nM DEX + 10 nM insulin. After 24 hrs of glucose treatment, cells were harvested for RNA extraction and elongase, desaturase and L-pyruvate kinase (L-PK) mRNA expression was quantified by qRT-PCR (transcript/cyclophilin). Results are represented as mRNA Abundance, Relative to Glucose Treatment, mean $\pm$ SD,n=4. These results are representative of 2 separate studies. *$p<0.001$ vs glucose treated cells, *ANOVA*.

specificity was examined using saturate (16:0), monounsaturated (16:1,n-7), and polyunsaturated (18:3,n-6, 20:5,n-3 and 22:5,n-3) fatty acids. Ad-Luc infected cells served as a control for basal elongase activity using the various substrates. Ad-Elovl2 infected hepatocytes elongate only 20:5,n-3 and 22:5,n-3 (Fig. 3.6). Ad-Elovl5 infected cells elongated 16:1,n-7, 18:3,n-6 and 20:5,n-3. Ad-Elovl6 infected cells elongated only 16:0 and 16:1,n-7 (Fig. 3.6).

Expression of hepatic elongases and desaturases is controlled by PPARα, SREBP-1 and ChREBP/MLX (Fig. 3.2-3.5). To determine if changes in elongase and desaturase activity impact hepatocyte fatty acid composition, primary hepatocytes incubated with insulin (induces SREBP-1), PPARα agonists and glucose (induces ChREBP) were examined for the capacity to elongate and desaturate ^{14}C-16:0. Primary hepatocytes were maintained in Williams E media with lactate and no insulin overnight. This treatment effectively lowers SREBP-1 and ChREBP nuclear abundance [59,179]. The next day cells were treated with lactate or glucose containing media in the absence or presence of insulin and WY14643. All cells received 100 μM ^{14}C-16:0. After 24 hrs of treatment, cells were harvested for lipid extraction and analysis of elongation and desaturation products by RP-HPLC.

Cells maintained in lactate media with no insulin or WY14643 do not desaturate 16:0 (Fig. 3.7B, C & D), but elongate 16:0 to 18:0. Addition of insulin or WY14643 to lactate-treated cells induces formation of 16:1,n-7, while only WY14643 induces formation of 18:1 (n-7/n-9). The combination of these treatments has no additive effect.

In cells switched to glucose-containing media, 16:0 desaturation to 16:1,n-7 and the elongation and desaturation of 16:0 to 18:1,n-7/n-9 is induced when compared to the

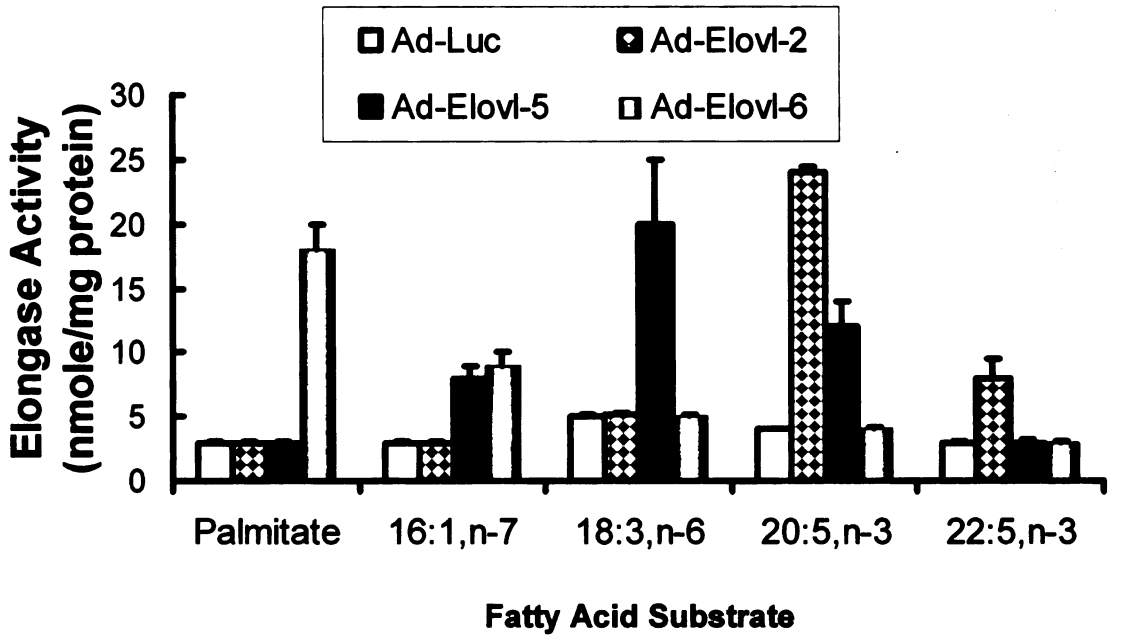


Figure 3. 6. Substrate specificity of hepatic fatty acid elongases. Recombinant adenovirus expressing luciferase (Luc) [white bar], Elovl2 [checked bar], Elovl5 [black bar] and Elovl6 [gray bar] were used to infect primary hepatocytes (5 PFU/cell). After 24 hrs, cells were harvested for elongase activity using various fatty acid substrates (see Methods and Materials). Results are expressed as Elongase Activity (nmoles ^{14}C-malonyl CoA assimilated into fatty acid/mg protein). Results are represented as mean $\pm$ SD,n=3. Results are representative of 2 separate studies.

lactate-treated cells. The combination of glucose + insulin or glucose + WY14643 induced formation of both 16:1,n-7 and 18:1,n-7/n-9 synergistically. Glucose, insulin and WY14643 have no apparent effect on the elongation of 16:0 to 18:0. Since 18:0 is a substrate for Δ^9D, measuring changes in 18:0 may not accurately reflect effects of these treatments on elongation activity. In an effort to reveal an effect on elongation, we examined the effect of glucose, insulin and WY14643 on the 18:1 to 16:1 ratio (Fig. 3.7E). This ratio would remain constant if these treatments did not regulate elongation. The most impressive effect on the 18:1/16:1 ratio is seen in cells maintained in lactate-containing medium supplemented with WY14643. Such studies indicate that hepatocyte levels of 18:1 (n-7/n-9) are controlled by both elongation and desaturation pathways. The elongation pathway is not a constitutive pathway. Cellular levels of 18:1 are not determined solely by regulating Δ^9D expression.

3.3.5. Regulation of elongase and desaturase expression in animal models of metabolic disease.

PPARα [188], SREBP-1 [189] and glucose metabolism, ChREBP & MLX [186] and LXR [190] play an important role in metabolic diseases like diabetes and obesity . Herein, we will determine if changes in hepatic lipid metabolism and composition induced by diabetes and obesity can be attributed to changes in elongase and desaturase expression. Three metabolic disorders were examined, streptozotocin-induced diabetes, glucose intolerance induced by high fat diets, obesity induced by leptin deficiency. Nuclear levels of SREBP-1, ChREBP, MLX and HNF-4α were monitored to correlate changes in nuclear content of these transcription factors with changes in elongase and desaturase expression.

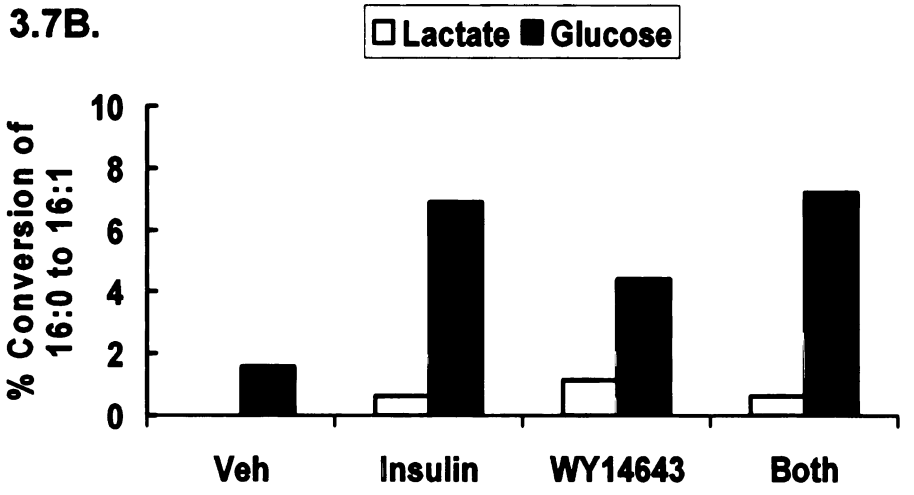
3.7A. ^{14}C-16:0 —*Elovl-6*→ 18:0 —Δ^9*D*→ 18:1,n-9

Δ^9*D* ↓

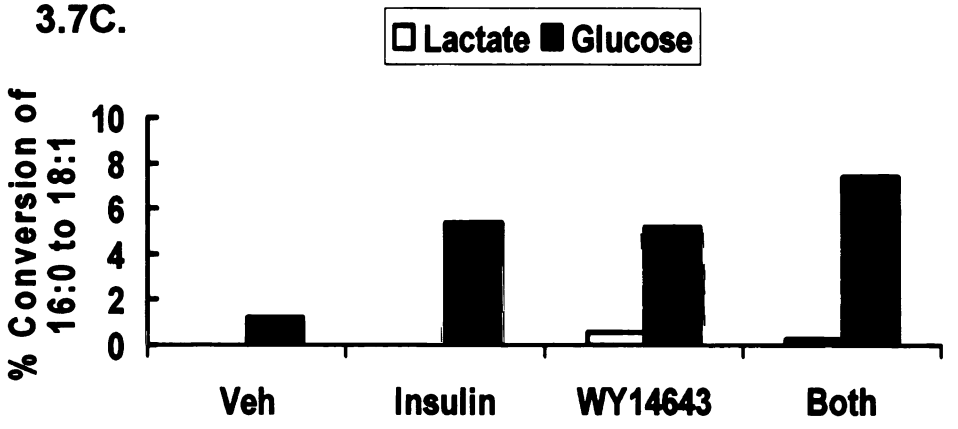
16:1,n-7 —*Elovl5*→ 18:1,n-7

Elovl-6

3.7B.



3.7C.



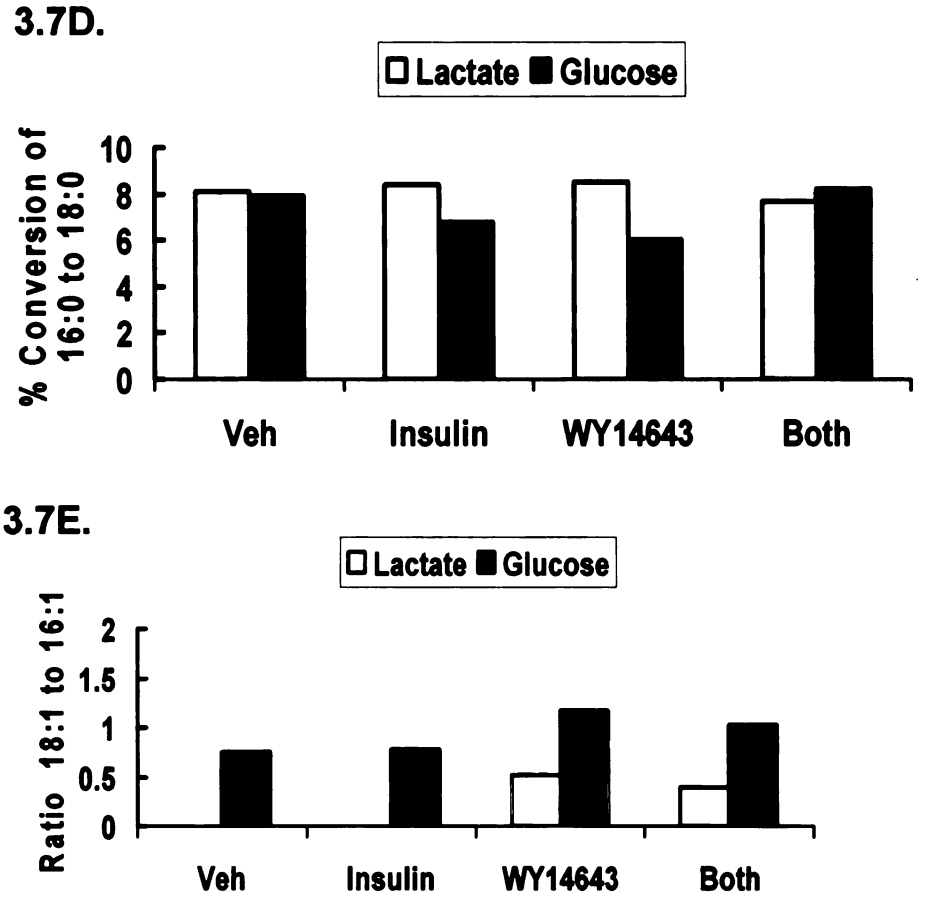


Figure 3. 7. Effects of insulin and WY14643 on monounsaturated fatty acid synthesis. A. The pathway for conversion of 16:0 to 16:1,n-7, 18:1,n-7 and 18:1,n-9 by Elovl5, Elovl6 and Δ^9D is diagrammed. B-E. Primary rat hepatocytes in cultured in Williams E + 10 nM Dex + 10 mM lactate overnight were maintained in the same medium or switched to Williams E + 10 nM Dex + 25 mM glucose in the absence or presence of insulin (100 nM) or WY14643 (50 μM). Cells received 100 μM ^{14}C-16:0 + 20 μM bovine serum albumin for 24 hrs. After 24 hrs of treatment, cells were extracted for total lipid and the lipid was saponified and the distribution of 14C-amongst 16:0, 16:1,n-7, 18:0 and 18:1,n-7 and n-9 was measured by RP-HPLC and a flow-through β-scintillation counter. Technical limitations preclude resolution of 18:1,n-7 and 18:1,n-9 by RP-HPLC. As such, the results are reported as 18:1. Results are reported as (B) % Conversion of ^{14}C-16:0 to ^{14}C-16:1; (C) % Conversion of ^{14}C-16:0 to ^{14}C-18:1; (D) % Conversion of ^{14}C-16:0 to ^{14}C-18:0; (E) ratio of ^{14}C-18:1 to ^{14}C-16:1, an index of elongation. Results are the mean of duplicate sample. Results are representative of 2 separate studies.

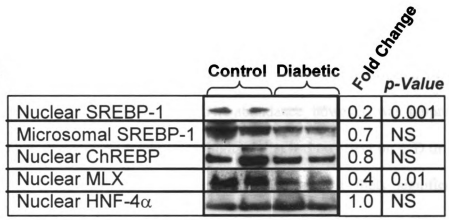
Streptozotocin-Induced Diabetes.

Rats made diabetic using streptozotocin have elevated blood glucose (378 ± 21 mg/dl) when compared to control animals (77.9 ± 5.2 mg/dl). Liver nuclei from diabetic rats contained little detectable nuclear SREBP-1 and suppressed levels of MLX, but no significant change in ChREBP or HNF-4α (Fig. 3.8A). Diabetes suppressed the expression of lipogenic and glycolytic genes, e.g., fatty acid synthase and L-PK, by ≥70% [176], while expression of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PepCk) was induced 3-fold (Fig. 3.8). The decline in the nuclear abundance of SREBP-1 and MLX correlated with a ~45% decline in Elovl6 and a >95% decline in Δ^9D mRNA abundance. Other elongases and desaturases remained unaffected. Examination of hepatic lipid composition revealed a significant 30% decline in 16:0 in diabetic animals, but no change in other saturated, mono- or polyunsaturated fatty acids. The decline in 16:0 is consistent with the decline in *de novo* lipogenesis in livers of diabetic rats [176].

Glucose-Intolerance Induced by High Fat Diets.

High fat diets induce glucose intolerance, insulin resistance, fatty liver and altered hepatic metabolism [191,192]. To examine the effects of diet induced diabetes on elongase and desaturase expression, male C57BL/6 mice were fed a diet with 10% of the calories as fat (low fat, lard diet) or 60% of the calories as fat (high fat, lard diet). Animals fed the low fat diet are 29 g and have blood glucose and insulin levels within the normal range, 121 mg/dl and 0.5 ng/ml (Table 2). Animals fed the high fat diet are ~44 g, with elevated blood glucose (152 mg/dl) and insulin (3.9 ng/ml). When compared to the low fat fed group, high fat fed animals were glucose intolerant (Fig. 3.9A) and have fatty

3.8A



3.8B

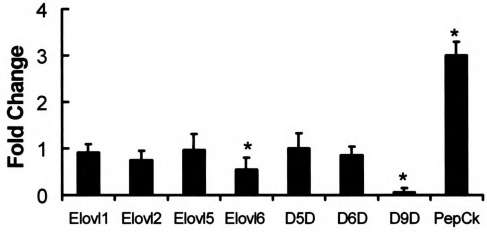


Figure 3. 8. Effect of streptozotocin-induced diabetes on hepatic elongase and desaturase expression. Male Spraque-Dawley rats were made diabetic using streptozotocin as described in Materials and Methods. Livers from control and diabetic animals were used for the isolation of nuclear and microsomal proteins for immunoblotting (A) and RNA extraction for qRT-PCR (B). [A] Effect of diabetes on SREBP-1 (microsomal [pSREBP-1] and nuclear [nSREBP-1]), and nuclear ChREBP, MLX and HNF-4α abundance. Protein levels were measured by immunoblot (Materials and Methods). Duplicate samples for each treatment are shown. The effect of diabetes on the abundance of these proteins was quantified and expressed as Fold Change (mean,n=5) induced by diabetes. Statistical significance (p-value) was assessed by *Student's T-test*. [B] Effect of diabetes on elongase, desaturase and phosphoenolpyruvate carboxykinase (PepCk) expression. Transcripts encoding fatty acid elongases, fatty acid desaturases and cyclophilin were quantified by qRT-PCR, while PepCk was quantified by northern blot analysis. Results are expressed as Fold Change in expression (transcript/cyclophilin) induced by diabetes. *$p<0.05$ vs control; N=5/group, *Students T-test*.

livers [191]. Nuclear levels of SREBP-1 and MLX were suppressed 60% and 50% (Fig. 3.9B), while ChREBP and HNF-4α nuclear levels remained unchanged.

Expression levels of Elovl1 and Elovl2 were not significantly affected by high dietary fat (Fig. 3.9C). Elovl5 and Elovl6 mRNA abundance, however, was suppressed by 50% and 75% with the high fat intake. The high fat diet had no effect on Δ^5D or Δ^6D, but significantly (≥80%) suppressed Δ^9D expression. High fat diets significantly suppressed fatty acid elongation of 16:0-CoA and 20:4-CoA, but not 24:0-CoA (Fig. 3.9D).

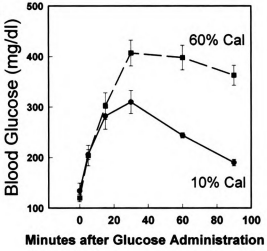
Palmitic (16:0), stearic (18:0), oleic (18:1,n-9) and linoleic (18:2,n-6) acids represent the major fatty acids in both diets. Analysis of hepatic lipid composition indicates that 18:2,n-6 accumulates relative to 20:4,n-6 in livers of high fat fed animals (Fig. 3.9E). 18:2,n-6, an essential fatty acid, is converted to 20:4,n-6. Since neither diet contains 20:4,n-6, the appearance of 20:4,n-6 in the livers requires conversion from 18:2,n-6 to 20:4,n-6 by elongation (Elovl2 and Elovl5) and desaturation (Δ^5D and Δ^6D). In the low fat diet, the ratio of 20:4,n-6 to 18:2,n-6 is 0.75. In the high fat diet the ratio is 0.3. Failure to convert 18:2,n-6 to 20:4,n-6 is consistent with a decline an Elovl5 expression, a key enzyme involved in PUFA synthesis.

Elongase and Desaturase Expression in Livers of Lean ($Lep^{ob/+}$) and Obese ($Lep^{ob/ob}$) Mice.

Defective leptin expression in C57BL/6J-*Lep*$^{ob/ob}$ mice leads to hyperphagia, hyperinsulinemia, insulin resistance and obesity [193]. When compared to the lean (*Lep*$^{ob/+}$) littermates, obese animals are heavier and have elevated blood levels of glucose and insulin (Table 3.2). The livers of obese mice are massively engorged with lipid,

Figure 3. 9. Effect of high fat diets on hepatic elongase and desaturase expression. Male C57BL/6 mice were fed 10% or 60% lard diets for 10 weeks as described in (Materials and Methods) [191]. [A] After nine weeks on the low and high fat diets, mice were assessed for glucose tolerance (Materials and Methods). Blood glucose was measured at the times indicated: 10% Cal (as fat), solid line-filled circle, 60% Cal (as fat), dashed line-filled square. Results are expressed as Blood Glucose (mg/dl), mean $\pm$ SD,n=8 in each group. [B]. Effect of dietary fat on SREBP (microsomal [pSREBP-1] and nuclear [nSREBP-1]), ChREBP, MLX and HNF-4α. After ten weeks on the diet, livers were recovered for nuclear and microsomal protein and RNA extraction as in Fig. 8. Nuclear and microsomal proteins were measured as described in Materials and Methods. Representative immunoblots from duplicate samples for each treatment are shown. The effect of dietary fat on the abundance of these proteins was quantified and expressed as Fold Change (mean,n=4 animals/group) induced by the high fat diet. Statistical significance (p-value) was assessed by *Student's T-test.* [C] Effect of dietary fat on elongase and desaturase expression. RNA was extracted and used for qRT-PCR analysis of elongase and desaturase expression. Results are expressed as Fold Change (transcript/cyclophilin), mean $\pm$ SD,n=8. [D] Effect of dietary fat on elongase activity. Hepatic microsomal preparations were used for fatty acid elongase assays (Materials and Methods). Results are expressed as Elongase Activity, (nmoles ^{14}C-malonyl CoA assimilated into fatty acids/mg protein). Mean $\pm$ SD,n=8. *$p \leq 0.05$ vs 10% Calories as fat, *Student's T-Test.* [E] Effect of dietary fat on hepatic lipid composition. Total lipids were extracted and saponified; fatty acid levels were quantified by RP-HPLC (Materials and Methods). Results are expressed as Fatty Acid Mole%, mean $\pm$ SD,n=8/group; *$p \leq 0.05$ vs 10% lard diet, *Students T-test.* [10% Calories as Fat (open bars) and 60% Calories as Fat (filled bars)].

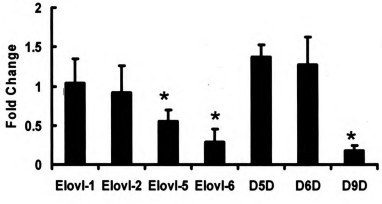
3.9A



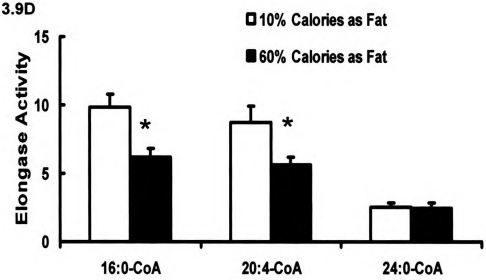
3.9B.

	10% Cal 60% Cal	Fold Change	p-value
Nuclear SREBP-1		0.4	0.01
Microsomal SREBP-1		1.0	NS
Nuclear ChREBP		1.1	NS
Nuclear MLX		0.5	0.01
Nuclear HNF-4α		0.8	NS

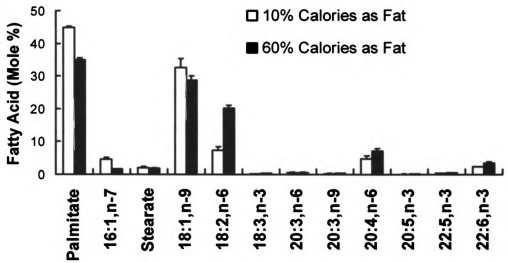
3.9C



3.9D


10% Calories as Fat
60% Calories as Fat
Elongase Activity
15
10
5
0
*
*
16:0-CoA
20:4-CoA
24:0-CoA

3.9E.

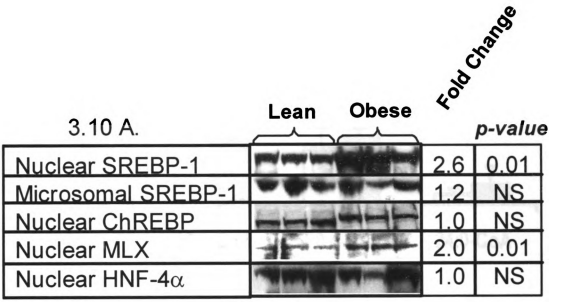

10% Calories as Fat
60% Calories as Fat
Fatty Acid (Mole %)
50
40
30
20
10
0
Palmitate
16:1,n-7
Stearate
18:1,n-9
18:2,n-6
18:3,n-3
20:3,n-6
20:3,n-9
20:4,n-6
20:5,n-3
22:5,n-3
22:6,n-3

predominantly as neutral lipid. Hepatic nuclei derived from obese mice have a 2.6- and 2-fold increase in SREBP-1 and MLX nuclear abundance, but no change in ChREBP or HNF-4α when compared to the lean littermates. Expression levels of Elovl5, Elovl6, Δ^5D, Δ^6D and Δ^9D are increased in livers of obese mice when compared to the lean littermates. Obesity also results in the induction of lipogenic gene expression (ACC, FAS) and PPARα target genes, e.g., Cyp4A and AOX (not shown). Fatty acid elongation of 16:0-CoA is increased 2-fold, while elongation of 20:4-CoA and 24:0-CoA was not affected (Fig. 3.10C).

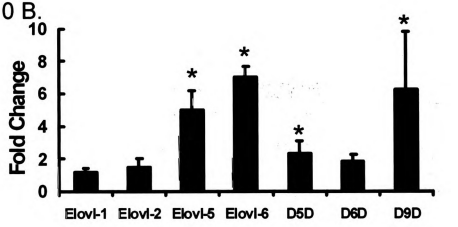
Fatty acid analysis of livers from lean and obese mice reveals a ~9-fold increase in total esterified fatty acid. Much of this increase is in the form of neutral lipid esterified with 18:1 (n-7 and n-9) (Fig. 3.10D). While monounsaturated fatty acid abundance increased, hepatic PUFA content, i.e., 18:2,n-6, 20:4,n-6 and 22:6,n-3, declined. This might appear inconsistent with the observed increase in fatty acid elongase activity and elongase and desaturase expression. *Lep*$^{ob/ob}$ mice, however, are hyperphagic; ingestion of excessive calories as carbohydrate elevates plasma insulin (Table 2) and enhances *de novo* lipogenesis and monounsaturated fatty acid synthesis. Induction of Elovl5, Elovl6 and Δ^9D expression by activated PPARα and elevated SREBP-1 and MLX nuclear content facilitates monounsaturated fatty acid (18:1,n-7 and 18:1,n-9) synthesis which is assimilated into neutral lipid. Elongation, as well as desaturation, are key steps for converting the end products of *de novo* lipogenesis to monounsaturated fatty acids. These studies indicate that fatty acid elongation is induced along with *de novo* lipogenesis and Δ^9-desaturation. Induction of these pathways contributes to the fatty liver phenotype characteristic of *Lep*$^{ob/ob}$ mice.

Figure 3. 10. Effect of leptin deficiency on hepatic elongase and desaturase expression. Male lean C57BL/6J-*Lep*$^{ob/+}$ and obese (C57BL/6J-*Lep*$^{ob/ob}$) mice (B6.V-Lep ob/J , #000632, Jackson Labs) were maintained on a Harlan-Teklad laboratory chow (#8640) diet and water *ad lib*. Livers from these mice were used for the isolation of nuclear and microsomal protein for immunoblotting (A) and RNA extraction for qRT-PCR (B). [A] Effect of obesity on SREBP-1 (microsomal [pSREBP-1] and nuclear [nSREBP-1]) and nuclear ChREBP, MLX and HNF-4α abundance. Protein levels were measured by immunoblot (Materials and Methods). Triplicate samples for each phenotype are shown. The effect of leptin deficiency on the abundance of these proteins was quantified and expressed as Fold Change (mean,n=4) induced by leptin deficiency. Statistical significance (p-value) was assessed by *Student's T-test*. [B] Effect of leptin deficiency on elongase and desaturase expression. RNA was extracted and used for qRT-PCR analysis of elongase and desaturase expression. Results are expressed as Fold Change (transcript/cyclophilin), mean ± SD,n=4. *$p<0.001$ vs Lean; *Students T-test*. [C] Effect of leptin deficiency on elongase activity. Hepatic microsomal preparations were used for fatty acid elongase assays (Materials and Methods). Results are expressed as Elongase Activity, (nmoles ^{14}C-malonyl CoA assimilated into fatty acids/mg protein). Mean ± SD,n=4. *$p\leq0.01$ vs Lean, *Student's T-Test*. D. Effect of leptin deficiency on hepatic lipid composition. Total lipids were extracted and saponified; fatty acid levels were quantified by RP-HPLC (Materials and Methods). Results are expressed as Fatty Acid Mole%, mean ± SD,n=4/group; *$p\leq0.01$ vs Lean animals, *Students T-test*. [Lean (open bars) and Obese (filled bars)].

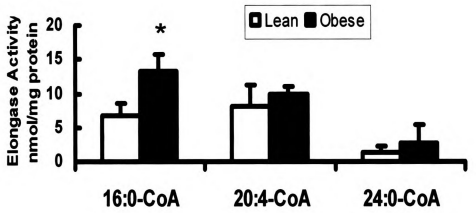
3.10 A.


Lean
Obese
Fold Change
p-value
Nuclear SREBP-1
2.6
0.01
Microsomal SREBP-1
1.2
NS
Nuclear ChREBP
1.0
NS
Nuclear MLX
2.0
0.01
Nuclear HNF-4α
1.0
NS

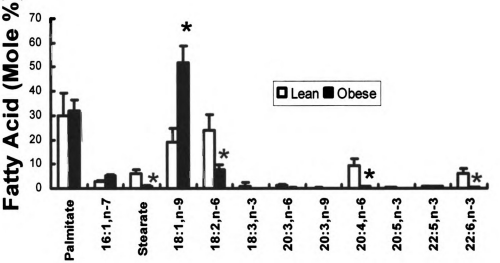
3.10 B.


Fold Change
10
8
6
4
2
0
*
*
*
*
Elovl-1
Elovl-2
Elovl-5
Elovl-6
D5D
D6D
D9D

3.10 C.


Elongase Activity
nmol/mg protein
20
15
10
5
0
*
Lean
Obese
16:0-CoA
20:4-CoA
24:0-CoA

3.10 D.


Fatty Acid (Mole %)
70
60
50
40
30
20
10
0
*
Lean
Obese
Palmitate
16:1,n-7
Stearate
18:1,n-9
18:2,n-6
18:3,n-3
20:3,n-6
20:3,n-9
20:4,n-6
20:5,n-3
22:5,n-3
22:6,n-3

Elongase and Desaturase Expression in Livers of TCDD treated mice.

TCDD is a toxic compound causing controversial human health effects by altering gene expression via the activation of the aryl hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix-PAS (bHLH-PAS) family [194]. Hepatotoxicity is the severe result of TCDD exposure and is characterized by hepatomegaly accompanied by hepatocyte hypertrophy, fat accumulation, immune infiltration, necrosis, and alterations in liver enzymes [195]. Expression level of Elovl5 is increased, whereas Elovl-2, Elovl-6 and Δ^5D are suppressed by 50% to 60% in livers of TCDD treated mice when compared to the control mice (Fig. 3.11 A). The TCDD treatment had no effect on Elovl1, Δ^6D or Δ9D expression. Moreover, TCDD treatment significantly decreases fatty acid elongation of 16:0-CoA, but not 20:4-CoA and 24:0-CoA (Fig. 3.11 B). This is due to the suppressed expression of Elovl6 by TCDD treatment.

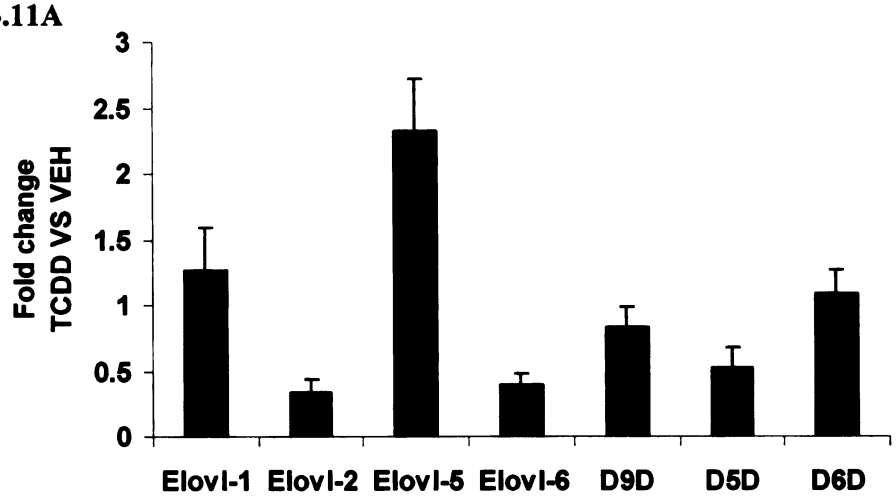
Fatty acid analysis of livers from TCDD treated mice reveals a ~2-fold increase in total esterified fatty acid. Much of this increase is due to the accumulation of 16:0, 18:1n-9 and 18:2,n-6 (Fig. 3.11C). Conversion of 18:2,n-6 to 20:4,n-6 is suppressed by 50% in TCDD treated mice, which is not consistent with an increase in Elovl5 expression (Fig. 3.11D). The decreased expression of Δ^5D may account for it.

3.4. Discussion

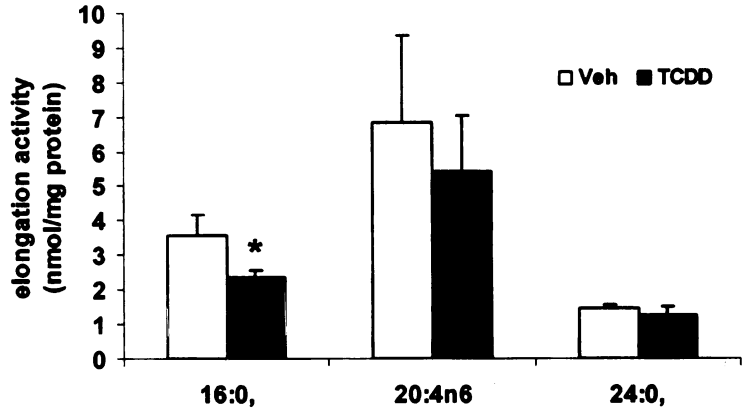
Fatty acid elongation and desaturation are two key metabolic routes for the synthesis of saturated, mono- and polyunsaturated fatty acids. Of these, fatty acid desaturases have received considerable attention for their regulation by hormones and nutrient and their capacity to generate specific unsaturated fatty acids. The outcome of

Figure 3. 11. Effect of TCDD treatment on hepatic elongase and desaturase expression. Male C57BL/6 mice mice were treated by gavage with 0.1 ml of sesame oil for a nominal dose of 0 (vehicle control) or 30 μg/kg body weight of TCDD for 168hr as described in (Materials and Methods) [200]. [A] Effect of TCDD on elongase and desaturase expression. RNA was extracted and used for qRT-PCR analysis of elongase and desaturase expression. Results are expressed as Fold Change (TCDD/VEH), mean ± SD,n=4. [B] Effect of TCDD on elongase activity. Hepatic microsomal preparations were used for fatty acid elongase assays (Materials and Methods). Results are expressed as Elongase Activity, (nmoles ^{14}C-malonyl CoA assimilated into fatty acids/mg protein). Mean ± SD,n=4. *$p \leq 0.05$ vs Veh, *Student's T-Test*. [C.D] Effect of TCDD on hepatic lipid composition. Total lipids were extracted and saponified; fatty acid levels were quantified by RP-HPLC (Materials and Methods). Results are expressed as Fatty Acid Mole%, mean ± SD,n=4/group.

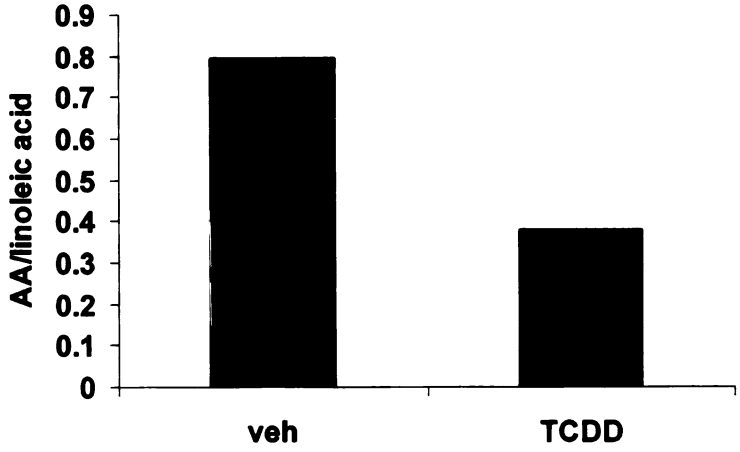
3.11A



3.11B



3.11C



these studies indicates that desaturases are well-regulated enzymes that play an important role in cellular and whole body lipid composition [9,160]. One of these enzymes, Δ^9D (stearoyl CoA desaturase-1), has emerged as a key enzyme in the control of whole body lipid composition [196].

In contrast to the desaturases, fatty acid elongase have only recently been recognized as proteins regulated at the pretranslational level [139,187,197]. Our previous studies indicated that fatty acid elongases are regulated by tissue-specific and nutritional factors and during postnatal development [26]. Those studies implicated certain transcription factors, like SREBP-1 and PPARα, as regulators of both elongase and desaturase expression. The current report extends those previous observations by evaluating the role of several hormones (insulin, T_3, glucocorticoids and leptin), transcription factors (SREBP-1c, PPARα, LXR, ChREBP and MLX) and nutrients (glucose and fat) in the control of hepatic elongase and desaturase gene expression, fatty acid elongase activity and lipid composition. Finding the involvement of these factors in the control of elongase expression prompted studies to evaluate how these enzymes were regulated in metabolic disease. The outcome of these studies has provided new information on how changes in both elongase and desaturase expression in metabolic disease contributes to hepatic lipid composition.

Seven fatty acid elongase subtypes (Elovl1 to Elovl7) have been identified in the genomes of the rat, mouse and human genomes (www.ensembl.org). Of these, 4 elongase subtypes are expressed in rat & mouse liver (Fig. 3.1). The hierarchy for hepatic expression of these enzymes is similar in these two species:

Table 3. 2. Dietary composition and physiological parameters of C57BL/6 mice

	Low Fat versus High Fat		Lean versus Obese	
Parameter	Low Fat	High Fat	Chow	Chow
Calories as fat (%)	10	60	20.3	20.3
Calories as carbohydrate (%)	70	20	55.4	55.4
Calories as protein (%)	20	20	24.4	24.4
Body weight (g)	29.0 ± 1.0	44.0 ± 1.0	23.7 ± 1.5	55.1 ± 2.8
Fasting blood glucose (mg/dl)	121.0 ± 1.0	152.0 ± 0.3	112.0 ± 11	266.0 ± 69.0
Plasma insulin (ng/ml)	0.5 ± 0.05	3.9 ± 0.4	0.2 + 0.06	15.4 ± 1.04
Animals per group	8	8	4	4

Elovl5>Elovl1=Elovl2=Elovl6. Analysis of elongase activity indicates that mouse liver has the highest elongase activity.

Elovl1. Elovl1 is a low abundance elongase in liver of rat and mouse. Based on studies in yeast, Elovl1 elongates a broad array of saturated and monounsaturated fatty acids. Elovl1 expression, however, is not regulated by any physiological manipulation used so far in this or our previous study [26]. Thus, changes in hepatic lipid composition induced during postnatal development, fasting and refeeding, diabetes, obesity, dietary fat, LXR or PPARα agonist can not be attributed to changes in Elovl1 activity. Hepatic Elovl1 appears to be expressed constitutively.

Elovl2. Elovl2 is also a low abundance elongase in liver of rat and mouse. In contrast to other elongases, Elovl2 has a very narrow substrate preference; it elongates C20 and C22 PUFA (Fig. 3.6). As such, Elovl2 participates in the conversion of essential fatty acid precursors to end products of PUFA synthesis, i.e., 20:4,n-6 and 22:6,n-3. Like Elovl1, Elovl2 is not regulated by any factors examined in this report or our previous report. The exception to this is the induction of Elovl2 mRNA following over expression of SREBP-1c (Fig. 3.3). Since insulin, LXR agonist and glucose fail to induce this transcript, we feel the induction of Elovl2 by overexpressed SREBP-1c may have limited physiological significance *in vivo*.

Elovl5. Elovl5 is the most abundant elongase transcript in rat and mouse. It also is expressed in many tissues, induced during postnatal development and suppressed by feeding rats n-3 PUFA enriched diets [26]. Several hormones (insulin, T3, glucocorticoids and leptin) and transcription factors (SREBP-1c, LXR, ChREBP and MLX) have no impact on hepatic Elovl5 expression. Only PPARα, n-3 PUFA-enriched

[26], high fat diets (Fig. 3.9) and obesity (Fig. 3.10) affect Elovl5 expression. Regulation of Elovl5 has physiological significance. Feeding rats a high carbohydrate diet supplemented with olive oil plus WY14643 significantly increased mead acid (20:3,n-9) production [26]. Mead acid is an elongation and desaturation product of 18:1,n-9, the predominant fatty acid in olive oil. It is our view that WY14643 induction of Elovl5 contributes to the formation of 20:3,n-9. Elovl5 also converts 16:1,n-7, but not 16:0, to an 18-carbon monounsaturated fatty acid (18:1,n-7) (Fig. 3.6) as well as elongating an intermediate (18:3,n-6) in the pathway for n-6 PUFA synthesis (20:4,n-6) (Fig. 3.6). Suppression of Elovl5 in high fat-fed mice correlates with decreased hepatic 20:4,n-6/18:2,n-6 ratio (Fig. 3.9). Enhanced Elovl5 expression correlates with the elevated content of 18-carbon monounsaturated fatty acids in livers of obese mice (*Lep*$^{ob/ob}$) (Fig. 3.10). Many PPARα-regulated transcripts, like acyl CoA oxidase and CYP4A, are induced in livers of *Lep*$^{ob/ob}$ mice (not shown). Induction of Elovl5 in livers of obese mice is likely due to PPARα activation. Mice exposed to TCDD, leading to fatty liver, have elevated Elovl-5 expression and enzyme activity (Fig. 3.11).

Despite the role Elovl5 plays in PUFA synthesis and its elevation in livers of obese mice, hepatic lipids in obese animals are not enriched in PUFA. In fact, obese livers are depleted of PUFA relative to other fatty acids like 18:1 (Fig. 3.10). Δ^5D, Δ^6D and Δ^9D are induced in livers of obese mice, but to differing extents (Fig. 3.10). Δ^5D and Δ^6D are induced in obese liver as a result of elevated nuclear abundance of SREBP-1 and activation of PPARα. Δ^9D is induced by these same transcription factors, but also by elevated nuclear ChREBP/MLX (Fig. 3.10). Thus, hyperphagia resulting from defective leptin production, coupled with the ingestion of the high carbohydrate diet stimulates *de*

novo lipogenesis and monounsaturated fatty acid synthesis. In this instance, Elovl5 substrates, in particular 16:1,n-7 (Fig. 3.6) are end products of *de novo* lipogenesis and Δ^9D. Elevated expression of Elovl5, Elovl6 and Δ^9D, coupled with enhanced production of end products of *de novo* lipogenesis yields elevates 18:1,n-7 and 18:1,n-9 production.

Elovl6. Elovl6 is a low abundance enzyme in livers of rat and mouse. Like Elovl2, Elovl6 has a narrow substrate preference, i.e., C12 and C16 saturated and unsaturated fatty acids (Fig. 3.6) [117]. In contrast to the other elongases, Elovl6 is regulated by multiple regulatory factors. Insulin and LXR agonist induce SREBP-1 which induce Elovl6 expression (Fig. 3.3 & 3.4). Insulin-induced glucose metabolism induces ChREBP nuclear content. ChREBP and MLX regulate glucose-regulated genes including L-PK, FAS and Δ^9D. Elovl6 is amongst these glucose-regulated genes (Fig. 3.5). Activation of PPARα also induces Elovl6 (Fig. 3.6). Elovl6 is regulated during postnatal development, but unlike Elovl5, Elovl6 expression falls at birth and is induced at weaning. Elovl6 expression during early postnatal development parallels SREBP-1 nuclear abundance [26]. Finding that both Elovl6 and Δ^9D are induced along with L-PK and FAS (Fig. 3.10) indicates that these enzymes play a role in the hepatic response to excess carbohydrate consumption.

Excess carbohydrate is channeled to *de novo* lipogenesis via enhanced L-PK activity. Insulin-stimulated glucose metabolism induces ChREBP translocation into hepatic nuclei [186,198,199]. ChREBP and MLX heterodimerize and bind ChoREs in promoters of responsive genes, like LPK, ACC and FAS. Insulin also increases SREBP-1 nuclear abundance leading to increased promoter occupancy of SREBP-1 on SRE in target genes, e.g., ACC, FAS and Δ^9D. Consistent with this scenario is the increased

nuclear abundance of SREBP-1 and MLX in livers derived from obese animals (Fig. 3.10). The end product of *de novo* lipogenesis, 16:0, is elongated (Elovl6) and desaturated (Δ^9D) to yield 18:1, the fatty acid accumulating in livers of obese mice. In this metabolic scheme, there appears to be a tight coordination between glycolysis, *de novo* lipogenesis, fatty acid elongation (Elovl6) and desaturation (Δ^9D) that involves 3 transcription factors, ChREBP, MLX and SREBP-1c.

Although these studies provide a link between ChREBP, MLX, SREBP-1 and PPARα in the control of elongase expression, the mechanism(s) for this control remains undefined. Whether this control involves direct interaction of these transcription factors with regulatory elements in the promoters of the elongases or indirect control through other mechanisms will require detailed analysis of the promoters for Elovl5 and Elovl6. These studies are beyond the scope of this report.

In conclusion, we have established that specific hepatic fatty acid elongases, Elovl5 and Elovl6 are regulated in liver by nutrients (glucose and fat), hormones (insulin) and nuclear receptor agonist, i.e., LXR and PPARα agonist. ChREBP, MLX, SREBP-1, PPARα and LXR control elongase, as well as desaturase expression. Only Δ^9D is independently regulated by LXR. Metabolic diseases, like diabetes and obesity, induce changes in hepatic lipid composition by controlling the function of key transcription factors that impact elongase and desaturase expression. These studies support the notion that regulation of both fatty acid elongase and desaturase expression may play an important role in managing hepatic lipid composition in response to changes in dietary and hormonal status.

Chapter 4

Effect of Fatty Acid Elongases on Gene Expression and Lipid Metabolism in Rat Primary Hepatocytes

4.1. Introduction

Fatty acid elongases catalyze the regulated and rate limiting step in long chain fatty acid elongation. Fatty acid elongases work in concert with desaturases to produce fatty acids longer than 16 carbons. The fate of these fatty acids is to serve as substrate for: 1) phospholipids and membranes synthesis; 2) eicosanoid synthesis (prostaglandins, thromboxanes and leukotrienes); 3) β-oxidation and energy metabolism; 4) triglycerides and lipid storage. Disturbance in any of these metabolic pathways is linked to metabolic disorders such as hepatic steatosis, hyperlipidemia, inflammation and insulin resistance.

Seven fatty acid elongase (Elovl) isoforms, each the product of a separate gene, have been cloned and characterized in mammals [115]. Evidence for the importance of Elovl in physiology comes from mutant, knockout and transgenic mice. For example, Quaking and Jimpy are two mouse mutant strains that have dramatically decreased Elovl-1 mRNA level [126]. Elovl3 is induced in brown adipose tissue following exposure of animals to the cold [116]. The water barrier function was impaired in Elovl3 null mice. Elovl6 is induced in transgenic mice over expressing SREBP-1 [24,78,134].

Among the 7 isoforms, four expressed in rat and mouse liver (Elovl-1, -2, -5 and -6); Elovl-5 is the most abundant based on its mRNA abundance. Elovl-5 utilizes a broad array of fatty acids as substrates (C_{16-20}) for MUFA and PUFA synthesis [24,115,201]. It

is induced during postnatal development and suppressed by feeding rats n-3 PUFA enriched diets [175]. Recently, we reported PPARα agonist (WY14,643) and leptin-deficient obesity (*Lep*$^{ob/ob}$) induce Elovl-5 mRNA and enzyme activity leading to changes in hepatic and blood lipid composition [201]. In unpublished studies, we have also found that mice exposed to TCDD, leading to fatty liver, have elevated Elovl-5 expression and enzyme activity[5].

C20 PUFA are the most potent fatty acids activating PPARα and its target genes [53]. Both Elovl-2 and Elovl-5 elongate C20 fatty acids. Compared to Elovl-5, however, Elovl-2 is expressed at lower levels in liver; its hepatic expression is constitutive in all physiological and pathological conditions tested. Elovl-2 also has a narrow substrate preference, $C_{20\text{-}22}$ PUFA. As such, we hypothesize that changes in hepatic Elovl-2 or Elovl-5 might affect PUFA control PPARα-mediated gene expression. To test this hypothesis, we developed recombinant adenovirus harboring the Elovl-2 (Ad-Elovl-2) or Elovl-5 (Ad-Elovl-5). We used recombinant adenovirus technology to over express Elovl-2 and Elovl-5 in rat primary hepatocytes. The outcome of these studies show that altered expression of these enzymes significantly impacts hepatocyte lipid composition and PPARα-regulated gene expression.

4.2 Materials and Methods

Recombinant adenovirus. Cloning of cDNA for Elovl-2 and Elovl-5 was described previously [201]. Cloning of cDNA for ACS4 is performed as described [175]. Primers of acyl CoA synthetase-4 (ACS4) corresponding to its open reading frame (Forward: atggcaaagagaataaaagctaagc; Reverse:ttatttgcccccatacatc) were designed based on sequences obtained from Genbank and the location of the open reading frame was

determined using DNA-Star. Rat liver was used as template. The coding region for each transcript was ligated into Ad-Easy XL adenoviral vector system (Stratagene), recombined in BJ 5183 cells and propagated in XL10 Gold ultra-competent cells. Ad-DNA was packaged into adenoviral particles in Ad-293 cells (Strategene). The resultant adenovirus was titered using Adeno-X^{TM} rapid titer kit (BD Biosciences, Palo Alto, CA) and used to infect rat primary hepatocytes.

Rat primary hepatocyte isolation and adenovirus infection. Male Spraque Dawley rats (Charles River Laboratories, Kalamazoo, MI) were maintained on Harlan-Teklad laboratory chow (#8640) and water *ad lib*. Rat primary hepatocytes were prepared from Teklad chow-fed (*ad lib*) male Spraque-Dawley rats, cultured on BioCoat (type 1 collagen) plates (Beckon Dickinson, Belford, MA.[201]. For RNA and protein extraction, cells were plated onto 100 mm type I collagen-coated plates (BD Bioscience, Bedford, MA) at 10^7 cells/plate in Williams E (Gibco/Invitrogen, Carlsbad CA), 10 mM lactate, 10 nM dexamethasone (Sigma, St. Louis), and 10 % fetal bovine serum (Gibco/Invitrogen). For adenoviral infection studies, cells were plated in the same media onto 6 well type 1 collagen-coated plates at 1.5 x 10^6 cells/well. The ratio of culture media to cell number was maintained constant for the different plating conditions. For treatments, hepatocytes were incubated in medium (Williams E + 10 nM dexamethasone without or with 100 nM insulin and/or 25 mM glucose. Confluent primary hepatocytes were infected (5-10 plaque forming units [PFU]/cell). Using Ad-green fluorescent protein (Ad-GFP) as a control for infection, greater than 80% of primary hepatocytes expressed functional protein at the 5-10 PFU/cell level. The control virus used routinely for these studies, however, was Ad-

luciferase (Ad-Luc). Ad-Luc was obtained from C. Rhodes, Pacific Northwest Research Institute, Seattle, WA.

Acyl-CoA synthetase 4 (ACS4) activity assay Acyl-CoA synthetase activity for ACS4 was determined using an isotopic method [202]. The assay mix for the isotopic method included 300 mM Tris–HCl, pH 7.4, 150 mM $MgCl_2$, 2.25mM glutathione, 20 mM ATP, 0.2 mM CoA, 200 μM fatty acid, and 10 nCi [^{14}C]fatty acid in 0.1% Triton X-100 in a total volume of 100 μl. The reaction was started by adding 5 μg postnuclear homogenate protein and the mixture was incubated for 5 min at 37 °C. Reactions were terminated with 0.2 ml of Dole's solution, isopropanol: heptane: 0.5 M H_2SO_4 (40/10/1; v/v). Water (0.15 ml) was added and unreacted palmitate was removed in the organic phase by extracting 2–3 times with 0.3 ml heptane. [^{14}C]fatty acyl-CoA formed during the reaction was measured by scintillation counting.

RNA extraction, northern analysis and quantitative real time-PCR (qRT-PCR). Total RNA was extracted from primary hepatocytes and used for northern blot and qRT-PCR analysis as previously described [74,201]. ^{32}P[cDNA] for LPK, SREBP1, mtHMG-CoA synthase or CYP4A2 were as described previously [74,179]. Hybridization was quantified by PhosphorImager analysis (Molecular Dynamics). Specific primers for real time PCR for each gene (Table 4.1) were designed using Primer Express software (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized using the SuperScript II RNase H- Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific forward and reverse primers and subjected to real-time PCR quantification using the ABI PRISM 7700 Sequence Detection System (Applied

Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative C_T method (User Bulletin #2, Applied Biosystems). Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. Primers used for quantitative real time PCR are listed in Table 4.1.

Lipid extraction and quantitation of hepatic fatty acids composition. Total lipid was extracted from rat primary hepatocytes in chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene [175]. 7-nonadecenoic acid (19:1) was added as a recovery standard at the time of extraction. Protein (Bio-Rad, Hercules, CA) was measured in extracts after the initial homogenization step. Total lipids were saponified, fractionated and quantified by reverse phase HPLC (RP-HPLC) using a YMC J-Sphere (ODS-H80) column and a gradient starting at 77.5% acetonitrile + acetic acid (0.1%) and ending at 100 % acetonitrile + acetic acid (0.1%) over 90 mins with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the MSU Mass Spectrometry facility by GC/MS (www.bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for RP-HPLC were obtained from Nu-Chek Prep (Elysian, MN).

Immunoblotting. Total, cytosolic, microsomal and nuclear protein extracts from rat primary hepatocytes were prepared as described previously [159,175]. Proteins (50-100 μg) extracted from microsomal or nuclear fractions were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12% polyacrylamide Bis-Tris, Invitrogen) and transferred to nitrocellulose membranes. Membranes were incubated with

Table 4. 1.Primer sequences used for gene expression by northern blot and quantitative reverse transcription-PCR.

Gene	Forward	Reverse
INSIG 1	TGCAGATCCAGCGGAATGT	CCAGGCGGAGGAGAAGATG
INSIG 2A	GACGGATGTGTTGAAGGATTTCT	TGGACTGAAGCAGACCAATGTC
INSIG 2B	CCGGCAGAGCTCAGGATTT	AACTGTGGACTGAAGCAGACCAA
SCAP	ATGAGGAGCTGTGGAGGAAA	CCGTGGATTCAGGTGTAGTGT
CYCLOPHILIN	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG

antibodies for, SREBP-1 (IgG-2A4, sc-13551), SREBP-2(Santa Cruz Biotechnology, San Cruz, CA). Anti-mouse and anti-rabbit secondary antibodies were obtained from Bio-Rad (Hercules, CA). The SuperSignal West Pico chemiluminescence kit (Pierce) detection system was used.

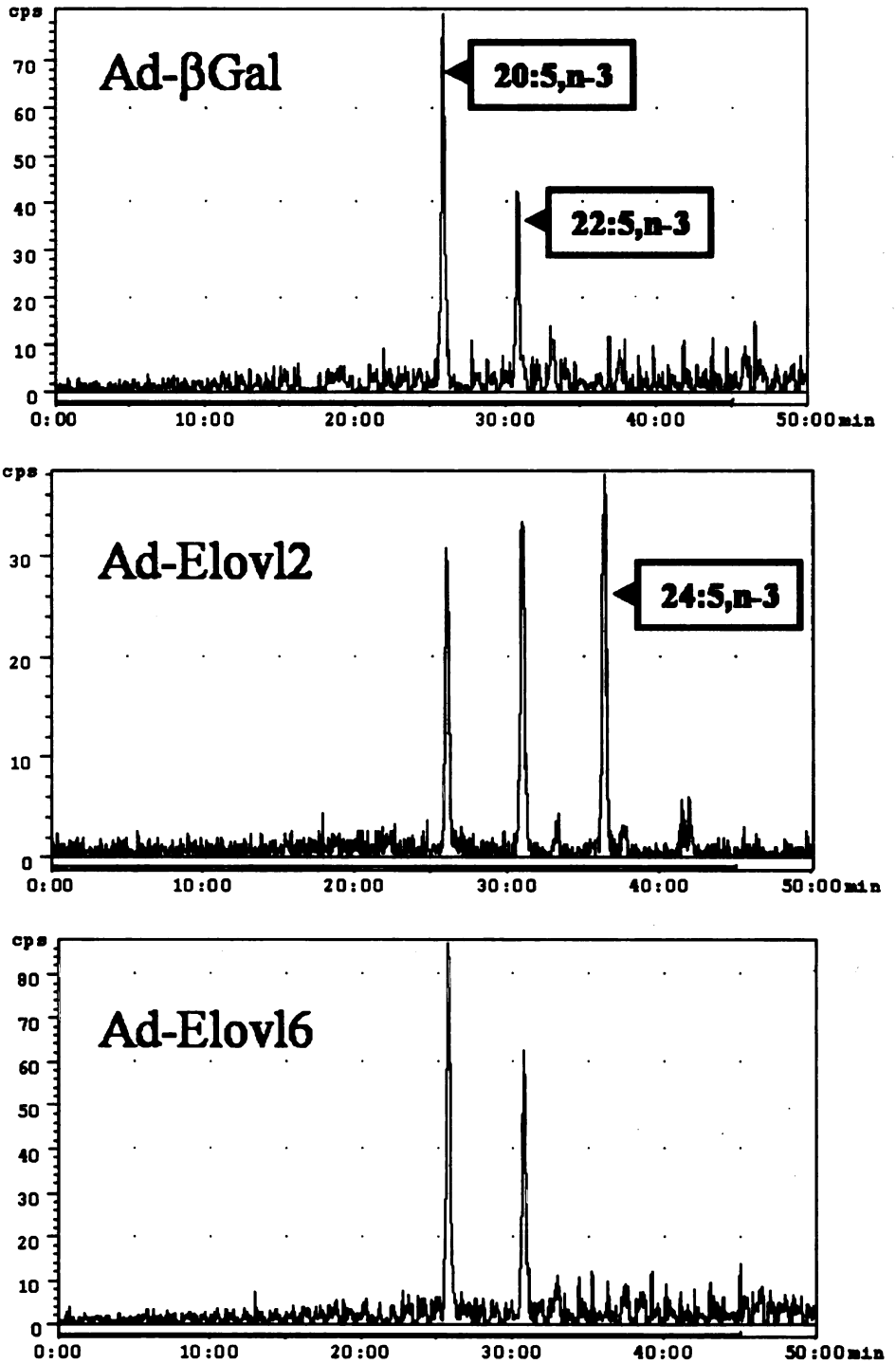
Statistical Analysis. Statistical analysis used Student's t-test and ANOVA plus post hoc Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html).

4.3 Result

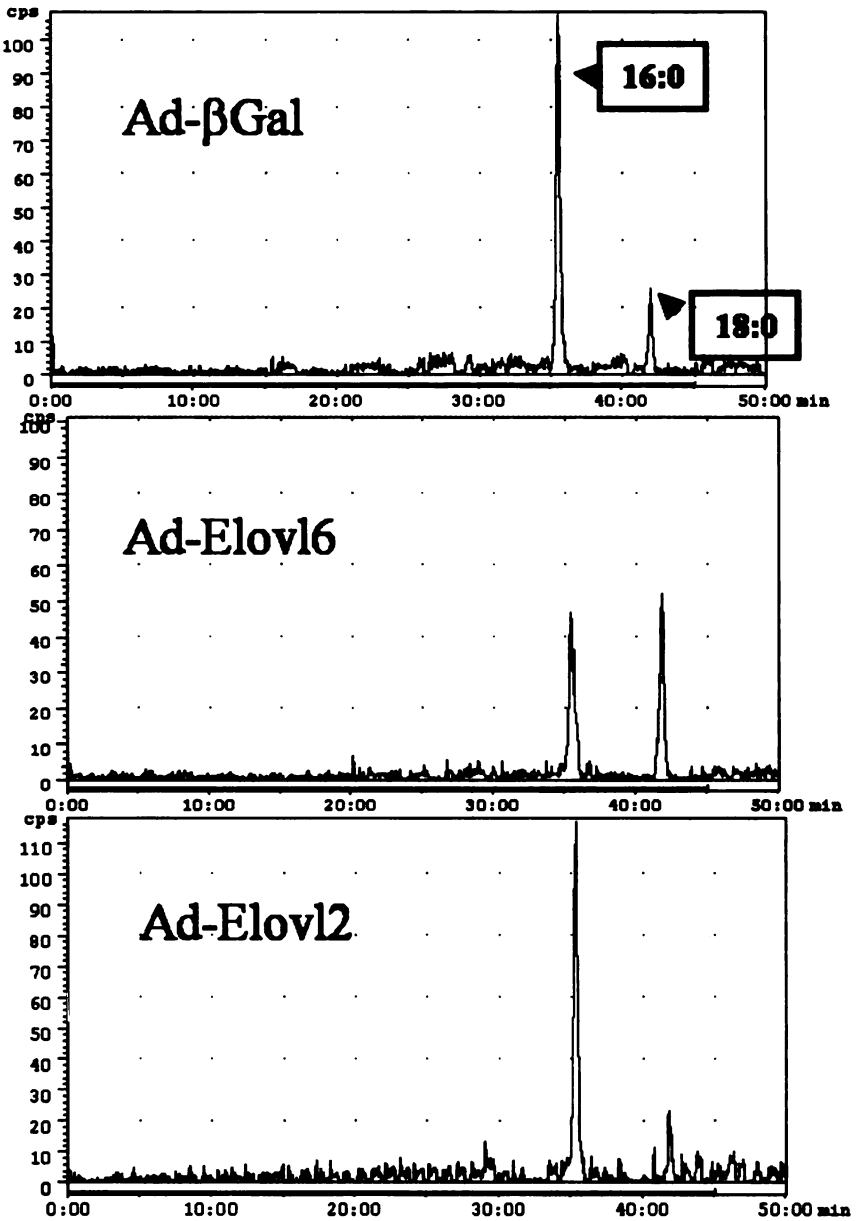
4.3.1. Efficacy of Ad-Elovl2 and Ad-Elovl5 infection in rat primary hepatocytes.

Rat primary hepatocytes were infected with Ad-Elovl2, Ad-Elovl5, Ad-Elovl6 Ad-Luc (Luciferase) or Ad-βGal (β-galactosidase) at 10 pfu/cell for 4 hrs. After infection, medium was replaced with fresh medium containing ^{14}C-20:5,n-3, ^{14}C-20:4,n-6 or ^{14}C-16:0 at 100 μM for an overnight incubation. The total lipid was extracted and saponified fatty acids were fractionated by RP-HPLC as described in Methods. The results showed that the cells infected with Ad-βGal or Ad-Elovl2 and treated with ^{14}C labeled 16:0 converted 25% of 16:0 to 18:0 (Fig 4.1 B). However, cells infected with Ad-Elovl6 converted 55% of 16:0 to 18:0 (Fig 4.1 B) and cells infected with Ad-Elovl5 converted 35% of 16:0 to 18:0 (Fig. 4.1 C). Primary hepatocytes typically converted 40% of exogenous C20 to C22. Infecting cells with Ad-βGal, or Ad-Luc did not affect this reaction. The cells infected with Ad-Elovl-2 and treated with ^{14}C labeled 20:5,n-3 converted 70% of 20:5,n-3 to 22:5,n-3 and 24:5,n-3 (Fig 4.1 A). The cells infected with Ad-Elovl-5 and treated with ^{14}C labeled 20:4,n-6 convert 60% of 20:4,n-6 to 22:4,n-6,

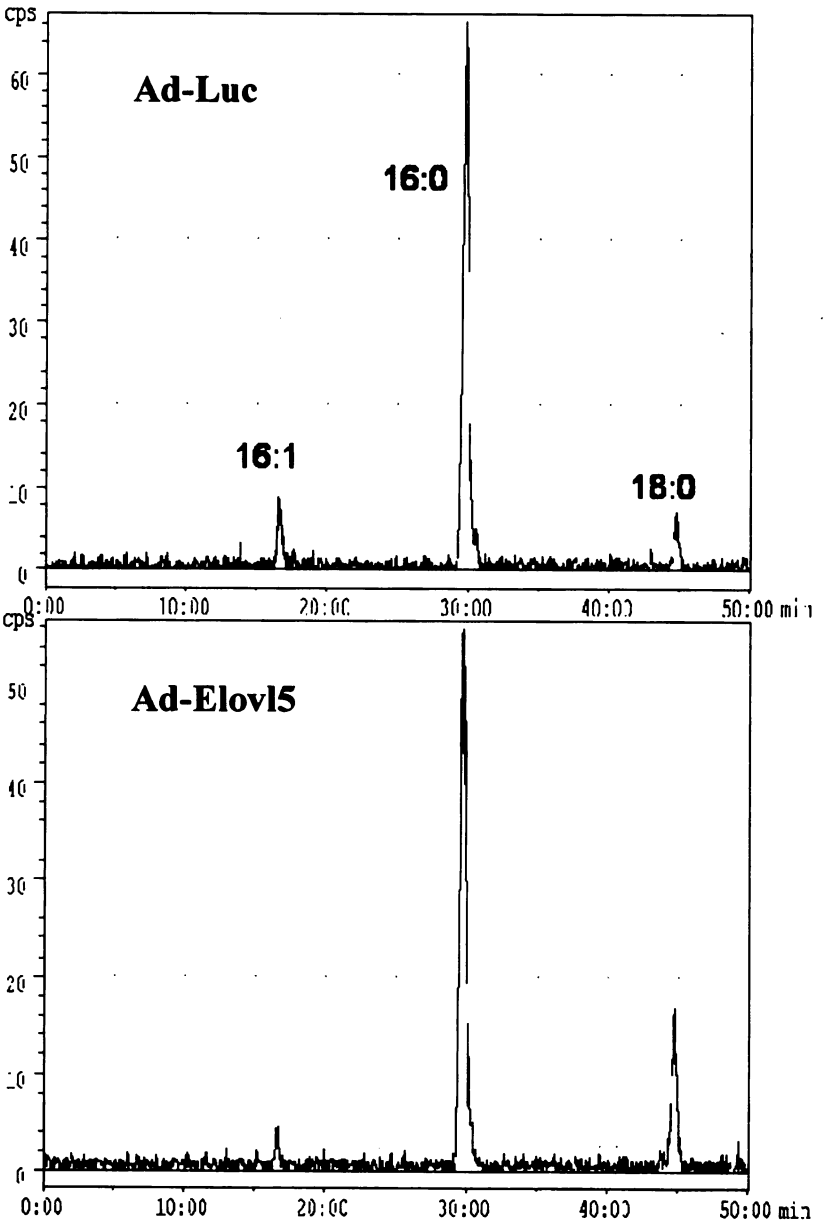
4.1A


cps
Ad-βGal
20:5,n-3
22:5,n-3
0:00
10:00
20:00
30:00
40:00
50:00min
Ad-Elovl2
24:5,n-3
Ad-Elovl6

4.1B.


cps
Ad-βGal
16:0
18:0
0:00
10:00
20:00
30:00
40:00
50:00 min
Ad-Elovl6
Ad-Elovl2

4.1C.


cps
Ad-Luc
16:0
16:1
18:0
0:00
10:00
20:00
30:00
40:00
50:00 min
cps
Ad-Elovl5
0:00
10:00
20:00
30:00
40:00
50:00 min

4.1D.

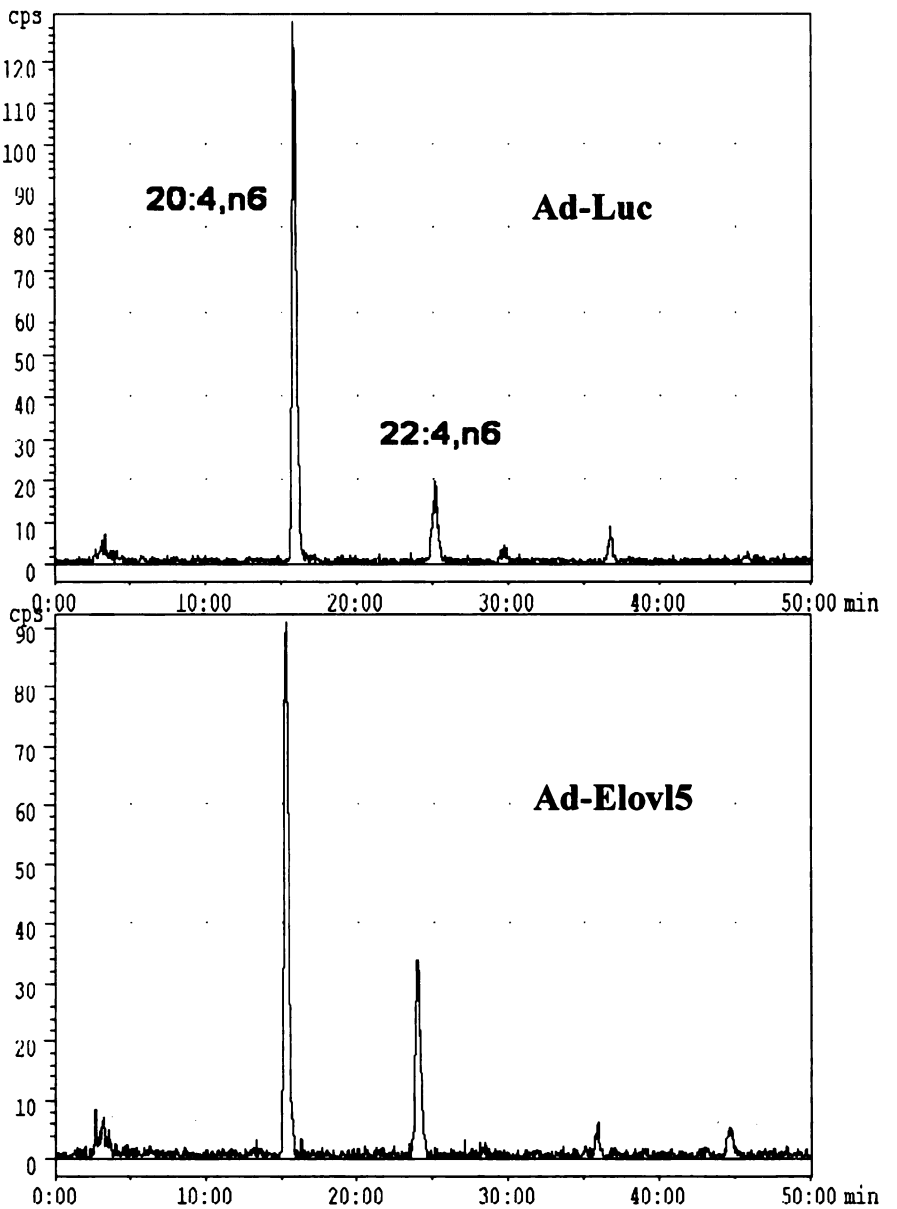


Figure 4. 1. Fatty acid analysis of hepatocytes overexpressed Elovl2, Elovl5, Elovl6, βGal and Luc. Primary hepatocytes were infected with 10 pfu/cell Ad-Elovl2, Ad-Elovl5, Ad-Elovl6, Ad-βGal and Ad-Luc respectively. Then, either ^{14}C-16:0 or ^{14}C-20:5*n*-3 or ^{14}C-20:4n6 (at 250 μM, 1.7 Ci/mole) were treated to these 4 group of cells. Total lipids were saponified, fractionated, and quantified by RP-HPLC. Distribution of ^{14}C-16:0 (*B and C)* and ^{14}C-20 (A and D) derivatives were fractionated by RP-HPLC. The level of radioactivity was quantified using a flow-through scintillation counter in line with the HPLC unit.

but not 24:4,n-6 (Fig. 4.1 D). This study shows that overexpression of Elovl2 and Elovl5 by recombinant adenovirus leads to increased conversion of their substrates to longer fatty acids. C_{20} and C_{22} are the substrates for the Elovl2. C_{16} and C_{20} unsaturated fatty acids are the substrates for Elovl5. This study indicates that recombinant adenovirus can be used to over express elongases in primary hepatocytes and change the lipid profile.

4.3.2. Effect of Elovl2 and Elovl5 on fatty acid metabolism in rat primary hepatocytes.

Further analysis of lipid profile in hepatocytes indicated that overexpression of either Elovl2 or Elovl5, reduced 20:5,n-3 level significantly, which is the most potent fatty acid activator of PPARα. Additionally, Elovl-2 increased 24:5,n-3 and Elovl-5 increased 22:5,n-3 level (Fig. 4.2 A and B).

4.3.3. Effect of Elovl2 and Elovl5 on gene expression in rat primary hepatocytes.

Prior to the addition of fatty acids to primary hepatocytes, intracellular non-esterified unsaturated fatty acid levels are very low, representing ≤0.1% of the total fatty acyl chains in the cell. The relative abundance of putative PPARα ligands in the NEFA pool is 20:4,n-6 = 18:2,n-6 = 18:1,n-9 > 22:6,n-3 > 18:3,n-3/6 = 20:5,n-3. When compared to all PUFA in primary hepatocytes, 20:5,n-3 is the most active ligand for activating PPARα [136]. Since both Elovl-2 and Elovl-5 can convert C_{20} fatty acids to C_{22} fatty acids, overexpression of Elovl-2 or Elovl-5 might attenuate 20:5,n-3 effects on PPARα-regulated gene transcript by rapidly reducing 20:5,n-3 mass in hepatocytes.

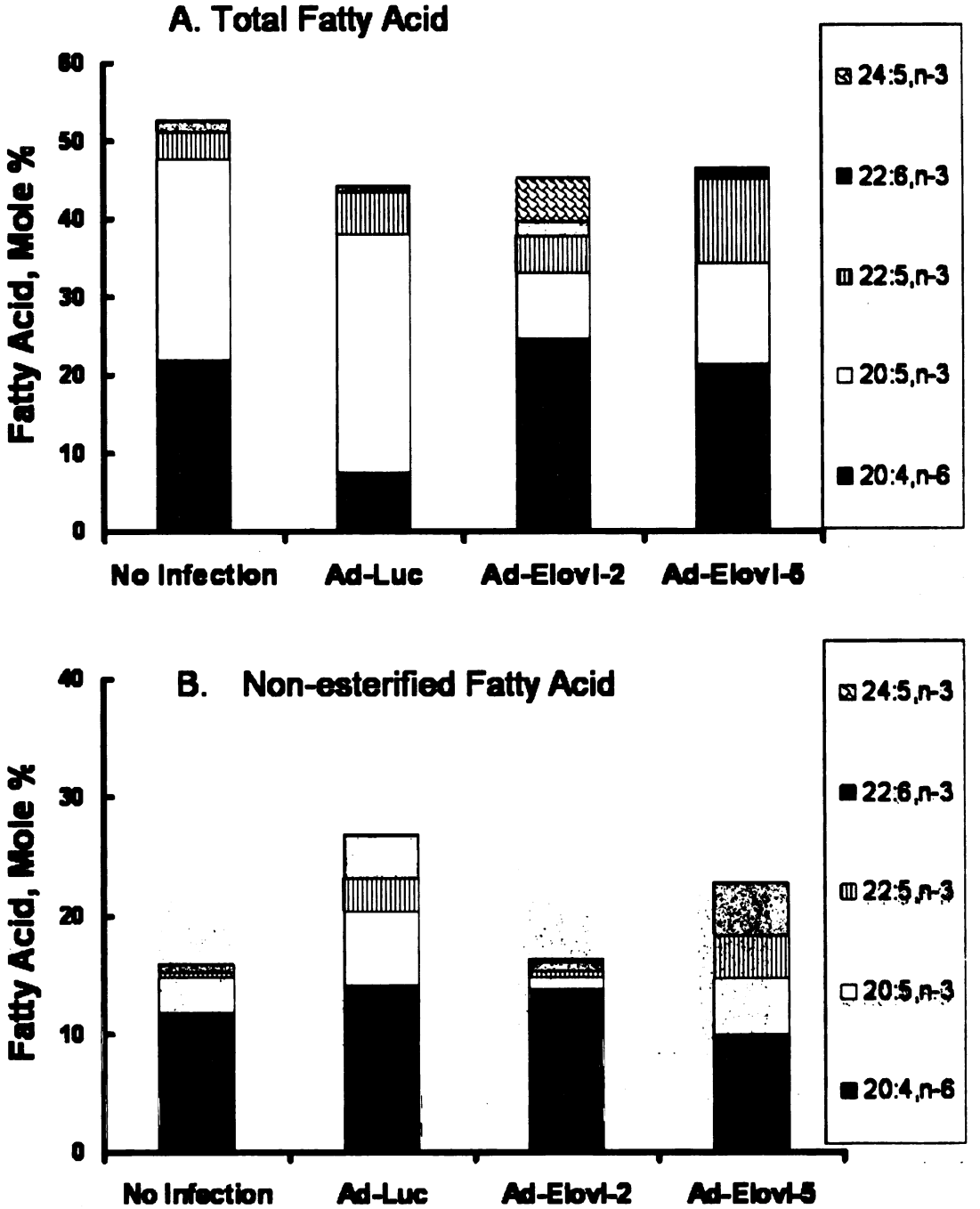


Figure 4. 2. Fatty acids analysis of rat primary hepatocytes overexpressing Elovl2 and Elovl5. Primary rat hepatocytes were infected with 10pfu/cell adenovirus expressing Elovl2, Elovl5 and luciferase (control) followed by 250uM 20:5n3 treatment. Cells were harvested at 24 hr after 20:5n3 treatment. A: Total lipids were extracted and saponified and analyzed by RP-HPLC. Each fatty acid was expressed as percentage of total fatty acid amount. B: NEFA were separated from total lipid. Each fatty acid was expressed as percentage of total fatty acid amount.

To test this hypothesis, hepatocytes were infected with either 10 pfu/cell Ad-Elovl-2, ad-Elovl-5 and Ad-acyl CoA synthetase-4 [Ad-ACS4 a control virus] overnight and treated with 250 μM 20:5,n-3 for 24 hr. Total RNA was isolated and used for northern analysis to examine the expression of PPARα target gene i.e., cytochrome P450-4A (CYP4A), cytosolic thioesterase I (CTE-I) and mitochondrial HMG-CoA synthase 2 (HMGCS2). The efficacy of Ad-ACS4 was indicated by enzyme assay as shown in Fig 4.4 A. The results show that overexpression of Elovl-2 significantly suppressed 20:5,n-3 induction on PPARα target genes CYP4A, CTE-I and HMGCS2, but did not affect 20:5,n-3 effects on the expression of $mRNA_{SREBP-1}$ or $mRNA_{LPK}$ (Fig. 4.3). Overexpression of Elovl-5 has the similar effect as Elovl-2 on PPARα target genes (Data not shown).

4.3.4. Effect of Elovl2 and Elovl5 on SREBPs nuclear content in rat primary hepatocytes.

The nuclear abundance of SREBP-1, but not SREBP-2, is suppressed by PUFA. Of the C18-22 PUFA found in primary hepatocytes, 22:6,n-3 is the most potent suppressor. The mechanism for this control involves 22:6-mediated activation of SREBP-1 26S proteasomal degradation [159]. Based on the differential effects of PUFA on SREBP-1 content, I next determined if changes in elongase activity affected SREBP-1 nuclear abundance. The experiment was designed as the gene expression study. Instead of RNA analysis, however, nuclear and microsomal protein extracts were isolated from infected primary hepatocytes to measure SREBP-1 and SREBP-2 by immunoblot analysis. Elevated Elovl-2 activity in the absence of exogenous PUFA had no effect on SREBP-1 or SREBP-2 nuclear abundance. Addition of 20:5,n-3, however, enhanced the

suppression of SREBP-1 nuclear content, without effects on SREBP-2 nuclear content. Over expressing Elovl5 or Elovl6, however, did not alter the 20:5,n-3 effect on SREBP-1 nuclear content (Fig. 4.4). Elovl6 did not utilize 20:5,n-3 as substrate. Although Elovl5 converts 20:5,n-3 to 22:5,n-3, Elovl5 does not convert 22:5,n-3 to 24:5,n-3. This study suggests that excessive formation of 24:5,n-3 impacts SREBP-1 nuclear abundance. Unfortunately, 24:5,n-3 is not commercially available, thus limiting a direct test of whether 24:5,n-3 or some other action of Elovl2 over expression affects SREBP-1 nuclear abundance.

We examined possible mechanisms for this effect of Elovl2 over expression on 20:5-mediated suppression of SREBP-1 nuclear abundance. As shown in Fig.4.3, infection of hepatocytes with Ad-Elovl2 did not enhance the effect of 20:5,n-3 on $mRNA_{SREBP1}$ abundance. 22:6,n-3 is a robust suppressor of hepatocyte SREBP-1 nuclear abundance [179]. The mechanism for this suppression involved 22:6-mediated 26S proteasomal degradation of nuclear SREBP-1 [179]. Treating Ad-Elovl2 infected hepatocytes with 26S proteasomal inhibitors (MG132 or lactacystin) did not block the enhance 20:5,n-3 suppression of mature SREBP-1 (Data not shown). I next examined the effects of these treatments on proteins involved in proteolytic maturation of SREBP. Analysis of Insig-1 and Insig-2 mRNA and SCAP protein revealed mRNA of Insig1 and Insig2a were induced by overexpression of Elovl2 in the cells treated with 20:5,n-3 compared to Ad-Luc group. There is no change of mRNA of Insig2b and SCAP by overexpression of Elovl2 (Data not shown). However, SCAP protein was suppressed by

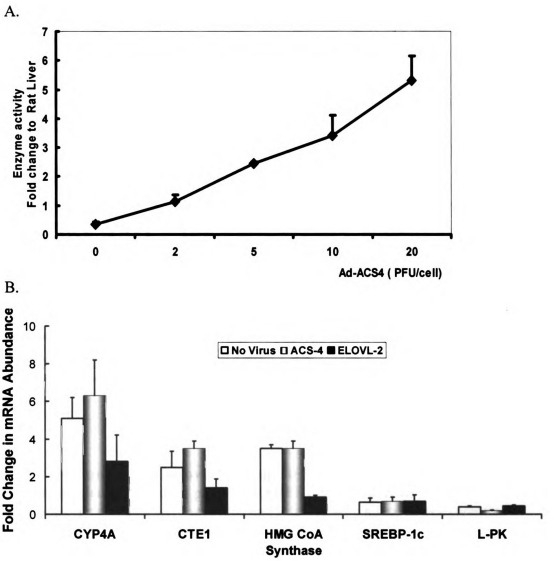


Figure 4. 3. Effect of Elovl2 on hepatic gene expression. A. Efficacy of Ad-ACS4 in rat primary hepatocytes. Postnuclear fraction was prepared by differential centrifugation from rat liver and the rat primary hepatocytes infected with different dosage of Ad-ACS4. Fractions (5μg protein) were assayed for ACS activity. B. Primary rat hepatocytes were infected with 10pfu/cell Ad-Elovl2 or Ad-ACS4 (control) followed by 250uM 20:5n3 treatment. RNA was harvested at 24 hours after treatment. Northern blot was performed to exam PPARα targeted genes (Cyp4A, CTE-1, HMG CoA Synthase) and SREBP-1c and L-PK gene expression. Mean ± SD,n=3. These results are representative of 3 separate studies.

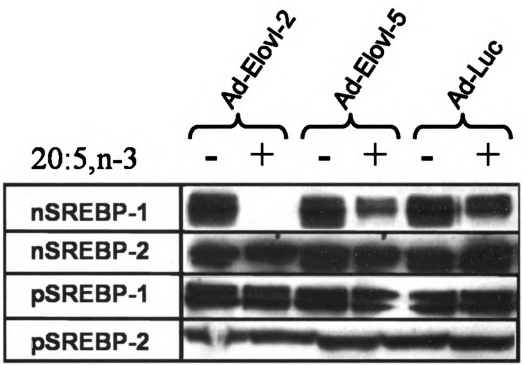


Figure 4. 4. Overexpression of Elovl2 effect on 20:5,n-3 regulation of rat hepatic sterol regulatory element binding protein 1 (SREBP-1) and SREBP-2 nuclear content. Primary hepatocytes were prepared and infected with adenovirus expressing Elovl2, Elovl5, Elovl6 and luciferase 24 hours. Followed by treatment of 250 μM 20:5,n-3 described before. Nuclear and microsomal proteins were isolated from cells after 24 hours fatty acid treatment. Western blot was performed and probed with antibodies for SREBP-1 and SREBP-2.

overexpression of Elovl2 followed by 20:5,n-3 treatment (Fig 4.5 C). Based on the outcome of these studies, elevated expression of Elovl2 coupled with the addition of 20:5,n-3 conversion to 24:5,n-3 inhibits the proteolytic maturation of SREBP-1, but not SREPB-2 in rat primary hepatocytes.

4.4. Discussion

My previous studies established that certain fatty acid elongases, like fatty acid desaturases, are regulated during development, by nutritional status and by hormones [175,201]. My goal in this study was to determine the effect of altered elongase expression on rat primary hepatocyte lipid metabolism. Since changes in hepatic lipid content are known to control two major transcriptional regulatory networks, I determined if altered elongase expression affected these networks, i.e., PPARα and SREBP-1. The outcome of these studies supports the hypothesis that fatty acid elongases affect both hepatocyte lipid content and gene expression.

Fatty acid metabolism in the liver is tightly regulated. Fatty acids are oxidized to meet energy needs, or esterified to produce triglycerides, which are either stored in the liver or exported to peripheral tissue by VLDL particles. Two classes of transcription factors, namely, SREBPs and PPARs play a major role in lipid metabolism and energy homeostasis. Our studies suggest that overexpression of hepatic Elovl2 or Elovl5 can alter fatty acid structure, which will affect the composition of intracellular NEFA. As such, these enzymes contribute to the availability of fatty acid metabolites controlling SREBP-1 and PPARα functions. Overexpression of both Elovl2 and Elovl5 enhance 20:5,n-3 conversion to 22:5,n-3 or 24:5,n-3, which leads to reduction of 20:5,n-3 and thus attenuates 20:5,n-3 mediated induction on PPARα regulated transcripts. Moreover,

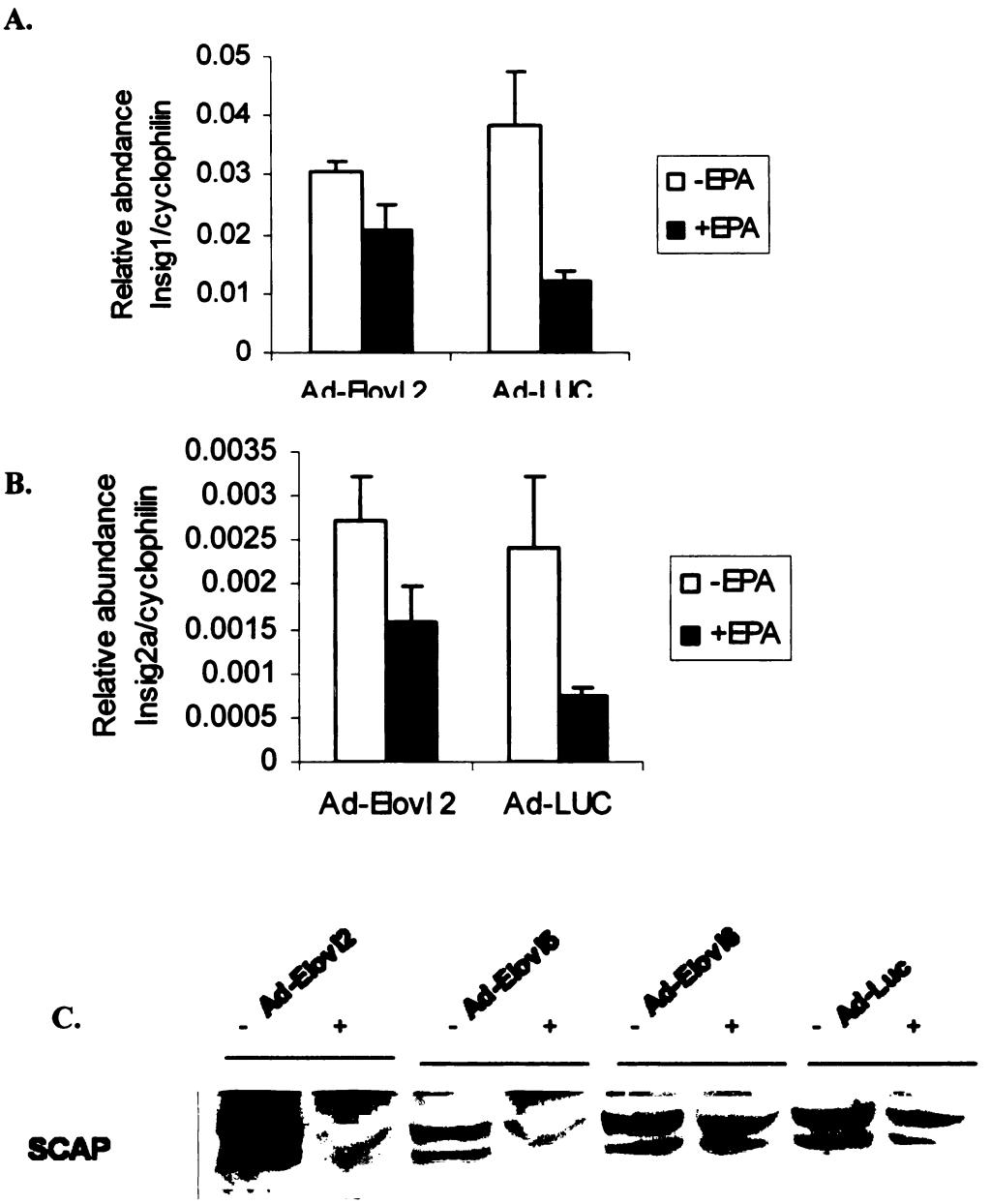


Figure 4. 5. Effect of overexpression of Elovl2 on mRNA of Insigs and SCAP protein. Primary rat hepatocytes were infected with 10pfu/cell Ad-Elovl2 or Ad-Luc (control) followed by 250uM 20:5n3 treatment. RNA and microsome protein were harvested at 24 hours after treatment. Real time PCR was performed to exam mRNA of insig1 (A), insig2a (B) and SCAP protein level (C). These results are representative of 3 separate studies.

overexpression of Elovl2, but not Elovl2, can enhance 20:5,n-3 mediated suppression of SREBP-1c nuclear content.

Over expressed Elovl2: In liver, Elovl2 is constitutively expressed at low levels. Its substrate preference is limited to $C_{20\text{-}22}$ PUFA. Over expression of Elovl-2 enhanced the capacity of hepatocytes to convert 20:5,n-3 to 22:5,n-3 and 24:5,n-3. Interestingly, this does not lead to elevated 22:6,n-3 formation. The likely explanation for this is the suppression of Δ^6-desaturase activity in cultured rat primary hepatocytes (unpublished observation). Over expression of Elovl2 lowers total and non-esterified 20:5,n-3 levels in hepatocytes. A consequence of this is the attenuation of 20:5-mediated induction of PPARα target genes, i.e., CYP4A, CTE1 and HMGCS2.

A second outcome of our analysis was finding that over expressed Elovl2 significantly enhanced the 20:5-mediated suppression of mature (nuclear) SREBP-1 in hepatocytes. The mechanism for this effect is attributed to effects on proteolytic maturation of SREBP-1. This interpretation is based on the following observations. First, over expressed Elovl2 did not enhance 20:5,n-3 suppression of $mRNA_{SREBP\text{-}1}$ in primary hepatocyte. Second, 26S proteasomal inhibitors did not block the 20:5,n-3/Ad-Elovl2 suppression of mature SREBP-1. Third, over expressed Elovl2 suppressed hepatic levels of SCAP and induce INSIG 1 or 2. Taken together, these finding support the notion that Elovl2 alters 20:5,n-3 metabolism in such a fashion to impact SREBP-1 maturation to the nuclear form.

Over expressed Elovl5: Like Elovl2, over expression of Elovl5 significantly lower hepatic levels of 20:5,n-3 (Fig. 4.1D) and attenuated 20:5,n-3 induction of PPARα target genes. Unlike Elovl2, Elovl5 does not elongate 20:5,n-3 to 24:5,n-3. Elovl5 also

does not enhance 20:5-mediated suppression of SREBP-1 nuclear abundance. Elovl5, in contrast to Elovl2, is the most abundant elongase in liver; it is also well regulated during post-natal develop, by diet, hormones drugs and toxins. As such, changes in Elovl5 activity in these various physiological and pathophysiological states will likely affect hepatic lipid composition. Elovl5 effects on gene expression, however, are likely to be not as widespread as those seen with Elovl2. Elovl5 effects may be restricted to the control of PPARα signaling. More studies will be required to verify this notion.

In summary, fatty acid elongases have the capacity to significantly impact hepatic lipid composition. In doing so, elongase-mediated changes in hepatocyte lipid composition affect at least two transcriptional regulatory networks known to be controlled by exogenous fatty acids, viz. PPARα and SREBP-1. Such findings reveal the importance of fatty acid metabolism on the control of these transcription factors. These studies, however, are in primary hepatocytes. Studies in the next chapter reveal effects of elongase over expression in vivo. These studies reveal even more effects of elongases on hepatic metabolism and gene expression.

Chapter 5

Effect of Elovl5 on Hepatic Function and Lipid Metabolism in Vivo

5.1. Introduction

Seven fatty acid elongase(Elovl) isoforms, each the product of a separate gene, have been idendified in the mammalian genome; 7 of these isoforms have been cloned and characterized in mammals [115]. Evidence for the importance of Elovls in physiology comes from transgenic mice studies. For examples, Quaking and Jimpy are the two mouse mutant strains have dramatically decreased Elovl-1 mRNA level [126]. Elovl-3 is induced in brown adipose tissue following exposure of animals to the cold [116]. The water barrier function was impaired in Elovl-3 null mice. Elovl-6 is induced in transgenic mice over expressing SREBP-1 [24,78,134]. Among these isoforms, there are four expressed in rat liver (Elovl-1, -2, -5 and -6), in which Elovl-5 is the most abundant. Elovl-5 utilizing a broad substrate array, C_{16-22}, involved in PUFA synthesis [24,115,201]. It is induced during postnatal development and suppressed by feeding rats n-3 PUFA enriched diets [175]. Recently, we reported PPARα, high fat diets and obesity affect Elovl-5 expression [201]. Over expressing Elovl-5 in rat primary hepatocytes alters hepatic gene expression, at least in part, by controlling ligand availability for key transcriptional factors, like PPARα.

Even though individual Elovl isoforms have different substrate preferences, tissue specific expression, and are regulated differently [115,133,175,201,203], the significance of this diversity is unknown. The regulation of elongase paralleling the change of hepatic fatty acid composition under pathological conditions leads us to test the specific role of individual elongase in hepatic function and onset of chronic disease. Here, I over express Elovl-5 in mouse liver by recombinant adenovirus and test the hypothesis that Elovl-5 will affect hepatic function by manipulating fatty acids structure.

5.2 Materials and Methods

Recombinant adenovirus. Cloning of cDNA for Elovl-5 was described previously [201]. The coding region for Elovl5 transcript was ligated into Ad-Easy XL adenoviral vector system (Stratagene), recombined in BJ5183 cells and propagated in XL10 Gold ultra-competent cells. Ad-DNA was packaged into adenoviral particles Ad-293 cells. The resultant adenovirus was amplified in HEK293 cells and purified by cesium chloride gradient centrifuge.

Animals. All animal procedures were done in accordance with Michigan State Universtiy Institutional Animal Care and Use committee approved guidelines. Six-week-old male C57BL/6 mice were purchased from Charles River, Kalamazoo and adapted to the environment for 1 week before study. All mice were housed in a specific pathogen-free environment with a 12-h light/dark cycle in a temperature-controlled environment. Mice had free access to water and regular chow diet. The adenovirus was intravenously administrated (via the retro-orbital sinus) into 3-4 groups animal: saline (n=8), Ad-LUC(n=8), Ad-Elovl-5(n=8). All treated animals were injected with 2 X 10^{11} virus particles/animal in a final volume of 200 µl sterile PBS + 1% sucrose; saline injected

mice received the PBS + 1% sucrose alone. Mice were given TekLad chow and water, ad lib, and were weighed daily. The 3rd day after injection, all mice were fasted overnight. The next day, 4 mice from each group were then refed with chow diet for 4 hrs before anesthesia. All animals were euthanized at noon – 2 PM on the 4th day post injection.

Immunohistology: At euthansia, a sample of liver was taken for histology: a) formalin-fixed, paraffin-embedded for hematoxylin & eosin (H&E) stain and periodic acid-Schiff (PAS) staining to detect glycogen; a second sample was fixed in OCT, frozen (liquid nitrogen/-80°C) and stained with Oil Red O (ORO) to detect lipid. Embedding, sectioning, staining and slide preparation was done in the Investigative HistoPathology Laboratory Staff, Department of Physiology-MSU.

Blood glucose measurement and plasma preparation: Blood was rapidly drawn from the vena cava (~1 ml) into a heparin syringe. Glucose measurement was performed at the time of blood collection using a hand-held glucose meter (One touch II, Life Scan Inc.). The remaining blood was centrifuged at 800 rpm for 10min, and the plasma was stored in -80°C for subsequent assays for cholesterol, triglycerides and saponified fatty acid profile.

Measurement of liver glycogen content: Liver (~0.1 g) was homogenized in PBS, adjusted to 0.5 M KOH, and incubated at 95°C for 1 hr. The homogenate was cooled, neutralized with 0.1X vol. 6% Na_2SO_4; glycogen was precipitated with 3 volumes of ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol and resuspended in 1 ml water, adjusted to 50 mM Na-acetate, pH 5.0. Glycogen was hydrolyzed with amyloglucosidase and the released glucose measured using a Wako Glucose assay kit; Standards were glucose (200 mg/dl) and glycogen (1 mg/ml) [204] Absorbance was read in a spectrophotometer (DU 640; Beckman) at 340 nm wavelength.

In vitro fatty acid elongation assay. Rat liver microsomes were isolated by differential centrifugation [201]. Elongation reactions were carried out with modifications to the procedure described by Moon et al [117]. Briefly, reaction mixtures contained 50 μg microsomal proteins in a total reaction volume of 100 μl. The reaction constituents are: 50 mM potassium phosphate buffer- pH 6.5, 5 μM rotenone (Sigma), 40 μM fatty acyl CoA (Avanti Polar Lipids, Alibaster, Al and Sigma, St. Louis, MO), 60 μM malonyl CoA (Sigma) [6.5 dpm/pmol] [2-^{14}C]-malonyl CoA (Perkin Elmer), 1 mM NADPH (Sigma) 20 μM BSA (fatty acid free). Reactions (at 37°C) were initiated with the addition of NADPH. When fatty acids were used as substrate,n-aOH neutralized fatty acid (40 μM) replaced fatty acyl CoA. CoASH (at 100 μM), MgCl2 (1 mM) and ATP (1 mM) were added to the reaction to generate fatty acyl-CoA. Elongase reactions were terminated after 20 minutes with the addition of 100 μl 5 N KOH + 10% methanol; lipids were saponified for 1 hr at 65°C. The saponification reaction was acidified with 100 μl 5 N HCl; 100 μl ethanol was added to aid hexane extraction of fatty acids. Elongated fatty acids were collected by 2 independent extractions with hexane (800 μl). Hexane extracts were pooled and ^{14}C-radioactivity was quantified by β-scintillation counting. Results are expressed as Elongase Activity Units,n-moles ^{14}C-malonyl CoA incorporated/mg protein/20 mins. Formation of reaction products was dependent on the presence of NADPH and the fatty acid CoA. Fatty acid elongation products were verified by reverse-phase-HPLC chromatography using a flow-through β-scintillation counter.

RNA extraction, northern analysis and quantitative real time-PCR (qRT-PCR). Total RNA was extracted from liver samples and used for template for qRT-PCR analysis as previously described [201]. Specific primers for each gene (Table 5.1) were designed

using Primer Express software (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized using the SuperScript II RNase H- Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific forward and reverse primers and subjected to real-time PCR quantification using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative C_T method (User Bulletin #2, Applied Biosystems). Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. Primers used for quantitative real time PCR are listed in Table 5.1.

Lipid extraction and quantitation of hepatic fatty acids composition. Total lipid was extracted from liver in chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene [175]. 7-nonadecenoic acid (19:1) was added as a recovery standard at the time of extraction. Protein (Bio-Rad, Hercules, CA) was measured in extracts after the initial homogenization step. Total lipids were saponified, fractionated and quantified by reverse phase HPLC (RP-HPLC) using a YMC J-Sphere (ODS-H80) column and a gradient starting at 77.5% acetonitrile + acetic acid (0.1%) and ending at 100 % acetonitrile + acetic acid (0.1%) over 90 mins with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the MSU Mass Spectrometry facility by GC/MS (www.bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for RP-HPLC were obtained from Nu-Chek Prep (Elysian, MN).

Table 5. 1.Primer sequences used for gene expression by quantitative reverse transcription-PCR for mouse.

Gene	Forward	Reverse
ACC	TGTCCGCACTGACTGTAACC	ATTTCCATAGCCGACTTCCA
FAS	CAAATACAATGGCACCCTGA	TGGCGAAGCCGTAGTTAGTT
SREBP-1	AGGAGAACCTGACCCTACGA	GGTAAGCGTCTCCACCACTT
CYP4A10	TGTTTGACCCTTCCAGGTTT	CAATCACCTTCAGCTCACTCA
HMGCS2	CCTTGAACGAGTGGATGAGA	CAGATGCTGTTTGGGTAGCA
AOX-1	CCCAACTGTGACTTCCATCA	ACGGATAGGGACAACAAAGG
L-PK	AGGAGTCTTCCCCTTGCTCT	ACCTGTCACCACAATCACCA
PEPCK	ACATTGCCTGGATGAAGTTTG	GGCATTTGGATTTGTCTTCAC
GLUT2	GAGGAAGTCAGGGCAAAGAA	AAGAGCTGGATCACGGAGAC
G6PC	TCGTGGCTGGAGTCTTGTC	GCGAAACCAAACAAGAAGATG
GCK	GAGATGGATGTGGTGGCAAT	ACCAGCTCCACATTCTGCAT
CHREBP	CTGGGGACCTAAACAGGAGC	GAAGCCACCCTATAGCTCCC
Δ9 D	TCAACTTCACCACGTTCTTCA	CTCCCGTCTCCAGTTCTCTT
Δ5 D	TGTGTGGGTGACACAGATGA	GTTGAAGGCTGATTGGTGAA
Δ6 D	CCACCGACATTTCCAACAC	GGGCAGGTATTTCAGCTTCTT
ELOVL5	GGTGGCTGTTCTTCCAGATT	CCCTTCAGGTGGTCTTTCC
ELOVL2	ACGCTGGTCATCCTGTTCTT	GCCACAATTAAGTGGGCTTT
CYCLOPHILIN	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG

Immunoblotting. Liver microsomal and nuclear extracts were prepared as described previously [159,175]. Proteins (50-100 μg) extracted from microsomal or nuclear fractions were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12% polyacrylamide Bis-Tris, Invitrogen) and transferred to nitrocellulose membranes. Membranes were incubated with antibodies for ChREBP (Novus Biologicals, Littleton, CO); MLX(N-17), SREBP-1 (IgG-2A4, sc-13551), SREBP-2, ERK1 (sc-94), and phosphor-ERK (sc-7383) (Santa Cruz Biotechnology, San Cruz, CA); GSK3β, phosphor-GSK3β (Ser9) and caspase 9 (Cell signaling technology, Beverly, MA). Anti-mouse, anti-goat and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The SuperSignal West Pico chemiluminescence kit (Pierce) detection system was used.

Statistical Analysis. Statistical analysis used Student's t-test and ANOVA plus post hoc Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html).

5.3 Result

5.3.1. Generation of the mice overexpressing Elovl5 in liver.

C57BL/6 mice were injected with saline, Ad-Luc (control virus) and Ad-Elovl-5, respectively. Liver and kidney RNA were examined for Elovl-5 gene expression in these mice. Elovl-5 mRNA level was elevated ~30 fold compared to Ad-luciferase and saline groups (data not shown) in mouse liver. There is no significant difference of Elovl-5 expression in kidney, heart and spleen among the 3 treatments (data not shown). This outcome indicated that the adenovirus were sequestered by the liver, not other tissues.

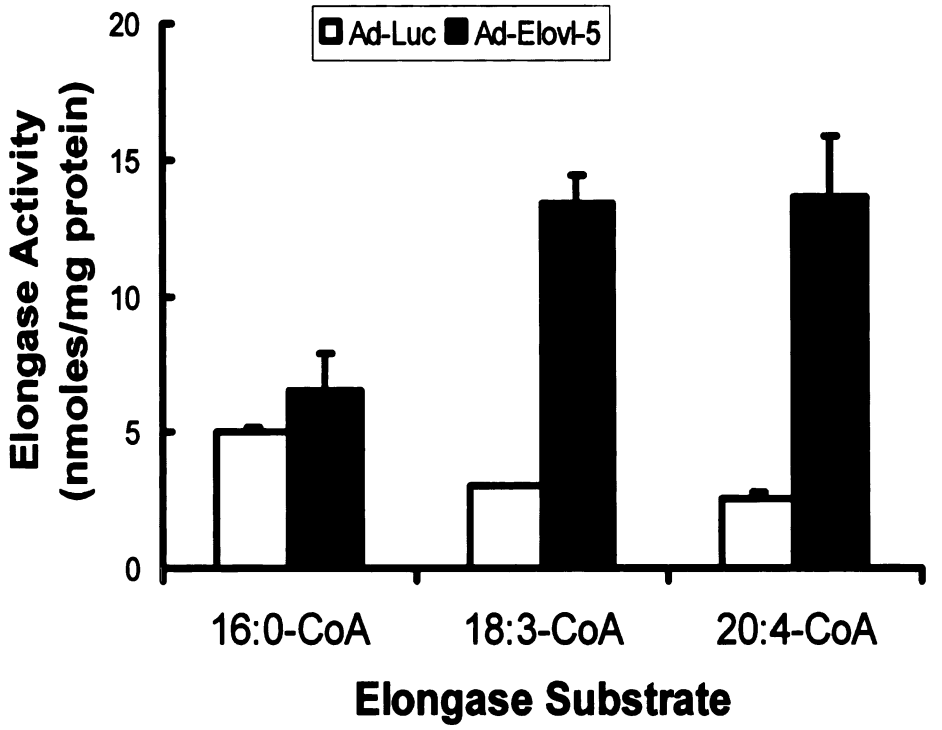


Figure 5. 1. Effect of overexpressing Elovl5 on elongase activity in mice liver. Male C57BL/6 mice were injected with Ad-Luc and Ad-Elovl5 and fed with chow diet for 4 days as described in (Materials and Methods). Hepatic microsomal preparations were used for fatty acid elongase assays (Materials and Methods). Results are expressed as Elongase Activity, (nmoles 14C-malonyl CoA assimilated into fatty acids/mg protein). Mean $\pm$ SD,n=8. *$p \leq 0.01$ vs Ad-Luc, *Student's T-Test.*

Liver elongase assay showed that enzyme activity of Elovl-5 was increased by 2-3 fold as compared to Ad-Luc and saline groups (Fig. 5.1). This level of Elovl-5 activity is seen in animals fed the peroxisome proliferator, WY14643, or in livers of *Lep*$^{ob/ob}$ mice [26,205].

5.3.2. Metabolic characterization of the mice overexpressing Elovl5 in liver.

On a normal chow diet, all Ad injected mice lose body weight and have decreased food intake in the first day after injection compared to the saline group (data not shown). Two days after injection, food intake resumes and body weight increases. By day 4, post injection, body weight and food intake between the saline and Ad-infected groups is not significantly different. All the Ad infected animals developed a modest, but insignificant increase in liver weight when compared to the saline group (data not shown). Livers of the Ad-Elovl-5 group have small white lesions compared to Ad-Luc infected groups (Fig. 5.2 A).

Surprisingly, the blood glucose measured at the time of euthansia decreased 30%-40% in the Ad-Elovl-5 groups compared to the control group (Ad-luc and saline groups) in both fasting and refeeding state (Fig. 5.3 A). Hepatic glycogen stores, determined by Periodic acid shift (PAS) staining, was significantly decreased in Ad-Elovl-5 groups compared to Ad-Luc group (Fig. 5.2 B). Direct measure of hepatic glycogen content (Methods) confirmed the PAS staining results (Fig. 5.3 B). The fold change of hepatic glycogen levels during fast and refeeding of the Ad-Elovl-5 infected mice was decreased 4 fold compared to Ad-Luc group. Hepatic lipid content, determined by oil red O staining (ORO), mirrored the quantitative hepatic triglyceride in all Ad groups (Fig. 5.2 B); Ad-

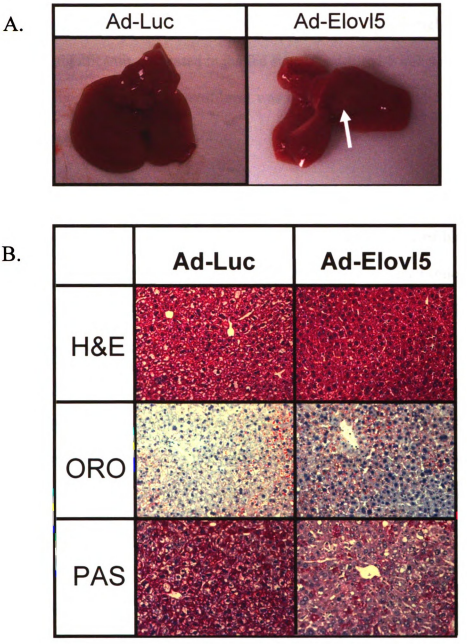


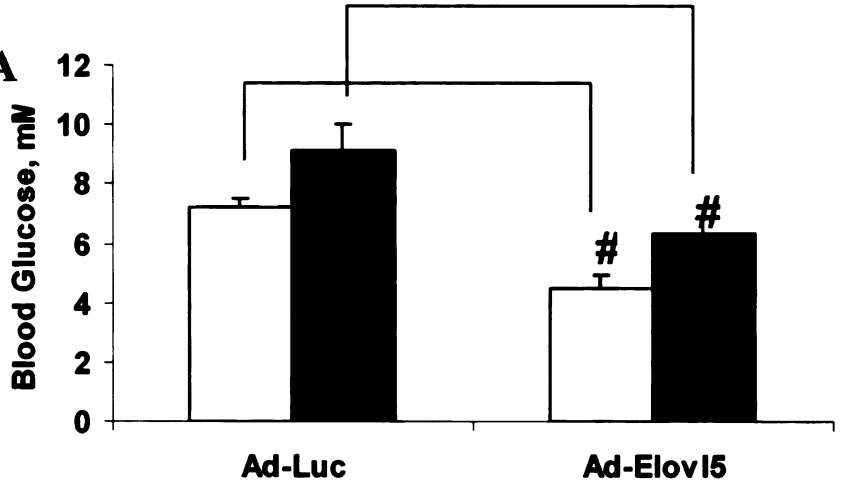
Figure 5. 2. Liver morphology and histology for Ad mice. A. Liver morphology of Ad injected mice. B. Liver histology of Ad mice. Liver sections from the mice overexpressing Luc and Elovl5 were stained with hepatoxylin and eosin (H&E) and oil red O (ORO) and (PAS), magnification 20X. Arrows indicated necrosis in Ad-Elovl5 liver. Sections are representive of several animals for each condition. Image in this figure is presented in color.

Elovl5 had not major effect on hepatic neutral lipid content. Blood triglyceride levels, however, were significantly decreased in the fed state of Ad-Elovl-5 infected animal. (Fig. 5.3 C). No change of blood cholesterol level was observed in Ad-Elovl-5 injected mice (Data not shown).

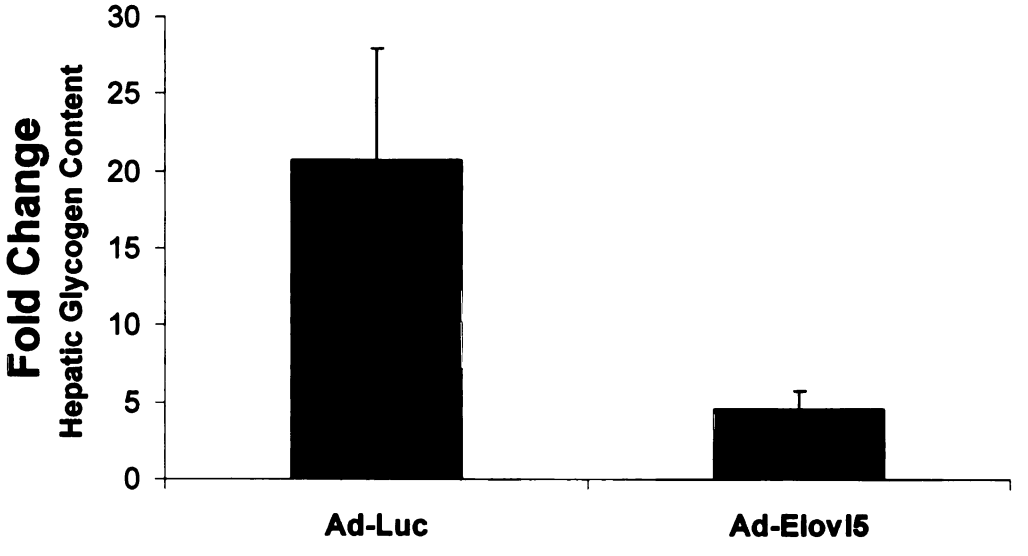
5.3.3. Alteration of glucose metabolism in Ad-Elovl5 injected mice.

Stores of glycogen in the liver are considered the main buffer of blood glucose levels. Since glycogen level is suppressed in Ad-Elovl-5 injected mice, we first hypothesized that overexpression of Elovl-5 altered hepatic glucose metabolism. Fasting induces glycogenolysis and gluconeogenesis, whereas refeeding induces glycolysis and glycogenesis. We first examined the mRNA expression and nuclear content of ChREBP, which is required for most glucose-regulated hepatic genes [55,62]. Results showed that $mRNA_{ChREBP}$ was decreased in the refed group overexpressing Elovl-5, but not in Ad-Luc group (Fig. 5.4 A). Immunoblotting of ChREBP and MLX in fed mice showed a decline in nuclear ChREBP, but no change of nuclear MLX, the ChREBP heterodimer partner. (Fig. 5.4 B). L-PK, a target of ChREBP/MLX and a major glycolytic enzyme, was significantly suppressed in livers of Ad-Elovl5 infected animals when compared to Ad-Luc infected animals (Fig. 5.5). A second ChREBP/MLX target gene was examined. Glut2 is a key hepatic glucose transporter. Like L-PK, Glut2 expression was also suppressed in Ad-Elovl-5 infected animals in both the fasted and fed state. The gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PepCk) and glucose-6-phosphatase (G6Pase) were examined. Elovl-5 significantly suppressed $mRNA_{PepCk}$ and $mRNA_{G6Pase}$ in both fasting and refeeding (Fig. 5.5).

3A


Blood Glucose, mM
12
10
8
6
4
2
0
#
#
Ad-Luc
Ad-Elovl5

3B


Fold Change
Hepatic Glycogen Content
30
25
20
15
10
5
0
Ad-Luc
Ad-Elovl5

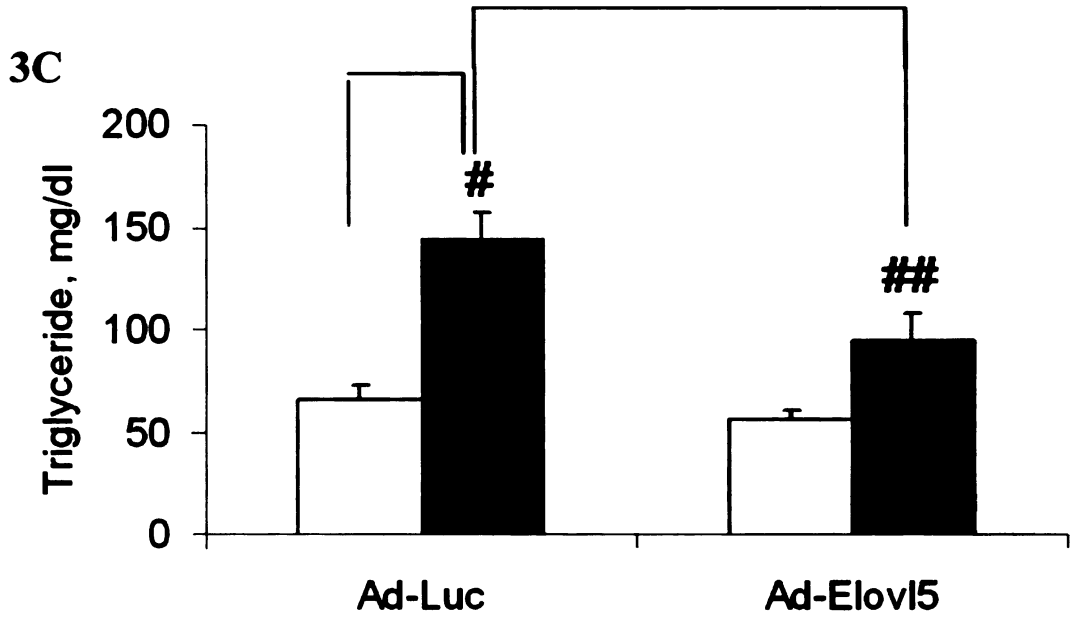


Figure. 5.3. Effect of overexpressing Elovl5 on blood glucose, triglyceride and hepatic glycogen level. A. Blood glucose level was measured by blood glucose meter (One touch II, Life Scan Inc.). The open bar represents the fast group; the filled bar represents the refeeding group; Mean $\pm$ SD,n=4. #P<0.05 vs fast group, anova. B. Liver glycogen level was measure as described in the method. Results are expressed as Fold Change (refeeding/fast), mean $\pm$ SD, n=4. C. Effect of overexpressing Elovl5 on blood triglyceride level. Blood triglyceride level was measured by Wako L-Type TG H test kit. The open bar represents the fast group; the filled bar represents the refeeding group; Mean $\pm$ SD,n=4. #P<.01,##P<.05.

Glycogen synthase kinase-3β (Gsk-3β) is a serine/threonine kinase that plays a key role in controlling glycogen synthesis by regulating the phosphorylation status of glycogen synthase [206]. Gsk-3β is a highly-regulated kinase; its activity is controlled by its phosphorylation status. Insulin suppresses Gsk-3β activity by inducing its phosphorylation through the PI3K-Akt pathway [207]. Inhibition of GSK3β releases glycogen synthase from inhibition and stimulate glycogen synthesis. I examined total and phospho-Gsk3β in livers of fed Ad-infected animals only. Contrary to my expectation, the phosphorylation status of Gsk-3β (ser-9) was significantly increased in Ad-Elovl-5 injected when compared to the saline or Ad-Luc infected groups (Fig. 5.6). As such, Elovl-5 over expression does not suppress hepatic glycogen content by activating Gsk-3β.

I also examined a second target of insulin action, Erk. Insulin rapidly, but transiently induces Erk phosphorylation in rat primary hepatocytes [83]. Over expressed Elovl-5 increased phospho-Erk with little effect on total Erk (Fig. 5.6). No adenovirus infection increased the formation of caspase 9 (37.5 kd), an indicator of intrinsic apoptosis [208](Fig. 5.6).

Taken together, these results indicate that elevated hepatic activity of Elovl-5 mimics some, but not all of the effects of insulin: a) induces Gsk-3β phosphorylation; b) induces Erk phosphorylation; c) suppresses PepCk gene expression. Unfortunately, however, there are contradictory outcomes; such as the suppression of hepatic glycogen content and expression levels for ChREBP, L-PK and Glut-2, with little effect on glucokinase. While my studies do not provide a complete explanation for the effects of over expressed Elovl-5 on hepatic glucose metabolism, these studies provide clear evidence that change in fatty acid elongase activity, alone, are sufficient to promote

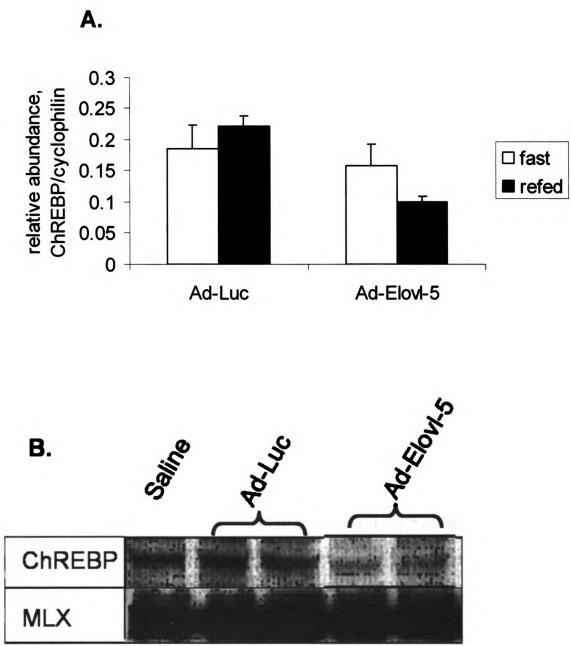
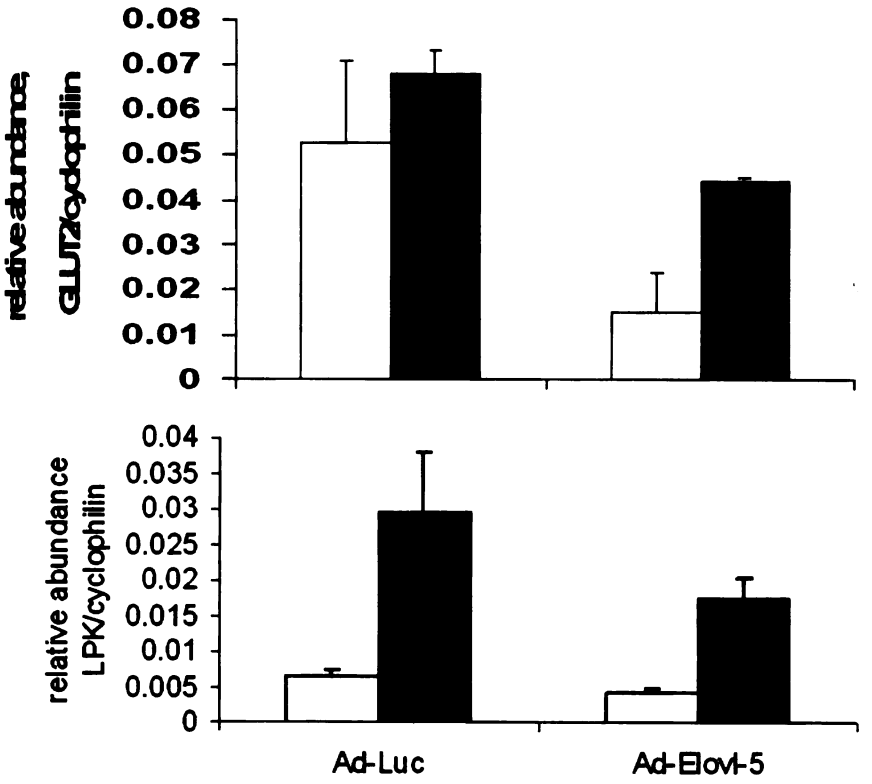


Figure. 5.4. Effect of overexpression of Elovl5 on hepatic ChREBP gene expression and nuclear content. A. Effect of overexpression of Elovl5 on hepatic ChREBP gene expression. RNA was extracted and used for qRT-PCR analysis of ChREBP gene. Results are expressed as Fold Change (transcript/cyclophilin), mean $\pm$ SD, n=4. B. Effect of overexpression of Ad-Elovl5 on nuclear ChREBP and MLX abundance. Livers from Ad-Luc and Ad-Elovl5 injected mice were used for the isolation of nuclear proteins for immunoblotting. Duplicate samples for each treatment are shown.

major changes in hepatic glucose metabolism and key signaling pathways that impact hepatic glucose metabolism.

5.3.4. Decreased expression of genes involved in lipid metabolism in Ad-Elovl5 injected mice.

Lipid metabolism is a second major hepatic metabolic pathway that is affected by dietary lipid composition. Herein, I examined changes in hepatic expression of genes involved in hepatic lipid metabolism. Elevated expression of Elovl5 (Ad-Elovl5) in fasted mice suppressed expression of PPARα target genes, CYP4A and HMGCS2 levels, but not AOX-1, by 70% (Fig. 5.7). There was no change in SREBP-1 gene expression, SREBP-1 nuclear content or SREBP1-target genes, ACC and FAS, in Ad-Elovl5 injected mice comparing Ad-Luc injected mice (Data not shown). However, these three genes responded nicely to the fast-refeeding. In addition, mRNA levels for SCD-1, the desaturase responsible for the production of monounsaturated fatty acids was significantly suppressed in liver of Ad-Elovl5 injected mice compared with Ad-Luc injected mice in refed states (Fig. 5.7). SCD1 expression is controlled by PPARα, SREBP-1 and ChREBP/MLX [205]. The decline in SCD1 expression in Ad-Elovl5 infected animals is likely due to disruption of the PPARα and ChREBP/MLX regulatory pathways.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Ad-Elovl-5

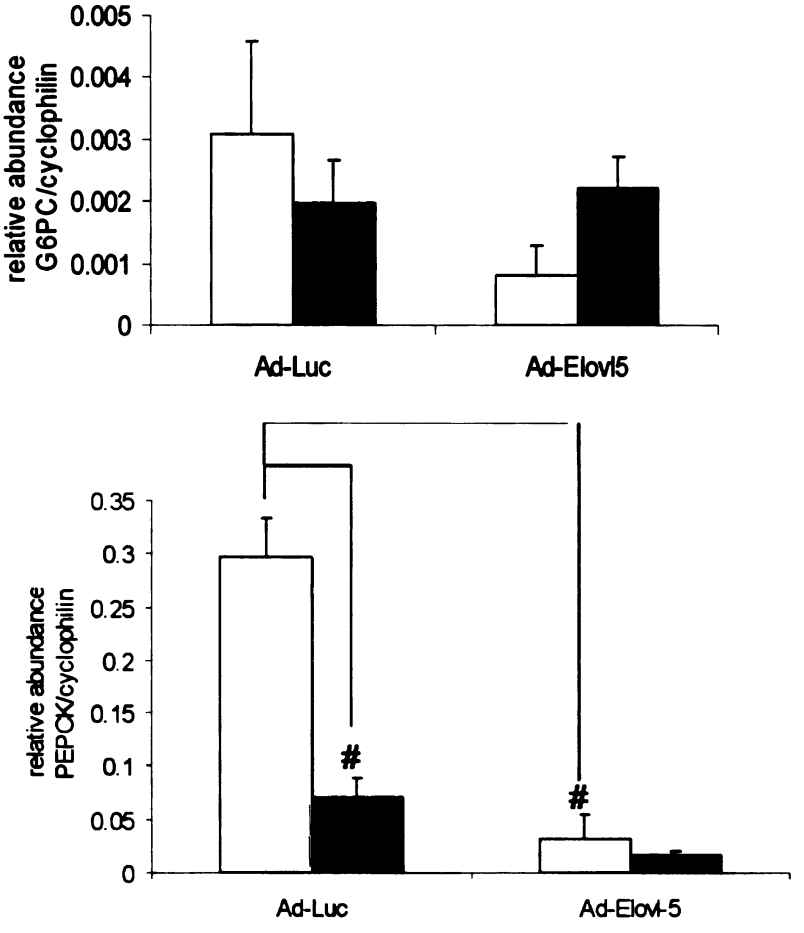

Figure. 5.5. Effect of overexpressing Elovl5 on gene expression involved in glucose metabolism. RNA was extracted and used for qRT-PCR analysis of gene involved in glucose metabolism. Results are expressed as Fold Change (transcript/cyclophilin), mean $\pm$ SD, n=4. #P<0.01.

5.3.5. Effect of Elovl5 overexpression in hepatic and plasma fatty acid profile.

Elovl5 utilizes a broad array of fatty acid substrates, C_{16-20}, to impact both MUFA and PUFA synthesis. I hypothesized that overexpression of Elovl5 can promote endogenous PUFA synthesis. To eliminate the contribution of dietary lipids from the absorptive phase, I examined hepatic and plasma fatty acid of fasted mice. The outcome of these studies provided unexpected results. The ratio of 20:4,n-6 to 18:2,n-6 is decreased in Ad-Elovl5 injected mice (Fig. 5.8). 18:2,n-6, an essential fatty acid, is converted to 20:4,n-6 by elongation (Elovl5) and desaturation (Δ^5D and Δ^6D). There was no increase in 22:4,n-6 in livers of Ad-Elovl5 infected animals. Since other enzymes involved in PUFA synthesis pathway, i.e., Δ^5D and Δ^6D desaturase, did not change, I speculate that over expressed Elovl5 stimulated the conversion of 20:4, n-6 to 22:4,n-6 and its peroxisomal degradation. PUFA of $\geq$22 carbons are preferred substrates for peroxisomal oxidation [209].

Two interesting observations were made in my analysis of the plasma fatty acid profile. Mice receiving Ad-Elovl5 had a higher (by 40%) level of PUFA in the plasma (Fig. 5.9). This is due to the accumulation of 18:2,n-6 in the plasma. Second, the level of 18:0, relative to 18:1, was increased (Fig. 5.9).

5.4. Discussion

The data from these in vivo studies support the hypothesis of this thesis. Altered expression of an elongase, alone, is sufficient to change hepatic gene expression. The outcome of these studies, however, revealed a more complicated change in hepatic

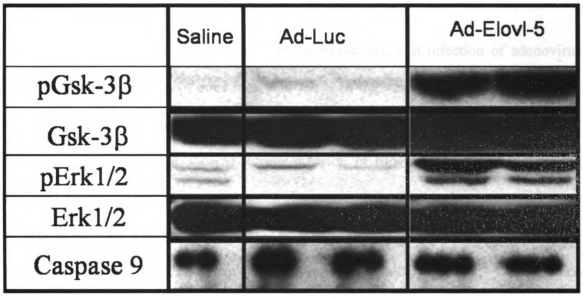

Figure. 5.6 Effect of overexpression of Elovl5 on regulating GSK3β and ERK. Total protein was extracted from liver as methods described. Protein levels were measured by immunoblot (Materials and Methods). Duplicate samples for each treatment are shown.

metabolism affecting both glucose and lipid metabolism. As such, these studies may have revealed novel linkages between fatty acid elongation and other facets of hepatic metabolism.

Several reports in the literature have demonstrated that infection of adenovirus into the retro-orbital sinus of mice results in >99% of the virus infecting liver [210,211], and as expected, we did not observe Elovl5 expression in other tissues, including kidney, heart, or spleen (data not shown). Overexpression of Elovl5 in mouse liver, by the recombinant adenoviral approach, elevated elongase activity 3-fold when compared to liver of animals infected with the Ad-Luc control or control (saline injected) animals. This level of expression is seen in other physiological states, e.g., *Lep*$^{ob/ob}$ obese mice [205], TCDD-treated mice (Fig 3.11 A) and WY14,643 treated rats [26]. As such, the change in elongase activity is within a physiological range.

Several key observations were made. First, there was ~40% decrease in blood glucose level in fasted and fed Ad-Elovl5 infected mice. The likely explanation for this is the decline in hepatic glycogen content and expression of genes involved in gluconeogenesis (PEPCK and Glc-6-P), coupled with a decline in genes involved in glucose transport (Glut2) and glycolysis (LPK). The outcome of these results implicated some change in hepatic insulin signaling. Analysis of two downstream targets of insulin action, namely, Gsk3β and Erk indicated that elevated Elovl-5 activity as associated with increased phosphorylation of these proteins. This same effect is observed in acute treatment of hepatocytes with insulin [83]. As such, elevated Elovl5 activity appears to mimic insulin action. Defects in liver and muscle glycogen synthesis are major factors contributing to postprandrial hyperglycemia in patients with type 2 diabetes [212]. Our

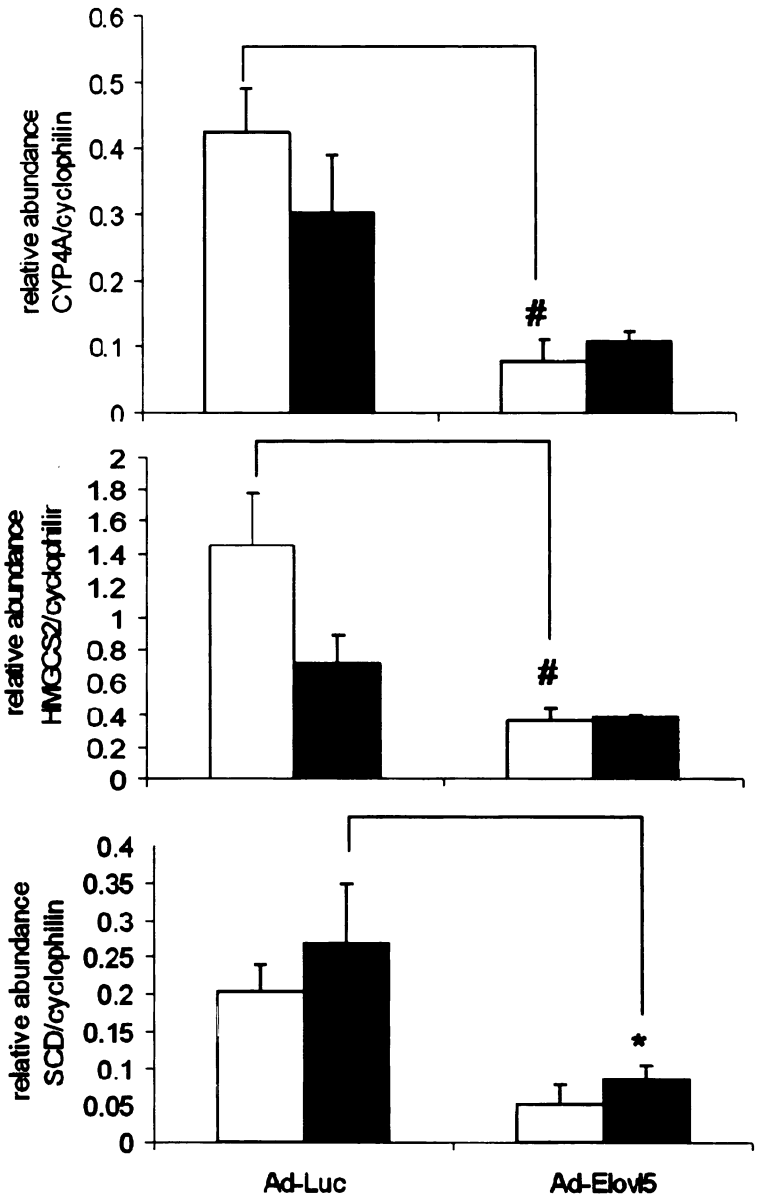

Figure. 5.7. Effect of overexpressing Elovl5 on PPARα target gene expression. RNA was extracted and used for qRT-PCR analysis of PPARα target gene, Cyp4A and HMGCS2 as well as SCD-1. Results are expressed as Fold Change (transcript/cyclophilin), mean ± SD, n=4. #p<.01, *p<.05.

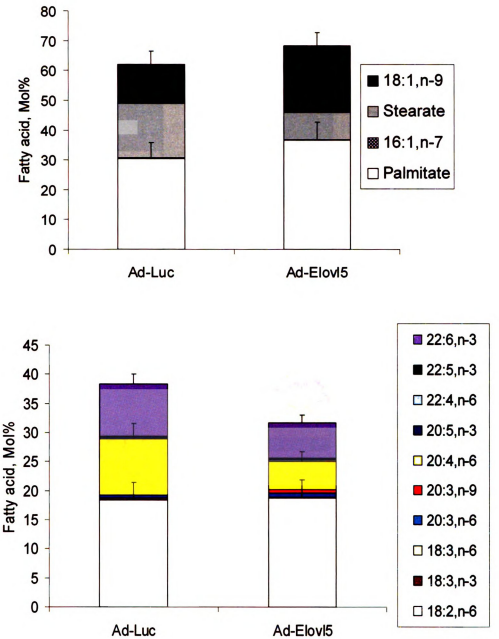

Figure. 5.8. Effect of overexpressing Elovl5 on hepatic lipid composition in the fasted mice. Total lipids were extracted and saponified; fatty acid levels were quantified by RP-HPLC (Materials and Methods). Results are expressed as Fatty Acid Mole%, mean $\pm$ SD, n=4/group.

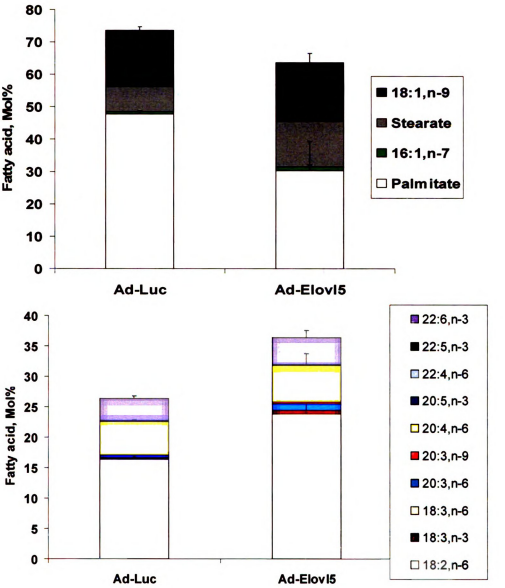

Figure. 5.9. Effect of overexpressing Elovl5 on plasma lipid composition in the fasted mice. Total lipids were extracted and saponified; fatty acid levels were quantified by RP-HPLC (Materials and Methods). Results are expressed as Fatty Acid Mole%, mean $\pm$ SD, n=4/group.

results suggest that elevated Elovl5 lowers blood glucose levels. We observed in high fat-fed animals, both glucose-intolerance and lower hepatic Elovl5 expression [205]. Perhaps alterations in hepatic Elovl5 levels might improve blood glucose levels in high-fat-fed mice. More analysis will be required to fully understand the underlying mechanism(s) by which elevated Elovl5 impacts hepatic glucose metabolism.

A second key observation is the effect of elevated hepatic Elovl5 activity on hepatic and plasma lipid composition. While overexpressed Elovl5 did not significantly impact SREBP-1, and its target genes FAS and ACC during fast and refeeding, over expressed Elovl5 did impact the expression of several PPARα target genes, CYP4A and HMGCS2. The effect of over expressed Elovl5 was most apparent in the fasted state.

Although total hepatic lipid content did not change, the type of fatty acids seen in liver and plasma did change. In liver, overexpressed Elovl5 resulted in a reduction of 20-22 carbon PUFA. We speculate this is due to enhanced degradation in the peroxisome. The oxidation reaction of fatty acyl-CoA is the initial and rate-limiting step of the peroxisomal beta-oxidation pathway. AOXs and pristanoyl-CoA oxidase (PCOX) can oxidize the CoA esters of straight-chain fatty acids. Interestingly, the expression of AOX-1, which is responsible for peroxisomal β-oxidation, was not affected by Elovl-5 overexpression. However, we need additional data on how overexpression of Elovl5 affects other subtypes of AOX and PCOX. Hepatic content of 18:1,n-9 increased in livers of mice receiving Ad-Elovl5. 18:1 is the preferred substrate for triglyceride and cholesterol ester synthesis [27]. The ratio of 18:1 to 18:0 has been implicated in the regulation of cell growth and differentiation through effects on membrane fluidity and

signal transduction [10]. With this outcome, I expected to see an elevation in plasma triglyceride content.

Overexpressed Elovl5 had three effects on plasma lipids; a) decreased triglyceride levels; b) decreased ratio of 18:1,n-9 to 18:0; and c) change in PUFA composition, mainly due to the accumulation of 18:2,n-6. Since 18:2,n-6 is only available from the diet, we speculate the accumulation of 18:2,n-6 in plasma is due to the decreased uptake of this fatty acid by liver or the increased efflux of 18:2,n-6 from the liver if the uptaked 18:2,n-6 is not converted to its CoA form rapidly. While my studies do not fully explain the mechanisms for these observations, they point out that change in hepatic lipid metabolism lead to changes in blood lipid composition. In studies not shown here, animals infected with Ad-Elovl2 show a remarkable decline in hepatic 20:4,n-6 levels. Whereas, in animals infected with Ad-Δ^6-desaturase show a significant increase in plasma 20:4,n-6 levels (data not shown). Taken together, these studies highlight the fact that hepatic PUFA synthesis has a major effect on blood lipids and likely will significantly impact lipid composition in other tissues.

Since both fatty acid and glucose metabolism are altered by Elovl5 overexpression, this led us to question how the early disruption of the fatty acid balance in the liver or plasma may influence subsequent abnormalities in glucose metabolism. Skeletal muscle also plays a major role in the development of metabolic disorders, through the regulation of insulin-induced glucose disposal. The increased PUFA amoumt in plasma may change the fatty acid composition in the membrane of skeletal muscle, which may modify insulin signaling pathway in skeletal muscle to impact blood glucose. More studies are needed to test this hypothesis.

In conclusion, the liver plays a central role in the regulation of whole body lipid and glucose homeostasis. My findings indicate that overexpression of Elovl5, alone, can alter hepatic function which leads to changes in whole body and hepatic glucose and lipid metabolism. These results strongly suggest that fatty acid elongases play an important role in management of glucose and fatty acid metabolism. Whether control of hepatic Elovl5 activity can be used to impact blood glucose and triglyceride in metabolic diseases remains an important unanswered question.

Chapter 6

Conclusions and Future Directions

In the last two decades, a growing number of articles emphasized the role of fatty acid in the control of gene expression. Fatty acids can directly bind to and regulate the activity of some transcriptional factors, such as, PPAR family [51,53]. In this case, fatty acids act like hydrophobic hormones to control the activity or abundance of key transcriptional factors. In contrast, fatty acids also regulate other transcriptional factors through indirect mechanism by changing their nuclear abundance and activity, such as SREBP-1c [14]. Whatever mechanisms are involved in fatty acid regulation of gene expression, the structure of fatty acid is the key determinant for the regulation of transcriptional factors [14,53,159].

During fatty acid metabolism, several steps are involved in the modification of fatty acid structure, including elongation/desaturation, β oxidation (mitochondrial or peroxisomal), or assimilation into complex lipids [14]. This suggests the enzymes in these metabolic pathways may influence gene expression by modifying fatty acid structures. My studies focus on the enzyme involved in the fatty acid elongation, which are called fatty acid elongase.

Four fatty acid elongases (Elovl1, 2, 5 and 6) and 3 desaturases (Δ5D, Δ6D and Δ9D) are expressed in liver. Among these enzymes, the desaturases are well studied, especially Δ9D. These enzymes are regulated by hormones, development and dietary lipids [5-10]. Δ9D knockout mice have shown low possibility to develop hepatic steatosis and improved insulin sensitivity in muscle [11-13]. As such, Δ9D had been recognized as

drug target for obesity treatment recently. Compared to the desaturases, we are still in the early stage of understanding how fatty acid elongases are regulated and what their roles are in physiology. My studies filled this gap with new information.

In the first part of these studies, I examined the nutritional and developmental regulation of rat hepatic Elovl expression and correlated changes in elongase expression with elongase enzymatic activity. Of the four elongase subtypes expressed in rat liver, Elovl5 is the most abundant elongase transcript. Rat hepatic Elovl5 expression is regulated at the pretranslational level by dietary n3 PUFAs and PPARα agonist and during postnatal development. These same factors regulate hepatic Elovl activity. Changes in the expression of the elongases (and their activity) and desaturases are sufficient to induce changes in tissue and plasma levels of specific fatty acids (e.g., 20:3,n-9). Unlike Δ^5, Δ^6, and Δ^9 desaturases, the elongases do not display a uniform response to fasting-refeeding, fish oil, or PPARα agonist. In this regard, changes in nuclear SREBP-1 levels correlate with changes in Elovl-6 expression but not other elongases. Although PPARα agonists induce all desaturases and three elongases (Elovl1, 5, and 6), the mechanism for this control remains unresolved.

Next, we further show the evidence that Elovl5 and Elovl6 are regulated in liver by nutrients (glucose and fat), hormones (insulin) and nuclear receptor agonist, i.e., LXR and PPARα agonist. From these studies, we conclude that ChREBP, MLX, SREBP-1, PPARα and LXR control elongase, as well as desaturase expression.

Metabolic diseases, like diabetes and obesity, induce changes in hepatic lipid composition by controlling the function of key transcription factors that impact elongase and desaturase expression. These studies support the notion that regulation of fatty acid

elongase, as well as desaturases, may play an important role in managing hepatic lipid composition in response to changes in dietary and hormonal status.

Individual Elovl isoforms have different substrate preferences, tissue specific expression, and are regulated differently [115,133,175,201,203]. The significance of this diversity is unknown. To get more information about the role of individual hepatic elongases, we amplify the individual elongase activity in hepatocytes and liver by recombinant adenovirus technology. The recombinant adenovirus approach elevated elongase expression and activity to 3 to 5 folds in rat primary hepatocytes. In addition, overexpression of Elovl2 and Elovl5 can suppress 20:5,n-3 PPARα regulated gene expression. I speculate this is due to the suppression of hepatocyte 20:5,n-3 levels and accumulation of 22:5,n-3 or 24:5,n-3. 22:5,n-3, and possibly 24:5,n-3, are weak activators of PPARα. Moreover, the overexpression of Elovl2, but not Elovl5, can enhance 20:5,n-3 suppression of nSREBP-1 content without affecting nSREBP-2. This strongly supports the notion that fatty elongase have the capacity to alter gene expression by controlling cellular content of fatty acids that impact transcription factor activity or nuclear abundance.

To broaden our view about the function of individual elongases in hepatic function and whole body lipid metabolism, I investigated the effect of overexpression of Elovl5 in mice in vivo. Elovl5 is the most abundant elongase expressed in rat liver. Moveover, it has broad substrate specificity. Overexpression of Elovl5 in mouse liver was achieved by use of adenoviral intravenous transfer to obtain liver-specific overexpression. Significant reduction in blood glucose in both fasting and refeeding as well as deficiency of glycogen storage during refeeding in Elovl5 overexpressed mice suggests Elovl5 play

an important role in glucose homeostasis. The suppressed gene expression of PEPCK during fasting can partly explain this observation; it suggests that gluconeogenesis is suppressed. Effects on GSK3β and ERK phosphorylation status were observed by overexpressing Elovl-5. These results mimic insulin effects on these signaling pathways.

Overexpression of Elovl5 also significantly reduced PPAR α target gene expression while having no significant effect on SREBP-1 and its targets genes. Moreover, overexpression of Elovl5 alters the fatty acid composition both in liver and in plasma. Most important, overexpression of Elovl5 decreased blood triglyceride level during refeeding. Taken together, these findings suggest that Elovl5 is also an important factor controlling both hepatic and whole body lipid metabolism.

In summary, this thesis provides novel information about hepatic fatty acid elongases, in particular Elovl5. The results indicate that Elovl5 plays an important role in hepatic function as well as whole body glucose and lipid metabolism. Abnormal expression of Elovl5 in pathological conditions can account for the fatty acid profile in both liver and plasma. These studies also suggest Elovl5 may be the potential target for treating common diseases such as hypertriglyceridemia, obesity, and insulin-resistant diabetes.

In future, the use of microarray methods will help expand the effects of Elovl5 overexpressed on liver function and may provide new clues to identify specific molecular events/pathways influenced by Elovl5. Moreover, modulating Elovl5 expression via siRNA knock down and adenoviral transduction will let us understand better and comprehensively the role of Elovl5 plays in glucose and lipid metabolism.

To investigate the role of Elovl5 on the onset of chronic disease, we can overexpress or knockdown Elovl5 in diabetic or other pathological animal models, such as the Zucker rat, $Lep^{ob/ob}$ mouse or the high fat-fed C57BL/6 (glucose intolerant) mouse, to test if changes in Elovl5 influence the onset and progression of metabolic disease.

We can also use the same methods to test special role of other hepatic elongases. By this, we will have comprehensive understanding about fatty acid elongase family. The discovery of the effect of fatty acid elongase on glucose homeostasis may identify novel pathways to combat diabetes, benefiting millions of such patients worldwide.

APPENDIX

PUBLICATIONS

Journal Articles

1. **Wang Y,** Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B,n-air M, Peters J, Busik J, Olson LK, Jump DB. Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. J Lipid Res. 47: 2028-2041 (2006).

2. Botolin D, **Wang Y**, Christian B, Jump DB. Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. J Lipid Res. 2006 Jan;47:181-92 (2005).

3. Jump, D.B.; Botolin, D.; **Wang, Y.**; Xu, J.; Christian, B. & Demeure, O. Fatty Acid regulation of hepatic gene transcription. J Nutr, 2005, 135, 2503-2506

4. **Wang Y**, Botolin D, Christian B, Busik J, **Xu J**, Jump DB. Tissue-specific,nutritional, and developmental regulation of rat fatty acid elongases. J Lipid Res. 46:706-15 (2005).

5. **Wang Y**, Botolin D, Christian B, Jump DB. Role of fatty acid elongases in hepatic lipid and glucose metabolism. Manuscript in preparation for cell metabolism.

Conference Abstracts

1. **Regulation of rat hepatic elongase and desaturase gene expression. Yun Wang**, Botolin D, Barbara Christian and Donald B. Jump.Regulation of hepatic elongase and desaturase gene expression. Poster presentation, EB 2006, San Francisco, California

2. **The impact of hepatic metabolism on fatty acid-regulated PPARα activity and SEEBP-1 nuclear content.** D.B. Jump and **Y. Wang.** FASEB Summer Conference: Nutrient Control of Gene Expression, July 30-Aug. 5, 2005, Tuscon, AZ

3. **Gene regulation in response to polyunsaturated fatty acids.** D.B. Jump and **Y.Wang**, and D. Botolin, J. Xu, O. Demeure, J. Busik, W. Chen and B. Christian. International Conference on the Bioscience of Lipids, September 20-24, 2005 in Ajaccio, Corsica.

4. **The impact of hepatic metabolism on fatty acid-regulated PPARα activity.** D.B. Jump and **Y. Wang**. Fatty acids and cell signaling conference, Paris, France, Sept. 28, 2005.

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