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GLUCOSE, N-3 POLYUNSATURATED FATTY ACIDS AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-α AGONIST REGULATE RAT LIVER-PYRUVATE KINASE GENE TRANSCRIPTION

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Glucose, N-3 Polyunsaturated Fatty Acids and Peroxisome Proliferator Activated Receptor-α Agonist Regulate Rat Liver-Pyruvate Kinase Gene Transcription

By

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Abstract

Glucose, N-3 Polyunsaturated Fatty Acids and Peroxisome Proliferator Activated Receptor-α Agonist Regulate Rat Liver-Pyruvate Kinase Gene Transcription

By

Jinghua Xu

Dietary carbohydrate and polyunsaturated fatty acids (PUFA) regulate hepatic metabolism through their effects on gene transcription. As a key regulatory enzyme in glycolysis and *de novo* lipogenesis, Liver-type pyruvate kinase (L-PK) transcription is induced by high glucose in the presence of insulin and inhibited by n-3 PUFA and peroxisome proliferator activated receptor α (PPARα) agonist WY14,643. The key transcription factors binding to the L-PK promoter are carbohydrate regulatory element binding protein (ChREBP), MAX-like factor X (Mlx) and hepatic nuclear factor-4α (HNF-4α).

In vivo feeding studies demonstrated that refeeding fasted rats induced hepatic L-PK mRNA accumulation, while fish oil and WY14,643 diets inhibited this response. Nuclear ChREBP content was increased by refeeding, while Mlx nuclear content was decreased by the n-3 PUFA diet. Treatment of rat primary hepatocytes demonstrated that glucose induced, while n-3 PUFA and WY14,643 inhibited L-PK mRNA accumulation and promoter activity. This response correlated with the change in histone H3 and H4 acetylation and the abundance of RNA polymerase II (RNA Pol II) on the L-PK promoter. Neither n-3 PUFA, nor WY14,643 had consistent

effect on ChREBP binding to the L-PK promoter. On the contrary, n-3 PUFA but not WY14,643 transiently decreased HNF-4α binding to the L-PK promoter.

Several mechanisms leading to the above changes were examined. The Mitogen Activated Protein Kinases (MAPK), AMP Activated Protein Kinase (AMPK) and oxidative stress were excluded from the fatty acid regulation of L-PK. The HNF-4α transactivation activity was not regulated by n-3 PUFA, but was inhibited by WY14,643. Overexpressed ChREBP did not relieve n-3 PUFA control, but partially reversed WY14,643 control of L-PK gene expression. While overexpressed Mlx fully abrogated n-3 PUFA suppression of L-PK promoter activity and L-PK mRNA abundance, it failed to relieve the suppression of L-PK gene expression by WY14,643. The regulation of Mlx nuclear protein level by n-3 PUFA is not through the CRM-1 nuclear export pathway. Instead, 26S proteasome appears to be involved in this regulation.

Finally, three chronic disease models were examined. Mlx nuclear protein abundance was reduced when L-PK mRNA decreased in streptozotocin-induced diabetic rats and high fat-induced insulin resistant mice. In obese mice, however, Mlx protein abundance was increased when L-PK mRNA was also induced. Neither ChREBP nor HNF-4α protein level were affected in these animals. These studies suggest that Mlx plays a key role in the metabolic regulation of L-PK gene transcription.

In summary, my studies provided new information to explain how glucose, n-3 PUFA and WY14,643 regulated hepatic L-PK gene transcription through their effects on L-PK promoter composition and explored the road to a new regulatory network centered on the transcription factor Mlx.

To my parents

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List of Abbreviations

ACC acetyl-CoA carboxylase

AF activation function

AICAR 5-amino-4-imidazolecarboxamide ribotide

AMPK AMP-activated Protein Kinase

ApoCIII apolipoprotein C III

bHLH/ZIP base helix-loop-helix/leucine zipper protein

cAMP cyclic adenosine-5'-monophosphate

CAR constitutive androstane receptor

ChIP chromatin immunoPrecipitation

ChREBP carbohydrate regulatory element binding protein

CYP cytochrome P450

DBD DNA binding domain

DHA docosahexaenoic acid

DR1 direct repeat 1

Elovl fatty acid elongase

EPA eicosapentaenoic acid

FABP fatty acid binding protein

FA-CoA fatty acyl-Coenzyme A

FAS fatty acid synthase

FATP fatty acid transport protein

G6P glucose-6-phosphate

GFP green fluorescent protein

GK glucokinase

GLUT2 glucose transporter 2

GRIP1 glucocorticoid receptor interacting protein-1

HNF1α hepatic nuclear factor 1 α

HNF-4α hepatocyte nuclear factor-4α

LBD ligand binding domain

L-PK liver-type pyruvate kinase

LXR liver X receptor

MAPK mitogen activated protein kinase

Mlx Max-Like factor X

NEFA non-esterified fatty acid

NF1 nuclear factor 1

NLS nuclear localization signal

PEP phosphoenopyruvate

PEPCK phosphoenopyruvate carboxykinase

PGC1 peroxisome-proliferator-activated receptor-gamma co-activator 1

PKA cAMP-dependant protein kinase

PP2A protein phosphotase 2 A

PPARα peroxisome proliferator activated receptor α

PUFA polyunsaturated fatty acid

ROS reactive oxygen specieses

RXR retinoid x receptor

SCD1 stearoyl CoA desaturase 1

SHP small heterodimer partner

SREBP sterol regulatory element binding protein

TAT tyrosine amino transferase

USF upstream stimulatory factors

WAT white adipose tissue

WBSCR14 Williams-Beuren syndrome critical region 14

INTRODUCTION

One of features of the American diet is the excess amount of saturated fat and carbohydrate. The high carbohydrate from diet will induce lipogenesis in liver and increase blood triglycerides. Together with the fat from diet, they increase the risk of obesity, diabetes, heart disease, and other chronic diseases. On the contrary, evidence has shown that polyunsaturated fatty acids (PUFA) can increase insulin sensitivity, decrease blood triglycerides and have beneficial effects on diabetes, obesity, cancer, and cardiovascular diseases [1]. PUFA have been considered "good fat" in this regard.

High carbohydrate diet induces glycolysis and lipogenesis in liver, while dietary PUFA decreases blood triglycerides by increasing fatty acid oxidation and decreasing hepatic lipogenesis. Dietary carbohydrate and PUFA control the expression of enzymes involved in these processes through transcription factors. The regulation of gene expression by dietary fat was first described in the 1990's. Many transcription factors have been identified as targets of fatty acid regulation, such as peroxisome proliferator activated receptors (PPAR), sterol regulatory element binding protein-1c (SREBP-1c), hepatic nuclear factor 4 (HNF-4), retinoid X receptor (RXR) and liver X receptor (LXR). PUFA can regulate these transcription factors' activity or nuclear abundance to control their target gene expression.

The glycolytic enzyme Liver-type Pyruvate Kinase (L-PK) is one of the carbohydrate and PUFA regulated genes. It catalyzes the final and regulatory step of

glycolysis and provides precursors for *de novo* lipogenesis. Its transcription is induced by high carbohydrate diet and inhibited by n-3 PUFA and PPARα agonist WY14,643 [2]. PUFA induce fatty acid oxidation by activating PPARα, and inhibits lipogenesis by reducing nuclear SREBP1c abundance [3]. However, the PUFA regulation of L-PK does not use these mechanisms. The key transcription factors binding to the L-PK promoter have been identified: Carbohydrate regulatory element binding protein (ChREBP)[4], MAX-like factor X (Mlx) [5]and hepatic nuclear factor-4α (HNF-4α) [2].

This thesis aims to identify the mechanism by which glucose, n-3 PUFA and PPARα agonist WY14,643 regulate L-PK transcription. The glucose, n-3 PUFA and WY14,643 effect on ChREBP, Mlx and HNF-4α nuclear protein abundance and L-PK promoter composition was studied, possible mechanisms involved in L-PK transcriptional regulation were examined and a PUFA targeted transcription factor was identified.

Chapter 1

Literature Review

1.1 Liver-Pyruvate Kinase (L-PK)

1.1.1 Pyruvate Kinase (PK)

L-PK is a tissue specific isoform of pyruvate kinase (PK). There are four isoforms of PK encoded by two different genes. PKL gene produces the L- and R-type isoforms by means of alternative promoters, and PKM gene encodes for the M1- and M2-types by alternative splicing. The expression of these genes is tissue-specific and under developmental, dietary and hormonal control [6]. L-PK is predominantly expressed in the liver, but is also present in the kidney, small intestine and pancreatic β-cells. R-PK has been found only in erythrocytes. M1-PK is expressed in the skeletal muscle, heart and brain; M2-PK is expressed in other tissues. The expression of the PK isoforms is developmentally regulated. M2-PK is the only detectable isoform in early fetal tissues. During development, it is gradually replaced by the L-, R-, or M1-types.

In contrast, in transformed cells and a regenerating liver, the tissue-specific isoforms are present at decreased levels or absent altogether and are replaced by the M2-type isoenzyme [6].

PK functions as a homotetramer in almost all organisms although it may exist as a monomer, homodimer and heterodimer, heterohexamer, or homodecamer [7]. PK catalyzes the last step of glycolysis in the cytosol. Glycolysis is the pathway through which glucose are broken into two molecules of pyruvate and generates two ATP. There are ten enzymatic reactions in this process. The last step of these reactions is catalyzed by PK:

$$\text{Phosphoenolpyruvate (PEP)} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$$

This reaction is irreversible and a key regulatory step in glycolysis, which makes PK an important player in glucose metabolism.

Pyruvate, the product of PK, can be directed into gluconeogenesis by the pyruvate carboxylase or be converted to acetyl-CoA by oxidative decarboxylation by pyruvate dehydrogenase. Acetyl-CoA is the starting material for fatty acid synthesis. Although fatty acid β-oxidation also produces acetyl-CoA, it is not the major source of acetyl-CoA for fatty acid biosynthesis, because the two processes are regulated reciprocally. In fact, the acetyl-CoA converted from pyruvate can enter the TCA cycle to be shuttled out of mitochondria as citrate and enters fatty acid synthesis. As such, PK is not only crucial in glucose metabolism, but also in *de novo* lipogenesis.

1.1.2 The Role of L-PK in Glucose and Lipid Metabolism

Liver is the body's central metabolic organ. It maintains the proper levels of circulating fuels for use by the brain, muscles and other tissues. One of the major functions of liver is to act as a blood glucose "buffer". It does so by taking up, releasing and breaking down glucose in response to hormones and the concentration of blood glucose. L-PK is the predominant form of PK in liver. It acts as a key player for hepatic glucose homeostasis and lipid metabolism.

After a carbohydrate-containing meal, when blood glucose reaches ~6 mM, the pancreatic β-cells secrete insulin and stimulate the liver to take up glucose and convert it into glucose-6-phosphate (G6P). G6P can be converted into glycogen and stored in liver. Alternatively, glucose will go through glycolysis, ending as pyruvate. Under this condition, L-PK is activated to produce pyruvate, which will be converted to acetyl-CoA by pyruvate dehydrogenase. When the body is in need of energy, acetyl-CoA will go through the citric acid cycle to generate ATP. When the body need of energy is low, acetyl-CoA will be used to synthesize fatty acids, phospholipid and cholesterol. Synthesized lipids will be secreted into the blood stream and taken up by adipose tissue.

On the other hand, when blood glucose concentration drops during fasting, the pancreatic α-cells release glucagon. Glucagon stimulates glycogen breakdown and the release of glucose from the liver. Glucagon also promotes gluconeogenesis to provide glucose to brain when the liver glycogen is depleted. The increase of glucagon and decrease of insulin also promote the mobilization of fatty acids from adipose tissue

and inhibit glycolytic enzymes including L-PK. As such, high carbohydrate diets can lead to obesity by increasing lipogenesis, which may cause insulin resistance and hyperglycemia, ultimately type II diabetes. This makes it important to understand the regulation of L-PK and how it affects glycolysis and lipogenesis [8].

1.2 Regulation of L-PK by Nutrients and Hormones

1.2.1 Acute Regulation

The mammalian L-PK is acutely activated by its substrate phosphoenolpyruvate (PEP) and its allosteric activator fructose-1,2-bisphosphate (F-1,6-BP)[6, 9]. Phosphorylation of L-PK decreases its binding to PEP and F-1,6-BP [9]. In rat primary hepatocytes, 5.5 mM glucose (low glucose) leads to the phosphorylation of L-PK and decreases its activity by 40%. Raising the glucose concentration to 28 mM (high glucose) can prevent or rapidly reverse the phosphorylation state of the enzyme [10].

Accumulation of cyclic adenosine 5'-monophosphate (cAMP) leads to activation of cAMP-dependent protein kinase (PKA), which will phosphorylate and inactivate pyruvate kinase [9]. Glucagon stimulates the adenylate cyclase to increase the accumulation of cAMP. On the other hand, insulin activates a phosphodiesterase that degrades cAMP to lower the induced cAMP level by glucagon[9, 11]. Thus insulin increases the L-PK activity while glucagon decreases it [12].

1.2.2 Chronic Regulation

Besides the acute regulation of enzyme activity, L-PK gene transcription is also regulated by nutrients and hormones. The accumulation of L-PK mRNA is suppressed by fasting and is stimulated by a high-carbohydrate diet [13, 14]. Both diet and hormones contribute to these regulations.

Glucose and insulin together stimulate transcription of L-PK, However, overexpression of glucokinase induces L-PK mRNA in fasting state. This indicates that L-PK is responding to glucose metabolism instead of insulin signaling [15]. Under physiological conditions, insulin is required to stimulate glucose metabolism and in turn stimulates L-PK transcription.

During fasting, increased secretion of glucagon by pancreatic α cells and increased cellular cAMP, which activates PKA, inhibits L-PK gene transcription in liver [11, 16, 17, 18]. The fasting status will increase cellular AMP/ATP level and activate AMP-activated protein kinase (AMPK), which can inhibit L-PK transcription [19].

Supplementation of fish oil, which is rich in n-3 PUFA, decreases the accumulation of hepatic L-PK mRNA when the rats are on high carbohydrate diet [20]. A peroxisome proliferator activated receptor agonist, WY14,643 also inhibits the accumulation of hepatic L-PK mRNA when added to high carbohydrate diet for rats [2]. The WY14,643 effect requires the presence of PPARα , while the PUFA effect does not [2]. Cis-regulatory elements in the promoter that are responsible for this regulation and factors that bind to these elements have been identified. Key cis-regulatory

elements controlling L-PK gene transcription are located between -197 to -125 bp upstream of the transcription starting site (Fig. 1.1).

The glucose/insulin response element of L-PK gene is a perfect palindrome located from -183 to -144 bp upstream from the transcription-starting site[21, 22, 23]. This region contains two imperfect E boxes (CANNTG) separated by 5 bases [24, 25], which is named carbohydrate regulatory element (ChoRE). It was found that carbohydrate regulatory element binding protein (ChREBP), a member of the basic helix-loop-helix/leucine zipper (bHLH/ZIP) family transcription factors, binds specifically to the ChoRE of the L-PK promoter [26, 27]. The binding of ChREBP to L-PK ChoRE needs another bHLH/ZIP protein, MAX-like factor X (Mlx), as the heterodimer partner [28, 5]. The presence of ChREBP/Mlx heterodimer is required for glucose induction of L-PK transcription [28, 29].

The full efficiency of ChoRE on the L-PK promoter requires cooperation with an imperfect direct repeat separated by one nucleotide (DR1) (TGGACTCTGGCCC) located from -145 bp to -120 bp [21, 22, 23, 30]. DR1 can be recognized by several nuclear factors such as PPARα and HNF-4α. Both PUFA and WY14,643 target this region and suppress L-PK gene expression [20, 2, 31]. WY14,643, but not PUFA, inhibit L-PK transcription through PPARα. However HNF-4, but not PPARα , binds to the L-PK promoter [2].

Cyclic AMP inhibits the L-PK gene transcription through the glucose response element and the HNF-4α binding site. This inhibitory effect was reported to require the contiguous connection of the two sites and the binding of HNF-4α [30].

While ChREBP and Mlx are required for glucose-regulated transcription of L-PK [21, 22, 23, 32, 33, 34, 25], acetyl CoA carboxylase [35], fatty acid synthase [36], S14 [37], stearoyl CoA desaturase-1, malic enzyme, and GLUT2 [28, 33], only glucose-regulated L-PK gene transcription requires a DR-1 element binding HNF-4 [20, 22], which makes it a unique regulatory system.

Besides these key regulatory factors, other transcription factors are present on the L-PK promoter and are essential for the expression of L-PK gene. Cooperative interactions between nuclear factor (NF) 1 family members and hepatocyte nuclear factor 1α (HNF1α) play an important role in hepatic L-PK transcription. NF1 and HNF1α bind to specific cis-regulatory elements on L-PK promoter at -115 to -96 bp and -96 to -66 bp respectively and synergistically activate the L-PK promoter [38]. However, no evidence has shown that these two factors are involved in glucose or fatty acid regulation of L-PK transcription.

The L-PK gene might have other unidentified cis-regulatory elements upstream of -180 bp. It was reported that positive regulatory elements responding to high carbohydrate feeding exist between -1065 bp and -945bp, and between -300 bp and -203 bp on the L-PK gene [39]. More studies will be needed to reveal the importance of these elements. The proximal promoter up to -197 bp is sufficient for full induction of L-PK promoter activity by glucose and full suppression of L-PK promoter activity by n-3 PUFA and WY14,643.[20], allowing me to focus on ChREBP, Mlx and HNF-4.

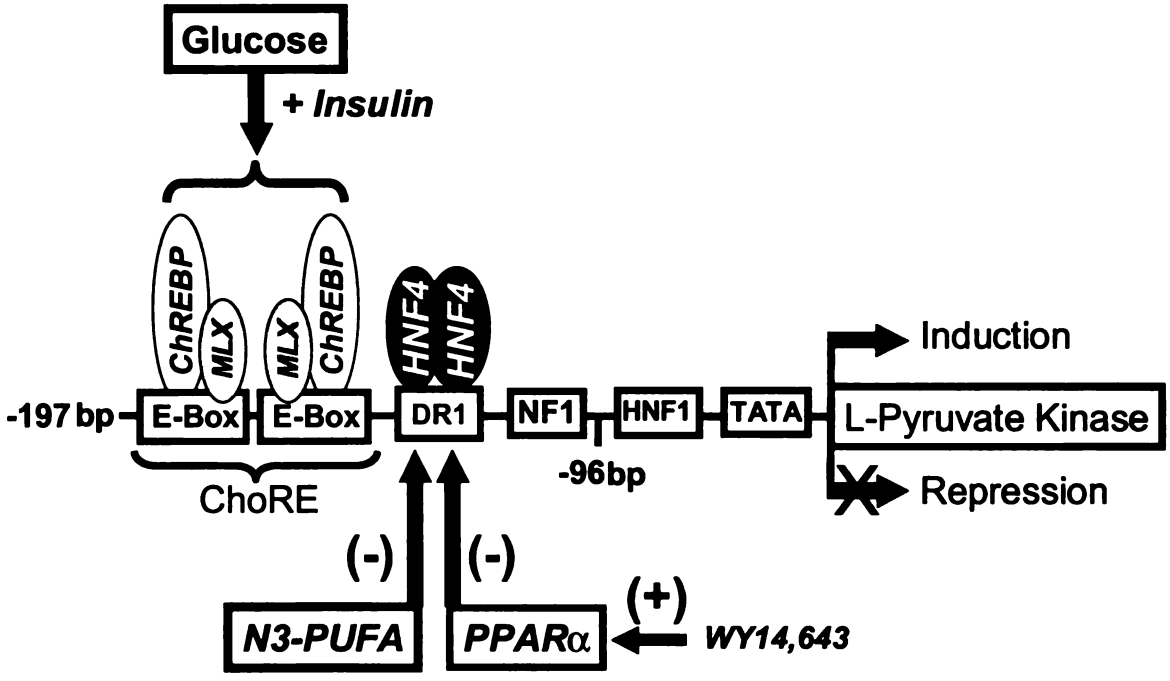


Figure 1.1: **Scheme of the L-PK promoter.** The diagram illustrates the locations of key transcription factor binding sites and their regulation by glucose, fatty acids and the PPARα agonist WY14,643. The key cis-regulatory elements controlling L-PK gene transcription are located between -197 to -125 bp upstream of the transcription start site. The two HLH-transcription factors, ChREBP and Mlx bind as a heterodimer to two E-boxes (CANNTG) separated by 5 nucleotides between -197 and -145 bp. The nuclear receptor HNF-4 binds the imperfect direct repeat (DR1,-143 TGGACTCTGGCCC -130) as homodimers.

1.3 Fatty Acid Regulation of Gene Transcription

Besides its roles in energy storage and as structural components for cells, dietary fat regulates genes involved in lipid, carbohydrate, and protein metabolism, as well as cell growth and differentiation. Imbalanced fatty acid pool in human body is related to the onset and progression of several chronic diseases, like coronary artery disease and atherosclerosis, dyslipidemia and inflammation, obesity and diabetes [40, 41, 42, 1]. Because of its impact on human health, considerable effort has been directed at understanding the mechanisms by which fatty acids control gene expression.

Fatty acids (FA) can be classified according to the number of double bonds in their carbon chains. Saturated fatty acids contain no double bond in their carbon chain, such as palmitic acid (Fig 1.2 A); monounsaturated fatty acids contain one double bond, such as oleic acid (Fig 1.2 B); polyunsaturated fatty acids (PUFA) contain two or more double bonds. There are two major classes of dietary PUFA, n-3 (or ω-3) and n-6 (or ω-6). They are named for the carbon involved in the first double bond from the methyl end (-CH3). Arachidonic acid is an n-6 PUFA (Fig 1.2 C), and eicosapentaenoic acid (Fig 1.2 D) is an n-3 PUFA. The precursors of these two classes of PUFA, linoleic acid and α-linolenic acid, respectively, cannot be synthesized in human and must be provided by diets. These two families cannot be inter-converted in mammals[43]. Because of this, they are essential fatty acids.

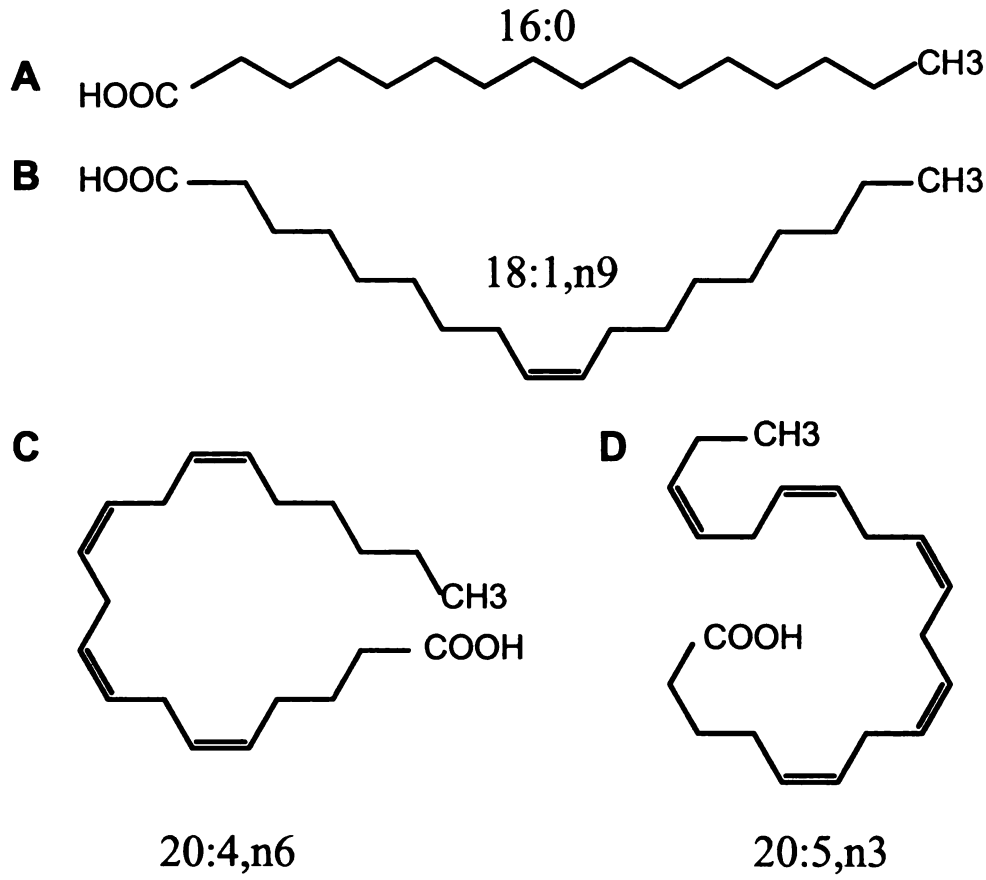


Figure 1.2: **Structures of fatty acids.** Fatty acids can be classified according to the number of double bonds. Saturated fatty acids contain no double bonds, such as palmitic acid [A]; monounsaturated fatty acids contain one double bond, such as oleic acid [B]; polyunsaturated fatty acids (PUFA) contain two or more double bonds, such as arachidonic acid [C], and eicosapentaenoic acid [D].

1.3.1 Dietary Lipid Metabolism and Cell Function

Fatty acids from the diet are carried in plasma in either esterified form or non-esterified form. The major esterified form is triacylglycerol, complexed in the lipoproteins. The non-esterified fatty acids (NEFA) are loosely bound to albumin. Blood lipoproteins are synthesized from dietary lipids after absorption and re-esterification in the intestine (chylomicron) or the liver (very low density lipoproteins). They are hydrolyzed by hepatic lipase or lipoprotein lipase to produce NEFA that are taken up by the liver, muscles or adipose tissue. On the other hand, circulating NEFA are produced mostly by hormone-sensitive lipase in white adipose tissue (WAT) in response to starvation resulting in lipolysis of the stored triacylglycerols. In lipogenic tissues such as liver and WAT, fatty acids can also be synthesized *de novo* from glucose and be esterified[44].

In cells, the signaling molecule is the free fatty acid (not bound to albumin). Fatty acids are taken up by a fatty acid transport protein (FATP) or other transport proteins, i.e. CD36. Fatty acids are converted to fatty acyl-CoA (FA-CoA) and can be elongated, desaturated, β-oxidized in mitochondria or peroxisome for energy production. FA can also be oxidized by peroxidative process or monooxidized in the microsome. Fatty acid can be assimilated into membrane lipids as phospholipid, sphingolipid or plasmalogens or serve as substrates for eicosanoid synthesis. Fatty acids, products of fatty acids metabolism or fatty acid-sensitive signal transduction mechanism can impact cell function [44].

In mammals, PUFA regulation of gene expression is found in brain, liver, heart and adipose tissues[45]. The modulation by PUFA can be in either a positive or negative manner. PUFA can transcriptionally or post-transcriptionally regulate gene expression. For the former, they can alter the transcription rate by acting on transcription factors. For the latter, they can change the stability of specific mRNAs[42, 46].

1.3.2 Fatty Acid Regulation of Transcription Factors

Liver plays a central role in whole body lipid synthesis and metabolism. Dietary fat can modulate hepatic gene expression leading to alterations in lipid and carbohydrate metabolism[42]. Fatty acid effects on gene expression are cell-specific and influenced by fatty acid structure and metabolism. Fatty acids regulate the hepatic gene expression through different mechanisms. They have impact membrane lipid/lipid raft composition, affect G-protein receptor or tyrosine kinase-linked receptor signaling [1, 45]; they can be covalently linked to proteins for post-translational modification, or acting as precursors of signaling molecules such as prostanoids and steroids [40, 41, 42]. More often, fatty acids can regulate the nuclear abundance of transcription factors; or bind directly to specific transcription factors, as fatty acids or their metabolites, to modify the transactivation activities of transcription factors.

Two well studied transcription factors involved in fatty acid regulation of hepatic gene expression are peroxisome proliferator activated receptor α (PPARα) and sterol regulatory element binding protein 1c (SREBP1c).

PPARs belong to the nuclear receptor superfamily. There are four members: α, β, $\gamma 1$ and $\gamma 2$. PPARα is the most abundant form found in the liver, with smaller amounts of the δ and γ forms also expressed [47, 48, 49, 50]. PPARs bind to DR1 elements as heterodimers with retinoid x receptor (RXR) [51]; PPAR is at 5' and RXR is downstream [52, 53].

PPARs are activated by fibrates, polyunsaturated fatty acids, eicosanoids, and various synthetic ligands[47, 48, 49, 50]. PPARα binds n-3 PUFA in its ligand binding domain. C20 PUFA are strong activators of PPARα, while C22 PUFA are weak PPARα activators. The synthetic compound WY14,643 is a strong activator of PPARα [51, 54, 55, 56]. Ligand binding to PPARα regulates its target genes involved in lipid oxidation and inflammation [47].

SREBPs are basic helix-loop-helix/leucine zipper transcription factors that control many genes involved in cholesterol and fatty acid synthesis. They bind DNA cis-regulatory elements called sterol-regulatory elements (SREs). Three isoforms, SREBP-1a, -1c, and -2, have different roles in lipid synthesis in differentiated tissues and organs. SREBP-2 plays a major role in regulation of cholesterol synthesis and cellular uptake. SREBP-1a is expressed in growing cells, providing both cholesterol and fatty acids that are required for membrane synthesis[57]. SREBP-1c is required to maintain basal levels of fatty acid synthesis. It is induced by insulin and LXR. Elevation of nuclear SREBP-1c abundance transcriptionally activates many genes involved in fatty acid synthesis [20].

The SREBPs are regulated at two levels. The first mechanism is proteolytic processing. SREBP precursors ($\sim$125 kD) are bound to the endoplasmic reticulum

when first synthesized. The N-terminal active portion (~65 kD) is cleaved by specific proteases and enters the nucleus to activate target genes involved in cholesterol and fatty acid synthesis [58]. This cleavage step is regulated by a putative sterol-sensing molecule, SREBP-activating protein (SCAP), that forms a complex with SREBPs and escorts SREBP translocation from the endoplasmic reticulum to the Golgi [20]. The Golgi contains 2 enzymes site-1 and site-2 protease that cleave SREBP generating the mature nuclear form. The second mechanism is the proteasomal degradation of nuclear SREBP. This process involves phosphorylation and ubiquitination of nuclear SREBP, which induces its degradation in the 26S proteasome [59]. SREBP1, but not SREBP2, is a major target of PUFA control in liver [1]. PUFA stimulate SREBP1 mRNA decay [60] and nSREBP1c proteasomal degradation and suppress SREBP-1c gene transcription. In all, PUFA lower the nuclear abundance of SREBP-1 thereby decreasing its stimulatory effect on lipogenic gene transcription [61].

1.4 Key Factors Involved in L-PK Transcriptional Regulation

Although the mechanisms by which PPARα and SREBP1c participate in PUFA control of genes transcription are well studied, the regulation of L-PK by n-3 PUFA does not use either of these mechanisms. Thus PUFA control of L-PK is a unique example of fatty acid control of hepatic gene expression. Considerable effort by our laboratory has been directed at defining the transcription factors involved in fatty acid regula-

tion of L-PK. The recent discovery of the role of ChREBP, Mlx and HNF-4 in L-PK has provided me the opportunity to evaluate the role these proteins play in PUFA regulation of L-PK.

1.4.1 Carbohydrate Regulatory Element Binding Protein (ChREBP)

Before ChREBP was identified, there was controversy, as to whether or not upstream stimulatory factors (USF) regulate the glucose responsiveness of L-PK promoter activity in hepatocytes. Some investigators suggested that USF-2 was required for glucose stimulation of L-PK promoter activity in single islet beta-cells and INS-1 cells[62]. Later studies showed that neither basal level nor glucose responsiveness of endogenous L-PK mRNA was affected by overexpression of USF-1 and -2. Similarly, L-PK expression was not affected by dominant-negative suppression of USF function [63].

Carbohydrate response element-binding protein (ChREBP) was recently shown to regulate glucose responsiveness of the L-PK promoter activity in hepatocytes [4]. ChREBP is encoded by a gene called Williams-Beuren Syndrome Critical Region 14 [4, 64]. Williams-Beuren Syndrome is a neurodevelopmental disorder caused by deletion of multiple neighboring genes, including WBSCR14, and characterized by congenital heart and vascular disease, hypertension, infantile hypercalcemia, and dental abnormalities [65, 66]. When WBSCR14 was first identified to encode a transcription factor, it was not related to glucose metabolism. In 2001, the Uyeda group identi-

fied a protein that was responsible for the glucose induction of L-PK transcription. Uyeda et al named the protein ChREBP [4]. ChREBP is expressed in liver, pancreas, adipose tissue, and other tissues [33, 63, 4, 67]. It is the principal glucose-regulated transcription factor controlling transcription of L-PK [25, 32, 23, 34, 22, 21] and other glucose-regulated genes, like acetyl CoA carboxylase [35], fatty acid synthase[36], S14 [37], stearoyl CoA desaturase-1, malic enzyme, and GLUT2 [28]. Chromatin Immunoprecipitation assay has shown its binding to ChoRE on ACC, FAS, L-PK promoters *in vivo*[29]. ChREBP knockout leads to the lost of glucose regulation on L-PK mRNA level and promoter activity [29].

ChREBP is a member of the basic helix-loop-helix/leucine zipper (bHLH/ZIP) family of transcription factors. It contains several functional domains including a nuclear localization signal (NLS), a proline-rich stretch (PRO) region, a bHLH/ZIP region, and a ZIP-like domain (Fig. 1.3). The NLS is located toward the N terminus (amino acids 155-174) of ChREBP. It is essential in translocating ChREBP into nuclei and for activation of L-PK transcription. The consensus phosphorylation site (Fig. 1.3 P1, 193RRSS196) located near the NLS domain is likely to participate in the regulation of ChREBP translocation [27]. The bHLH/ZIP domain is located in the C terminus and is responsible for dimerization and DNA binding[4].

ChREBP is regulated at the level of its entry into nucleus. Its nuclear exportation is thought to be through a CRM-1-dependant pathway by its binding to 14-3-3 proteins in a phosphorylation dependant manner [64]. However, this mechanism was not shown to be involved in glucose regulation.

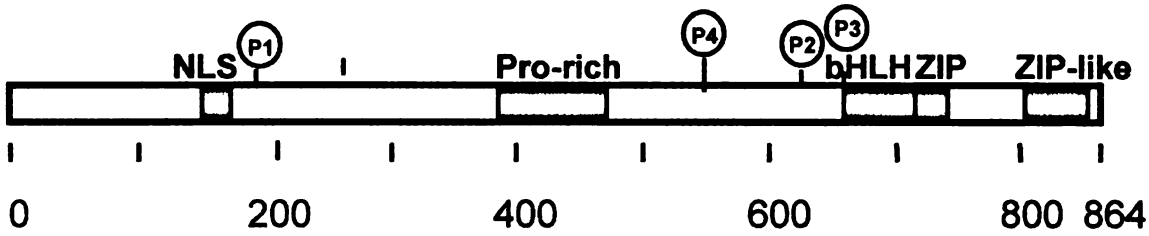


Figure 1.3: **Schematic representation of domain structure of mouse ChREBP.** The locations of NLS, proline-rich stretch, bHLH/ZIP, and ZIP-like domains are indicated. The locations of three phosphorylation sites of cAMP-dependent protein kinase (PKA) are indicated as P1, P2, and P3. The AMP-activated protein kinase (AMPK) site is indicated as P4 [27, 68].

In low glucose status ($\leq$5 mM), ChREBP is localized in the cytosolic compartment. As media glucose increases to over 8 mM, ChREBP enters the nucleus [26, 27]. This translocation is regulated by phosphorylation status of ChREBP. NLS and bHLH/ZIP domains of ChREBP are the targets of regulation by cAMP and glucose. cAMP induces PKA-mediated phosphorylation of Ser196 (near NLS) and inactivates nuclear import [27]. Insulin induces glucose metabolism, which include an increase in the pentose phosphate pathway and increased abundance of xylulose 5-phosphate (X5P). X5P activates a X5P-dependent Protein Phosphotase 2A. PP2A dephosphorylates Ser-196 of ChREBP in cytoplasm and induces ChREBP nuclear import [69, 27, 68].

This mechanism appears controversial. The phosphorylation status of ChREBP was not affected by high glucose, and mutants of PKA phosphorylation site can still respond to high glucose and cAMP, instead of being constantly active [70]. These observations suggested that mechanisms other than PKA, are involved in the glucose-induced translocation of ChREBP.

ChREBP DNA binding activity is also regulated by ChREBP phosphorylation status. PKA phosphorylation of Thr666 (within the bHLH site) inhibits the ChREBP DNA-binding [27]. Increased cellular AMP/ATP ratio activates AMPK. AMPK phosphorylation of ChREBP at Ser568 also reduces its DNA-binding activity [26]. When the glucose metabolite Xylulose 5-phosphate activates PP2A, it dephosphorylates Ser-568 and Thr-666 of ChREBP in the nucleus and activates ChREBP binding to ChoRE in target genes [69, 27, 68].

Since ChREBP is essential for glycolytic and lipogenic gene expression, investigators have asked if this protein is regulated by fatty acids. The Uyeda group first reported that fatty acid-inhibited glucose-induced L-PK transcription resulted from AMPK phosphorylation of ChREBP at Ser568, which inactivated its DNA binding activity. AMPK was activated by the increased cellular AMP that was generated by the fatty acid consumption of ATP during the formation of FA-CoA [26]. More recently, the Postic group reported that in mouse liver and hepatocytes, PUFA [linoleate (C18:2), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6)] suppressed ChREBP activity by increasing ChREBP mRNA decay and inhibiting ChREBP translocation from the cytosol to the nucleus. Postic et al claimed that these effects were independent of the AMPK activation. On the contrary, glucose metabolism via the pentose phosphate pathway was a determinant for ChREBP nuclear translocation. PUFA decreased X5P concentration leading to the inhibition of ChREBP translocation [71]. These controversies keep the mechanism of PUFA control of ChREBP unclear.

1.4.2 Max-Like Protein X (Mlx)

Mlx was first identified in 1999 as a Mad family partner [72]. Because it is structurally and functionally related to the Mad family heterodimer partner Max protein, this new protein was named Max-Like Protein X. Mad/Max heterodimers oppose the growth-promoting action of Myc/Max heterodimers, thus Mlx was first thought to be involved in the control of cell cycle[72, 73]. Mlx has broad expression in many tissues and a

long protein half-life. It can form heterodimers with bHLH/ZIP proteins and bind to E-boxes [72, 74].

Soon after the identification of Mlx, another bHLH/ZIP protein called MondoA was identified [75]. MondoA and Mlx can form heterodimers and translocate between cytoplasm and nucleus [75]. In fact, Mlx heterodimerization with MondoA and their nuclear localization was well studied before its role in glucose-induced gene expression was discovered by the Towle group [5]. One interesting point is that ChREBP structure is highly similar to that of MondoA, which gave ChREBP another name, MondoB. As a result, Mlx is now viewed as a partner of the Mondo family members [76].

MondoA forms homodimers weakly but associates with Mlx *in vivo*. MondoA/Mlx enter the nucleus via a nuclear localization signal and bind to E-boxes. The amino termini of the Mondo proteins are highly conserved among family members and contain separable and autonomous cytoplasmic localization and transcription activation domains [75]. Heterodimerization between MondoA and Mlx and a conserved domain in the N terminus of MondoA are important determinants for MondoA-Mlx subcellular localization [76]. In response to the nuclear export inhibitor, leptomycin B, MondoA-Mlx heterodimers accumulate in the nucleus [75]. MondoA and Mlx share sequence similarity in their bHLHZip domains and C termini [76]. Their C-terminal domains contains cytoplasmic localization domains that are required to keep Mlx monomers in the cytoplasm. As a dimerization interface, this C-terminal domain functions independently of the leucine zipper domain to mediate heterotypic interactions between MondoA and Mlx. Dimerization of the two inactivates their C termini

cytoplasmic localization activity so that the heterodimer complex can accumulate in the nucleus. MondoA-Mlx heterodimers, while poised for nuclear entry, are retained in the cytoplasm by conserved domains in the N terminus of MondoA. Mondo conserved regions (MCRs) II and III are CRM1-dependent nuclear export signal and binding site for 14-3-3 family members, respectively, which contribute to cytoplasmic localization of MondoA-Mlx (Fig. 1.4)[76].

Mlx was not connected to glucose metabolism until in 2004 when the Towle group reported that Mlx was the functional heterodimer partner of ChREBP [5]. Mlx is required for gene transcription mediated by ChREBP [28]. Mlx and ChREBP form heterodimers on the L-PK promoter as the principal targets for glucose activation of L-PK gene transcription [32, 23, 34, 22, 21, 25]. The Towle group also showed that Mlx is required for glucose induction of glycolytic and lipogenic genes transcription [28]. Overexpression of the dominant negative form of Mlx without DNA-binding activity totally eliminates the glucose induction of these genes [28].

The first identified heterodimer partner of Mlx, MondoA, is also reported to be a regulator of glycolysis [77]. Sans et. al. showed that MondoA and Mlx are associated to mitochondria in skeletal muscle cells. MondoA can translocate between mitochondria and nuclei to regulate glycolysis rate. MondoA can directly bind to E-boxes on glycolytic genes promoters and induce their expression. The targets of MondoA involved in glucose metabolism includes Hexokinase II (HKII), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and L-lactate dehydrogenase A (LDH-A) [77]. However, these studies were performed in skeletal muscle cells. Whether this mechanism functions in liver is unclear.

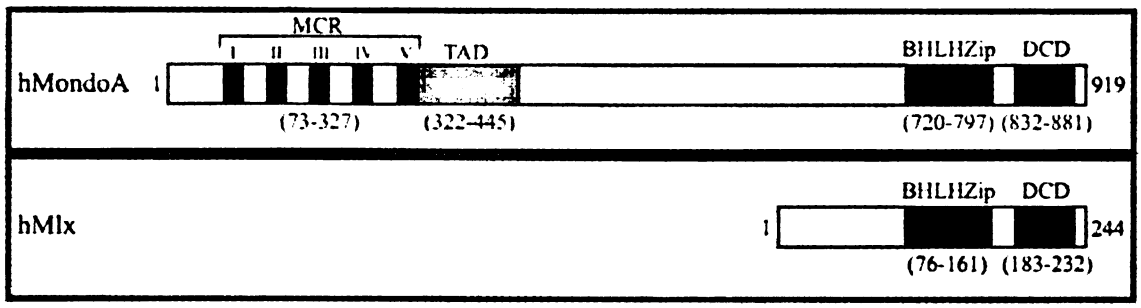


Figure 1.4: **Domain diagrams of human MondoA and human Mlx.** MCR, Mondo conserved region; TAD, transcriptional activation domain; bHLHZip, basic helix-loop-helix leucine zipper domain; DCD, dimerization and cytoplasmic localization domain [76].

Despite the fact that ChREBP/Mlx is required for glucose responses of hepatic glycolytic and lipogenesis genes and that ChREBP is possibly regulated by fatty acids, there has been no report about Mlx or MondoA participation in the fatty acid regulation of gene transcription.

1.4.3 Hepatic Nuclear Receptor 4 (HNF-4)

Another key protein involved in L-PK gene transcription is the hepatocyte nuclear factor-4 (HNF-4). HNF-4 belongs to the nuclear receptor superfamily, which is a group of ligand-activated transcription factors that regulate the expression of target genes, playing roles in reproduction, development, and general metabolism [52, 53]. Besides the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D [48], this superfamily also includes a large number of orphan nuclear receptors, including HNF-4, whose ligands, target genes, and physiological functions are not well understood [49]. Many of the orphan receptors respond to cellular lipid levels and lead to gene expression changes, ultimately protecting cells from lipid overload [47].

Two isoforms of HNF-4 have been identified in mammals. HNF-4α is expressed in liver, kidney, intestine and pancreas [78, 79, 80]. HNF-4γ has been found in pancreas, kidney, small intestine and testis[81], as well as in liver [82]. Most studies have focused on HNF-4α, little is known about the γ isoform.

HNF-4α has the common modular structure of most nuclear receptors [80] (Fig 1.5). The functional domains of HNF-4α consist of an N-terminal activation function

AF-1 (A/B domain), two zinc fingers responsible for DNA binding (C domain), a hinge region (D domain), a potential ligand binding domain (domain E) containing the activation function AF-2 and the F domain[80]. AF-1 consists of the extreme N-terminal 24 amino acids and functions as a constitutive autonomous activator of transcription. This short transactivation domain belongs to the class of acidic activators, and it is predicted to adopt an amphipathic alpha-helical structure. AF-2 transactivation domain within the LBD spans the 128-366 region of HNF-4α, and it cannot be further dissected without impairing activity. The 360-366 region of HNF-4α contains a motif that is highly conserved among transcriptionally active nuclear receptors, and it is essential for AF-2 activity, but it is not necessary for dimerization and DNA binding of HNF-4α. Thus, HNF-4α deletion mutants lacking the 361-465 region bind efficiently to DNA as homo- and heterodimers and behave as dominant negative mutants. Amino acid residues Ser-181 and Met-182 in H3, Leu-219 and Leu-220 and Arg-226 in H5, Ileu-338 in H10, and Ileu-346 in H11 are the key residues in the HNF-4α LBD pocket that have contact with the ligand. Mutations in these amino acids impair HNF-4 transactivation potential. These residues play a significant role in maintaining the structural integrity of the HNF-4α ligand binding pocket [83].The integrity of the hinge (D) domain of HNF-4α and the activation function (AF)-2 activation domain region are critical for coactivation [84]. The full transactivation activity of AF-2 is inhibited by the F region, spanning residues 371-465. The inhibitory effect of region F on the HNF-4α AF-2 activity is a unique feature among members of the nuclear receptor superfamily, and is proposed that it defines a distinct regulatory

mechanism of transcriptional activation by HNF-4[80]. Recently, the F domain was shown to affect the HNF-4α interaction with coactivator and corepressors[85, 86].

Hertz and Bar-Tana were the first to report that long chain fatty acids directly modulate the transcriptional activity of HNF-4α by binding as their fatty acyl-CoA thioesters to the HNF-4α LBD [87]. This binding may have positive or negative effect on the transcriptional activity of HNF-4α, depending on the length and degree of saturation of the fatty acid. Later, Hertz et. al showed that the CoA thioesters of amphipathic carboxylic hypolipidemic drugs such as clofibric acid analogues that are formed *in vivo*, can bind to HNF-4α, inhibit its transcriptional activity, and suppress the expression of HNF-4α-responsive genes [88]. More recently, it was shown that binding of fatty acyl-CoAs (FA-CoA) was specific as the binding affinities of the respective free fatty acids or free CoA (Kd values of 421-742 nM) were significantly lower. Fatty acyl-CoA binding significantly and differentially altered the HNF-4α LBD secondary structure. The CoA thioesters of some hypolipidemic peroxisome proliferators bind with high affinity (Kd values as low as 2.6 nM) to HNF-4α LBD, indicating that HNF-4α may serve as a target for these drugs [89]. In summary, one theory on the HNF-4α ligands is that high affinity binding to HNF-4α of fatty and xenobiotic acyl-CoAs in the physiological range results in significantly altered HNF-4α conformation and lead to the transcriptional activity modulation. However, structural analysis of the HNF-4α LBD indicate that fatty acyl-CoA thioesters, the proposed HNF-4α ligands, are not good candidates for HNF-4 ligand α [90].

Recently, free fatty acids were reported to bind HNF-4 LBD. The crystal structure of the HNF-4γLBD reveals a small ligand-binding site of only 625$\mathring{A}^3$, which is

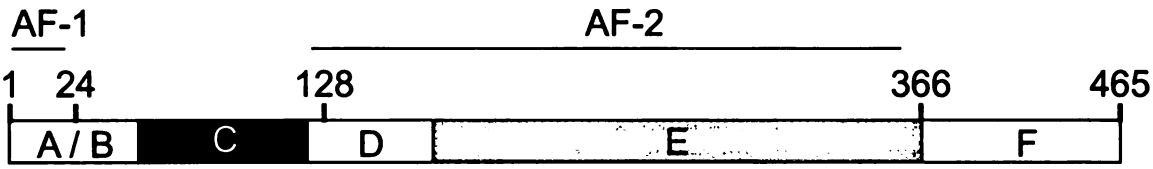


Figure 1.5: **Functional domains of HNF-4.** HNF-4 has several functional domains. The variable NH2-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The region C contains the highly conserved DNA-binding domain (DBD), which is responsible for the DNA recognition. The variable linker region D connects the DBD to the conserved E region that contains the ligand-binding domain (LBD). This region is responsible for dimerization and contains the ligand-dependent AF-2 transactivation domain within the COOH-terminal portion [80].

occupied entirely by a fatty acid that co-purifies with the HNF-4 protein [91]. The AF2 helix was in a conformation characteristic of a transcriptionally active nuclear receptor. GC/MS and NMR analysis of chloroform/methanol extracts from purified HNF-4α and HNF-4α LBDs identified mixtures of saturated and cis-monounsaturated C14-18 fatty acids. The purified HNF-4 LBDs interacted with nuclear receptor coactivators. Both HNF-4 subtypes showed high constitutive activity in transient transfection assays, which was reduced by mutations designed to interfere with fatty acid binding. The endogenous fatty acids did not readily exchange with radio-labeled palmitic acid, and all attempts to displace these bound fatty acid with exogenous fatty acids failed [92]. The Shoelson lab demonstrated that in the crystal structure of the HNF-4α LBD exists two conformational states. Each was a homodimer. One had an open form with α helix 12 (α12) extended and colinearized with α10. The other form was closed with α12 folded against the body of the domain. Although the protein was crystallized without exogenous ligands, the ligand binding pockets of both closed and open forms contained fatty acids. The carboxylic acid head group of the fatty acid ion paired with the guanidinium group of Arg (226) at one end of the ligand binding pocket, while the aliphatic chain fills a long, narrow channel that was lined with hydrophobic residues [93]. Taken together, these studies suggest that fatty acids are endogenous ligands, constitutively bound to HNF-4α.

There remains, however, arguments about the ligand for HNF-4. Later studies by the Bar-Tana group suggested that the F domain, instead of E domain (LBD), is responsible for the FA-CoA binding and regulation of HNF-4α conformation [94]. The absence of FA-CoA in crystalized HNF-4 LBD is due to the instability of FA-CoAs

in the buffers and the fact that HNF-4 actually helps FA-CoA degradation [95]. The same group also reported that Acyl-CoA Binding Protein (ACBP) is present in the nucleus and physically and functionally interacts with HNF-4 [96]. Thus, controversy over the ligands binding to and regulating HNF-4 remains.

HNF-4α homodimers bind to divergent DR1 sequences in promoters. The DNA binding activity of HNF-4α decreases due to the post-translational modification by phosphorylation[66]. HNF-4α potentially contains 21 serine, 6 threonine, and 7 tyrosine phosphorylation sites [97, 98]. The phosphorylation on Ser and Thr may modulate the DNA binding activity. In COS 7 cells, serine/threonine phosphorylation of HNF-4α increases affinity and specificity of DNA binding by altering its tertiary structure [98, 99]. The phosphorylation on Tyr is also important for its DNA binding, transactivation, and subnuclear localization [100]. Protein kinase A-dependent phosphorylation within the DBD inhibits DNA binding in HepG2 and COS 1 cells [101]. AMP-activated protein kinase (AMPK) mediated phosphorylation of HNF-4α on serine304 had a 2-fold effect, reducing the ability of the transcription factor to form homodimers and bind DNA and increasing its degradation rate in vivo [102]. Acetylation is another modification of HNF-4α that can affect its nuclear retention, DNA binding, and promoter activation in COS 1 and NIH 3T3 cells [98].

Nutrients might regulate gene transcription by modulating HNF-4α DNA binding activity. In the liver of protein-deprived animals, there is no change in the protein level of nuclear HNF-4α, but a 40% reduction in the DNA binding [103]. The binding affinity of HNF-4α for DNA is unaltered by dietary protein deprivation, while the number of HNF-4α molecules able to bind to DNA was reduced. This indicates that

protein deprivation inactivated or prevented HNF-4α from binding to DNA [103]. A similar mechanism might apply to the fatty acid regulation of hepatic genes transcription through HNF-4α.

HNF-4α regulates genes at the transcriptional level by interacting with the HNF-4α binding sites in the promoter or enhancer. It is one of several transcription factors essential for liver specific gene expression. HNF-4α plays critical roles in nutrition and metabolism from very early in embryonic development to the adult stage, which is conserved throughout evolution [89, 104]. HNF-4α is important in nutrient transportation [78, 105, 106, 107, 108, 109] and metabolism [110, 111, 112, 113, 114, 115]. HNF-4 plays a major role controlling expression of multiple genes involved in glucose and lipid homeostasis [116], nutrient transportation [78, 106, 117, 108, 107, 109], metabolism [110, 113, 111, 112, 115, 114], blood flow [118, 119, 120, 121], and gene transcription [122, 123, 124]. Most of these genes are not regulated by glucose. Of the many HNF-4 regulated genes, only L-PK, apolipoprotein A1 and CIII, and glucose-6-phosphatase have been reported to be affected fatty acids [3].

A variety of factors can interact with HNF-4 to regulate its target gene expression. Transcription factors such as HNF1α [125, 126], Hypoxia inducible factor 1 (HIF-1) [127, 128] and PPARα [129] pregnane X receptor (PXR) [130], can cooperately or competitively interfere with HNF-4 control of gene transcription. On the other hand, some transcription factors such as the forkhead in rhabdomyosarcoma (FKHR) [131] and small heterodimer partner (SHP)[132] block HNF-4 DNA binding ability by interaction with its DBD or LBD. Coactivator and corepressors such as

GRIP-1 and PGC1-α and constitutive androstane receptor (CAR) also participate in HNF-4 functions [133].

HNF-4α null mice die during embryogenesis [134]. In the Cre-loxP conditional knockout mice, the HNF-4α gene was liver-specifically disrupted. These animals accumulated lipids in the liver and exhibited greatly reduced serum cholesterol and triglyceride levels and increased serum bile acid concentrations. A wide array of hepatic mRNA levels were changed in the conditional knockout mice, indicating the central role of HNF-4α in hepatic genes expression and lipids homeostasis.[116]

Mutations in Factor IX promoter HNF-4α binding site cause the hemophilia B Leyden, an X chromosome-linked recessive bleeding disorder [118, 135]. Mutations in Factor VII promoter HNF-4α binding site cause the Factor VII deficiency, an autosomal recessive bleeding disorder [136]. HNF-4α is also known to play a very critical role in ApoCIII gene regulation. ApoCIII is synthesized in liver and secreted to bloodstream, associate with lipids to form plasma lipoproteins. Lipoproteins are responsible for delivering lipids to other tissues and return excess lipids to the liver. Thus, controlling apolipoprotein levels are important for atherosclerosis and coronary heart disease. HNF-4α is known to be involved in regulation of several apolipoproteins, although the inherited mutations in HNF-4α binding sites of these genes have not been identified [137].

By far, mutations in the HNF-4α gene itself are associated with only one disease, the subtype 1 of maturity-onset diabetes of the young (MODY1), a monogenic dominant inherited form of diabetes mellitus characterized by impaired insulin secretory response to glucose in pancreatic β cells [138, 139].

With in mind all the arguments about HNF-4 ligands and the variety of proteins that can interact with HNF-4, we know that a target on the L-PK promoter for PUFA control is on the HNF-4 binding site [2]. The question is "What is the regulatory mechanism for PUFA regulation of L-PK gene transcription?"

1.5 Hypothesis

While ChREBP is the key glucose-regulated transcription factor, Mlx and HNF-4 are required for full glucose-mediated activation of L-PK gene transcription [21, 28]. In contrast to Mlx [28], HNF-4 has not been reported to play a role in other glucose-regulated genes. PUFA and WY14,643 inhibit L-PK gene transcription [20, 2] by targeting the cis-regulatory region that binds HNF-4. While both WY14,643 and n-3 PUFA activate PPARα , PUFA inhibition of L-PK does not require PPARα [2]. Moreover, the PPARα/RXRα heterodimer does not bind the L-PK promoter[2] suggesting that PPARα control of L-PK does not involve direct interaction with the L-PK promoter. Based on the above facts, my hypothesis is that:

The ChREBP/Mlx heterodimer binds to the ChoRE (-183 bp to -144 bp from transcription starting site) of L-PK promoter. These two proteins respond to glucose induction and in turn cooperatively stimulate L-PK gene transcription. HNF-4 binds to the DR1 (-145 bp to -120 bp from transcription starting site) of L-PK promoter as a homodimer. HNF-4 is responsible for the n-3 PUFA and PPARα agonist WY14,643 inhibition of L-PK gene transcription.

To examine this hypothesis, the following aspects were studied in this thesis:

1) The dietary carbohydrate, n-3 PUFA and WY14,643 effect on the L-PK mRNA accumulation and of key transcription factors including ChREBP, Mlx and HNF-4α.

2) The time course study of L-PK expression, ChREBP, Mlx, HNF-4α nuclear protein level, as well as L-PK promoter composition changes in rat primary hepatocytes with treatments of glucose, n-3 PUFA and WY14,643.

3) The possible mechanisms that might contribute to the n-3 PUFA and WY14,643 regulation of L-PK transcription.

4) The L-PK expression in chronic diseases related to lipid and glucose metabolic disorders.

The outcome of my studies has provided a better understanding of glucose, n-3 PUFA and WY14,643 regulation of L-PK transcription. In addition, these studies have identified Mlx as a novel target for n-3 PUFA regulation.

Chapter 2

N-3 PUFA and WY14,643 Regulation of L-PK Transcription and ChREBP, Mlx, HNF-4α

2.1 Introduction

The glycolytic enzyme, liver-type pyruvate kinase (L-PK), plays a key role in hepatic glucose and lipid metabolism [8]. Phosphorylation and allosteric factors acutely regulate L-PK enzyme activity, whereas hormones and nutrients chronically control the abundance of L-PK protein by regulating L-PK gene transcription. High carbohydrate diets induce glycolysis, L-PK activity, *de novo* lipogenesis, and lipid storage, whereas n-3 polyunsaturated fatty acid (n-3 PUFA) supplemented diets inhibit these metabolic events [1, 3]. L-PK gene transcription is induced by insulin-

stimulated glucose metabolism [140] and inhibited by n-3 PUFA and activators of PPARα (WY14,643), PKA and AMPK [19, 20, 2, 141].

Key cis-regulatory elements controlling L-PK gene transcription are located between -197 and -125 bp upstream of the transcription start site (Fig. 1.1). Two basic helix-loop-helix transcription factors, ChREBP and Mlx bind as heterodimers to carbohydrate regulatory elements (ChoRE) located between -197 and -145 bp from the transcription start site. ChREBP and Mlx are required for glucose induced transcription of L-PK [25, 32, 23, 34, 22, 21, 33], as well as many other glucose responsive genes [27, 26]. The nuclear receptor, hepatic nuclear factor-4α (HNF-4α), binds an imperfect direct repeat (DR-1) between -143 and -130 bp from the transcription start site, which is adjacent to the ChoRE. Although this element is required for full glucose activation of L-PK gene transcription [20, 22], other glucose-regulated genes are not known to require HNF-4α. Thus, the role of HNF-4α in glucose-regulated gene expression appears unique to L-PK. The L-PK DR1 element is required for both n-3 PUFA and WY14,643 suppression of L-PK gene transcription [20, 2], indicating a role of HNF-4α in this regulation. Unlike WY14,643, n-3 PUFA suppression of L-PK gene transcription does not require functional PPARα [2].

In this chapter, I examined the glucose, n-3 PUFA, and WY14,643 regulation of hepatic nuclear abundance of ChREBP, Mlx, and HNF-4α *in vivo* and in cultured rat primary hepatocytes. The chromatin immunoprecipitation (ChIP) assay was used for a detailed analysis of L-PK promoter composition in response to glucose, n-3 PUFA, and WY14,643 challenge of rat primary hepatocytes. These studies have clarified

the effects of glucose, n-3 PUFA, and WY14,643 on L-PK promoter activity and composition.

2.2 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.

Feeding Studies: Male Spraque-Dawley rats (Charles River Laboratories, Kalamazoo, MI) were maintained on Harlan-Teklad laboratory chow (# 8640) and water *ad lib.* The high carbohydrate(90%) containing diets were supplemented with olive oil (10% w/w), fish oil (10% w/w), or olive (10% w/w) + WY14,643 (0.1% w/w). Rats were meal fed (8AM-12NOON) with olive oil diets for 7 days for meal training. After that, animals were separated to 3 groups on the different diets for another 7 days. On the day of the experiment, 3 of olive oil group rats were fasted overnight, the others were given meal still 8AM-12NOON. 2 hours after the meal, animals were sacrificed for recovery of blood and liver. The high carbohydrate-fat free diet (glucose replaces sucrose) was obtained from ICN Biomedicals, Inc (Aurora, OH). Fish oil is from Dyets (Bethlehem, PA), WY14,643 is from Chemsyn Laboratories, KS.

Primary Hepatocytes: Rat primary hepatocytes were prepared from male Spraque-Dawley rats fed Teklad chow *ad lib.* Rats are anesthetized by isoflurane inhalational anesthesia. The hepatic vain is cannulated with a 22-gauge cannula. The liver is first perfused with 400 mls of Buffer I (142 mM NaCl, 6.7 mM KCl, 10

mM HEPES, PH 7.4, 2.5 mM EGTA) at 42^{o}C followed by 400 mls of Buffer II (66.7 mM NaCl, 6.7 mM KCl, 100mM HEPES, 4.8 mM CaCl2) with 7mg/400ml liberase (Boehringer Mannheim, Indianapolis, IN) at 42^{o}C. Liberase is for digestion of the liver. After perfusion with Buffer II, the liver is extracted in cold Williams E (Gibco BRL of Life technology)/lactate (10 mM) media. Under the biosafety hood, primary hepatocytes are liberated and filtered through a nylon filter. The cells are centrifuged through a 40% Percoll TM (Amersham Pharmacia Biotech UK Limited) gradient twice. The cells are then suspended in Williams E lactate media with 10% fetal bovine serum, 1 μM insulin (Invitrogen, Carlsbad, CA), and 10 nM Dexamethasone, plated on BioCoat (collagen type I) plates (BD Biosciences, Bedford, MA). Cells were incubated for 4 hours before any treatment.

For the chromatin immunoprecipitation (CHIP) assay, RNA and protein extraction, cells were plated onto 100 mm type I collagen-coated plates (BD Bioscience, Bedford, MA) at 10^{7} cells/plate. For transfection studies, cells were plated in the same media onto 6-well type I 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 + 25 mM glucose, 10 nM dexamethasone, 1 μM insulin, 50 μM bovine serum albumin and no serum) in the absence and presence of 250 μM fatty acid (18:1,n-9 or 20:5,n-3) (Nu-Chek Prep, Elysian, MN) or 100 μM WY14,643 (Chemsyn Labs, KS). The bovine serum albumin (BSA) has very low endotoxin activity and is fatty acid-free (Serological Proteins, Inc., Kankakee, IL).

Reporter Plasmids: LPK-Luc was constructed by excising the -196 to +12 bp region from the LPKCAT[20] with BamHI and XhoI, subcloning into TOPOII (Invitrogen, Carlsbad, CA). After amplification, the TOPO-PK plasmid was digested with XhoI and SacI to release the L-PK promoter; the fragment was ligated into pGL3-basic vector (Promega, Madison, WI). phRG-Luc expressing Renilla luciferase was obtained from Promega (Madison, WS); phRG-Luc serves as an internal control for transfection efficiency.

Transfection & Fatty Acid Treatments, Luciferase Assay: Primary hepatocytes were plated in 6-well collagen-coated plates. Each well received 0.5 μg of reporter plasmid and 0.5 μg of receptor expression plasmid, plus Lipofectin (6.6 μl/μg DNA) or lipofectamine 2000 (1.5 μl/μg DNA) (Invitrogen, Carlsbad, CA) in serum-free Williams E-Lactate media (see above). After an overnight transfection, cells were treated with fatty acids for 24 hours in serum-free media containing BSA. After treatment, cells were lysed and assayed for luciferase activity using the dual luciferase assay kit (Promega, Madison, WI) with a dual channel Turner Luminometer. Protein concentration of the cell lysate was measured using the Bio-Rad Protein reagent (Hercules, CA) with BSA as standard. All experiments were run in triplicate and repeated at least one time.

RNA Extraction & Northern Blot, qRT-PCR: RNA was extracted from rat liver or primary hepatocytes with Trizol (Invitrogen, Carlsbad, CA). For Northern Blot, RNA was separated on denaturing (formaldehyde)agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-cDNAs [20]. Hybridization was visualized by Phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA). For quanti-

tative reverse transcriptase-polymerase chain reaction (qRT-PCR), RNA was reverse-transcribed using SuperScript II 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 7900 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative CT method (User Bulletin 2, Applied Biosystems) normalized to the abundance of cyclophilin mRNA.

Primers used for real time PCR:

L-PK:

Forward primer: 5'AGGAGTCTTCCCCTTGCTCT ;

Reverse primer: 5'ACCTGTCACCACAATCACCA.

Fatty acid synthase (FAS):

Forward primer: 5' GTGCACCCCATTGAAGGTTCC;

reverse primer: 5' GGTTTGGAATGCTGTCCAGGG.

S14 protein (S14):

Forward primer: CAAGGTGGCAGGCAATGAAG ;

reverse primer: ATGTGAGGAGGCTGGAGAAG .

Cyclophilin :

Forward Primer: TGGATGGCAAGCATGTGGTCTTTG ;

Reverse Primer: CTTCTTGCTGGTCTTGCCATTCCT.

Nuclear Protein Extraction & Western Blot: Hepatic total, cytoplasmic and nuclear proteins were extracted in the presence of protease inhibitors (P8340,

Protease Inhibitor Cocktail, Sigma, St. Louis, MO). Hepatocytes or rat livers were homogenized in Buffer A (0.25 M sucrose, 10 mM Tris-Cl, pH 7.5, 3 mM MgCl2) then centrifuged at 1500 g, 4^oC for 5 minutes. The supernatant contains cytosolic proteins. The nuclei pellet was resuspended in Buffer A, adjusted to 1% NP40 and homogenized. The homogenate was centrifuged at 3000 g, 4^oC for 5 mins. The nuclei pellet was resuspended in Buffer B (50 mM HEPES, pH 7.4, 0.1 M KCl, 3 mM MgCl2, 1 mM EDTA, 10% glycerol), incubated on ice for 10 min and centrifuged at 10,000 g. The supernatant was adjusted to 0.4 M ammonium sulfate and centrifuged at 25,000 g for 15 minutes. The nuclear proteins pellets were resuspended in NPDB (25 mM Tris Cl, pH7.5; 40mM KCl; 0.1 mM EDTA; 10% glycerol).

Cytoplasmic and nuclear proteins were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12% polyacrylamide Bis-Tris, Invitrogen, Carlsbad, CA) and transferred to nitrocellulose (BAS83, Schleicher & Schuell, Keene, NH). 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). The detection system used the Super Signal West Pico chemiluminescence kit (Pierce, Rockford, IL).

Chromatin Immunoprecipitation Assay: Rat primary hepatocytes were plated at 10^7 per 10 mm plate (type I collagen-coated) and treated with lactate, glucose, fatty acids or WY14,643. At the times indicated in the figures, cells were fixed by adding formaldehyde (1% final concentration) directly to the culture media and incubate at room temperature for 10 minutes (acetyl-histone-H4 and acetyl-histone-H3), 37^oC for 15 minutes (RNA Pol II and ChREBP) or 37^oC for 30 minutes (HNF-4α).

Cells were washed once with cold PBS containing protease inhibitors (P8340 Sigma, St. Louis), scraped in PBS, homogenized and transferred into conical tubes. Cells were pelleted and resuspended in 2 ml SDS lysis buffer (Upstate, Charlottesville, VA) with protease inhibitors (P8340 Sigma, St. Louis, MO). The cell lysate was placed on ice for 10 minutes. The lysate was sonicated with a Branson Sonifier 250 at power level 6, 60% output, 10 pulses for 10 times. Samples were kept on ice and cooled between pulses. After sonication, cell debris was removed by centrifugation and the supernatant was retained. Sonicated chromatin was aliquoted and kept in -80 ^{o}C for 1-2 months. 300 μl of sonicated chromatin was used for each immunoprecipitation (IP) reaction. The IP reaction was performed with Upstate Chromatin Immunoprecipitation assay kit (Charlottesville, VA). The acetyl-histone-H3, acetyl-histone-H4 and RNA Polymerase II antibodies were from Upstate. The HNF-4α (H-171) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The ChREBP antibody was from Novus Biologicals, Inc (Littleton, CO). 300 μl of sample was diluted into 2 ml with IP dilution buffer (Upstate); 20 μl was withdrawn for input DNA. After pre-incubation with protein A-agarose beads (Invitrogen, Carlsbad, CA) for 30 minutes and brief centrifuge, the supernatant was incubated with antibody (10 μl for Ac-H3, Ac-H4 or Pol II, 20 μg for HNF-4α, 5 μl for ChREBP) at 4 ^{o}C overnight. Antibody/protein/DNA complex was pulled down with protein A/agarose beads. The complex was washed sequentially with low salt, high salt, lithium washing buffers one time each and 2 times with 10 mM Tris-EDTA, pH 8.0. Protein/DNA complex was eluted by incubation in elution buffer (0.1 M NaHCO3, 1% SDS made freshly) at room temperature for 15 minutes twice. Supernatants were pooled and the DNA-

protein crosslinks reversed in 200 mM NaCl (final concentration) at 65 ^{o}C for 4 hours. Proteins were digested by proteinase K (Boehringer-Mannheim, Indianapolis, IN) at 45 ^{o}C for 1 hour. DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation. Glycogen was used to facilitate DNA precipitation. DNA pellets were resuspended in 50 μl sterile milli-Q water. 5 μl of the DNA samples were used as template for the polymerase chain reaction (PCR).

HNF-4 binding site on the TAT enhancer is -3062 to -3579 bp[142]. The S14 ChoRE is -1.4 and -1.35 kb. The fatty acid synthase ChoRE is -7.2 and -7.1 kb.

Primers for PCR were:

L-pyruvate kinase (L-PK) promoter between -288 and + 12 bp:

Forward: 5' AGAGATGGAGGCCTTGTGGGG;

Reverse: 5' TACGTTGCTTACCTGCTGTGT;

Tyrosine aminotransferase (TAT) enhancer between -3730 and 3431 bp,

Forward: 5': GATGAAGGTGTGTTCGTGGCA;

Reverse: 5' ACTGTGTTCTTGAAGCATCTG.

Phosphoenolpyruvate carboxykinase promoter: between -550 and + 22 bp,

Forward: 5' GATCCAGCAGACACCTAGTGG;

Reverse: 5' ATCTCAGAGCGTCTCGCC;

Fatty acid synthase (FAS) ChoRE between -7221 and -7125 kb,

Forward: 5' CTTCCTGCATGTGCCACAGGCGTGTCACCCTC;

Reverse: 5' GGAGGTTTGGCCAATGACCCCTTTGG;

S14 ChoRE, between -1485 and -1350 kb,

Forward: 5' AGCTCTCCCAGCCCTGAC

Reverse: 5' CCTGGTTGTGTAACTCCCTTTG.

S14 proximal promoter between -290 and + 16 bp,

Forward: 5' ATATGCCTGCAGTCAAGTGTACTGGGT;

Reverse: 5' ATATATCTCGAGGTGCTTCCTTTCTCAGAG;

b-actin coding region between 2383-3091 bp,

Forward: 5' TACTCCTGCTTGCTGATCCAC;

Reverse: 5' GGCTACAGCTTCACCACCAC.

Quantitation of Hepatic Fatty Acid Composition: Total lipids were extracted from liver in chloroform-methanol (2:1) plus 1 mM butylated hydroxytoluene. 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 using a YMCJ-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 min with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both ultraviolet light absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the Michigan State University mass spectrometry facility by GC-MS (www. bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for reverse-phase HPLC were obtained from Nu-Chek Prep.

Fatty Acid Metabolism in Primary Hepatocytes: Rat primary hepatocytes were treated with different fatty acids as described above. For metabolic labeling studies, the cells were treated with ^{14}C-labeled fatty acids (PerkinElmer

Life Sciences) in medium containing 250 μM fatty acid (0.5 μCi, 1.7 Ci/mol). After fatty acid treatment, the medium was collected, the cells were washed one time with phosphate-buffered saline and 40 μM BSA, washed one time with phosphate-buffered saline, and resuspended in 500 μl of 40% methanol. This washing method minimizes contamination of cellular lipids with unincorporated free fatty acids. The methanol extract of cells was acidified with HCl to 0.25 N, and lipids were extracted with chloroform:methanol (2:1) containing 1 mM butylated hydroxytoluene (BHT). The protein and aqueous phases were re-extracted with chloroform. The organic phases were pooled, dried under nitrogen, resuspended in chloroform and 1 mM BHT, and stored at -80 ^{o}C. ^{14}C-Labeled lipid extracts were further fractionated by thin layer chromatography (TLC)(LK6D Silica G 60A; Whatman) and developed in hexane:diethyl ether:acetic acid (90:30:1). The distribution of ^{14}C-fatty acids in various lipid fractions was visualized by exposure of the TLC to a phosphorimaging screen (Amersham Biosciences), and the levels of radioactivity were quantified. The location of lipids was compared with authentic standards for triacylglycerol, diacylglycerol, cholesterol ester, fatty acids, fatty acid (wax) esters, and glycerol- and sphino-phospholipids (Avanti Polar Lipids). The uptake of ^{14}C-fatty acids into cells and the organic fraction was quantified by scintillation counting. The depletion of ^{14}C-labeled fatty acids from medium was quantified by scintillation counting and TLC followed by phosphorimaging analysis as described above.

The NEFA fraction in total cellular lipids was fractionated on aminopropyl columns (Alltech Associates, Deerfield, IL). Lipid extracts in chloroform and 1 mM BHT were applied to amino-propyl columns (100 mg) and washed extensively with

chloroform:isopropanol (2:1) to remove neutral lipids. NEFA were eluted with diethyl ether and 2% acetic acid. Phospholipids were retained on the column. The diethyl ether, 2% acetic acid fraction was dried under nitrogen, resuspended in methanol and 10 μM BHT, and used directly for RP-HPLC fractionation and quantification of unsaturated fatty acids.

For RP-HPLC analysis of unsaturated fatty acids, the total extracted lipids were saponified (0.4 N KOH in 80% methanol for 1 h at 50^{o}C), neutralized, extracted in diethyl ether and 1% acetic acid, dried, and resuspended in methanol and 0.1 mM BHT for RP-HPLC analysis (reverse phase C18 column; Symmetry Shield, 2487 UV detector set to 192 nm with a 600 Controller; Waters Corp., Milford, MA). A linear gradient of 22 to 100% acetonitrile and 0.1% acetic acid over 40 min was used to fractionate unsaturated fatty acids. Verification and quantification of unsaturated fatty acids by RP-HPLC used authentic fatty acid standards (NuChek Prep) and Win-flow Radio HPLC software (IN/US Systems, Inc, Tampa, FL). The identity of specific fatty acids was verified by gas chromatography/mass spectrometry at the mass spectrometry facility at Michigan State University.

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

2.3 Results

2.3.1 Glucose, Fatty Acid and WY14,643 Effects on L-PK mRNA

Dietary regulation of L-PK mRNA.

L-PK gene transcription is induced by insulin-stimulated glucose metabolism and inhibited by n-3 PUFA and PPARα agonist [2]. In agreement with these previous studies, fasting rats 24 hours lowers L-PK mRNA. Refeeding fasted rats for 4 hours is sufficient to induce hepatic L-PK mRNA nearly 6-fold (Fig. 2.1). With rats on a high carbohydrate diet supplemented with olive oil, fish oil or olive oil + WY14,643 for 7 days, fish oil and olive oil + WY14,643 decreased liver L-PK mRNA by 50% and 70% compared to olive oil.

Lipid composition in liver.

The only difference between the olive oil and fish oil diets is in fatty acid composition. The olive oil diet contains 14% saturated fatty acid, 74% monounsaturated fatty acid, and only 8.5% PUFA. While the fish oil diet contains 26% saturated fatty acid, 21% monounsaturated fatty acid, and 43% PUFA (including 16.1% 20:5,n-3 and 11.2% 22:6,n-3) [143].

The liver total lipid analysis revealed that feeding the fish oil diet leads to over 10-fold increase in 20:5,n-3 and 2-fold increase in 22:6,n-3, compared to liver from rats on the olive oil diet. Hepatic content of saturated and monounsaturated fatty acids

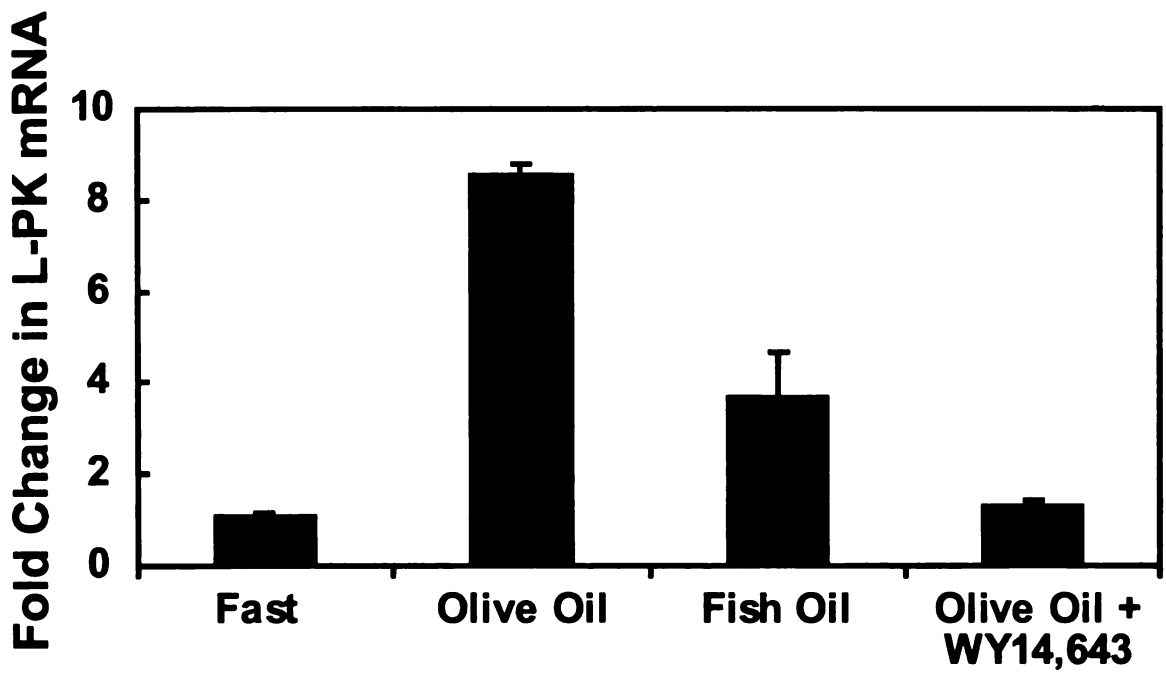


Figure 2.1: **Regulation of L-PK mRNA by high carbohydrate diet supplemented with olive oil, fish oil or olive oil + WY14,643.** Rats were fed a high carbohydrate diet supplemented with olive oil, fish oil or olive oil + WY14,643 for 7 days. On the 7th day, rats were fasted for 24 hours. One olive oil fed group was sacrificed fasted, the other groups were refed olive oil, fish oil or olive oil + WY14,643 diet for 4 hours and sacrificed. Total RNA was extracted and analyzed by qRT-PCR. Quantified results are expressed as fold change in L-PK mRNA to fasted group, mean ± SD with an n=3.

were not significantly changed by the diet (Fig. 2.2)[61]. This analysis supported the concept that the suppression of L-PK mRNA was caused by the different fatty acid component in the diet, specifically, the n-3 PUFA.

Time course of fatty acid and WY14,643 effect on L-PK mRNA accumulation.

After the examination of the dietary regulation of hepatic L-PK expression *in vivo*, rat primary hepatocytes were used to examine the time course for glucose, fatty acid and WY14,643 control of L-PK mRNA (Fig. 2.3). Primary rat hepatocytes were maintained in Williams E medium containing lactate and insulin overnight, then switched to Williams E medium containing glucose, insulin and either fatty acids (oleic acid, 18:1,n-9 or eicosapentaenoic acid, 20:5,n-3) at 250 μM or WY14,643 at 100 μM. Cells were harvested at various times afterward to examine hepatocyte abundance of L-PK mRNA (Fig. 2.3).

The treatments had no significant effect on L-PK mRNA abundance at 1.5 hours after the start of glucose treatment. By 6 hours of treatment, L-PK mRNA was induced 12-fold by glucose and glucose + 18:1,n-9 (Fig. 2.3). After 24 hours of treatment, L-PK mRNA was induced 20- to 25-fold by glucose or glucose + 18:1,n-9. Thus, 18:1,n-9 had no significant effect on the glucose induction of L-PK. Both 20:5,n-3 and WY14,643 significantly attenuate the glucose induction of L-PK mRNA at 6 hours by 50%. By 24 hours, 20:5,n-3 and WY14,643 had suppressed the glucose-stimulated accumulation of L-PK mRNA by 60% and 80%, respectively. In parallel experiments with transient transfection of L-PK-LUC in hepatocytes, similar effects

were seen with the L-PK promoter activity after 24 hours of treatment with glucose, 20:5,n-3 or WY14,643.

Previous studies established that glucose, fatty acid and WY14,643 regulated L-PK mRNA by controlling L-PK gene transcription [2]. These studies confirmed the effects of glucose, fatty acids and WY14,643 on L-PK mRNA abundance [2]. In addition, they provide key time course information on the response of the L-PK gene to glucose, fatty acid and WY14,643 challenge. In particular, glucose, fatty acids and WY14,643 had no significant effect on L-PK mRNA for at least 1.5 hours after the start of glucose stimulation.

Lipid composition in rat primary hepatocytes.

As shown in Fig. 2.2, fish oil diet leads to significant increase in 20:5,n-3 and 22:6,n-3 in rat livers, compared to liver from rats on olive oil diets [61]. To see if the time course of lipid content change correlates with the L-PK mRNA response, a fatty acid metabolism study was performed in rat primary hepatocytes. The results showed that a difference in fatty acid clearance and assimilation into the organic extracts (total cellular lipids) was observed only at the 1.5-h time point (Fig. 2.4 A)[144], when 140 nmol/mg protein of 18:1,n9 and 230 nmol/mg protein of 20:5,n-3 accumulated in cells. By 6 hours, the mass of exogenous 18:1n-9 exceeded 20:5n-3 by 15%, and by 24 h, there was no difference. This indicated that the take-up of fatty acids by primary hepatocytes is a fast event. A between 18:1,n9 and 20:5,n-3 happens within 1.5 hours.

The total cellular lipids are mostly complex lipids, including neutral lipids (cholesterol esters, triacylglycerols or diacylglycerols) and polar lipids (glycerophspho- and sphingo-lipids). The NEFA pool accounts for less than 0.5% of the total lipid (Fig. 2.4 B)[144]. When primary hepatocytes were treated with 18:1,n9, 18:1,n9 within the NEFA fraction did not change. This result means that 18:1,n9 is actively assimilated into complex lipids. However, in the 20:5,n-3 treated cells, the 20:5,n-3 in the NEFA pool increased from 0.1 nmol/mg protein to 1 nmol/mg protein within 1.5 hours and dropped to 0.5 nmol/mg protein by 24 hours. The 18:1,n9 treatment did not change the total NEFA amount, while the 20:5,n-3 treatment led to 25% increase in the total NEFA mass within 90 minutes. By 24 hours, the total mass of NEFA was back to pretreatment level.

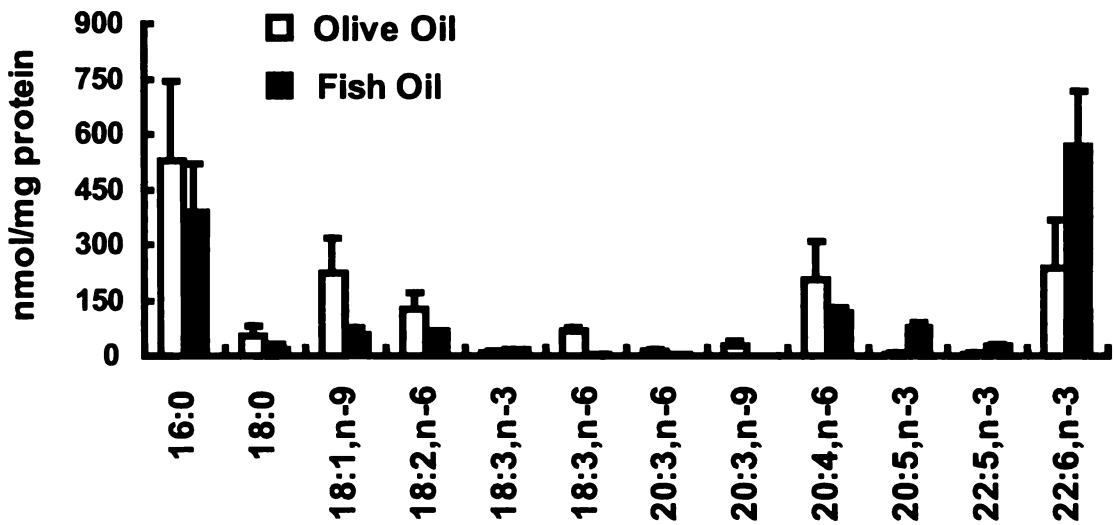


Figure 2.2: **Comparison of fatty acid content between olive oil and fish oil fed rat livers.** Male Sprague-Dawley rats were meal fed for 7 days with a high-carbohydrate diet supplemented with either olive oil or fish oil at 10% (w/w)(see Materials and Methods). Total hepatic lipid was extracted, saponified, and quantified (see Materials and Methods). Results were obtained from three separate animals per group (mean ± SD). Statistical analysis used Students t-test, and one-tailed P values were calculated (http://faculty.vassar.edu/lowry/ VassarStats.html) [61].

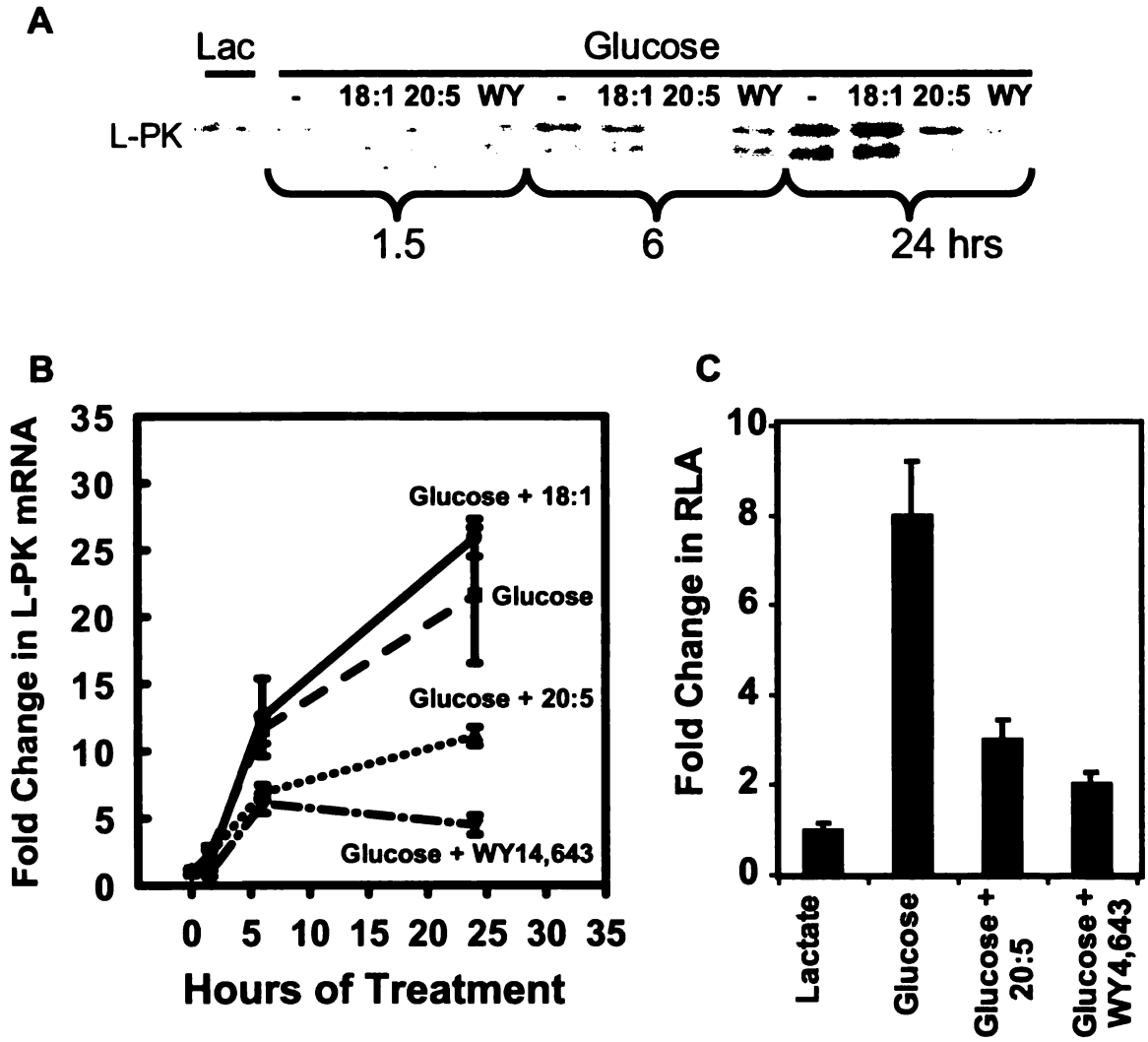
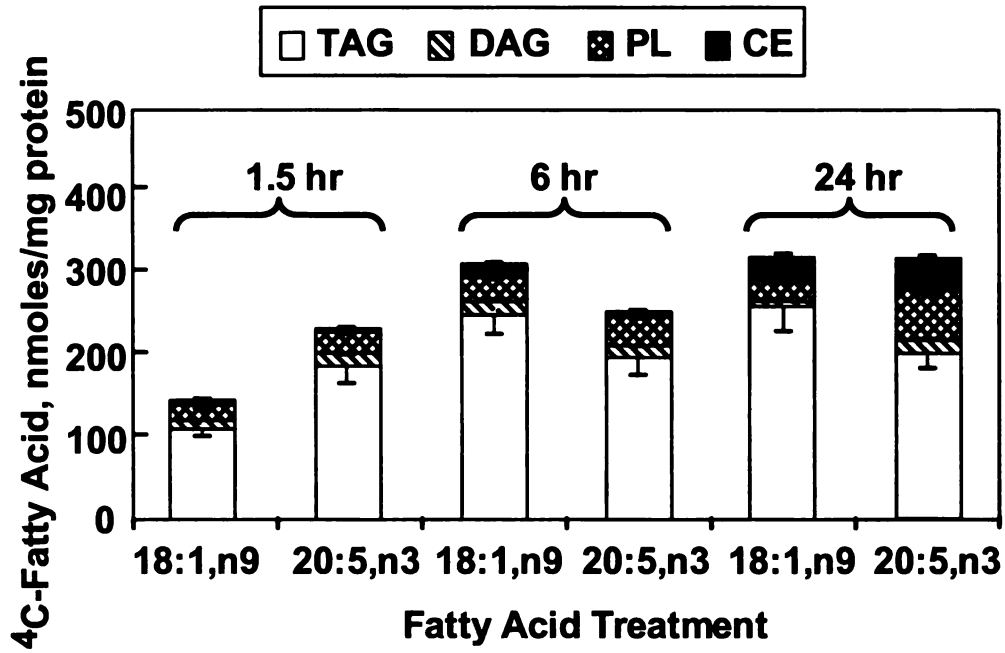


Figure 2.3: **Regulation of L-PK mRNA in primary hepatocytes by glucose, 18:1,n-9, 20:5,n-3 and WY14,643.** Rat primary hepatocytes were maintained in Williams E medium + 10 mM lactate + 1 μM insulin or switched to Williams E containing 25 mM glucose + 1 μM insulin in the absence or presence of 250 μM 18:1,n-9, 20:5,n-3 or 100 μM WY14,643. Cells were harvested at the times indicated for total RNA extraction. L-PK mRNA was detected by northern analysis [A] and quantified [B]. Quantified results are expressed as Fold Change in L-PK mRNA, mean ± SD with an n=4. [C] primary hepatocytes in lactate-containing medium were transfected with L-PK-Luc and treated as above with glucose, fatty acids, or WY14,643 for 24 hours. Cells were harvested for luciferase assays. Results are expressed as the fold change in relative luciferase activity (RLA) from lactate-treated cells, mean ± SD with an n=4.

A


TAG
DAG
PL
CE
500
400
300
200
100
0
1.5 hr
6 hr
24 hr
18:1,n9
20:5,n3
18:1,n9
20:5,n3
18:1,n9
20:5,n3
14C-Fatty Acid, nmoles/mg protein
Fatty Acid Treatment

B

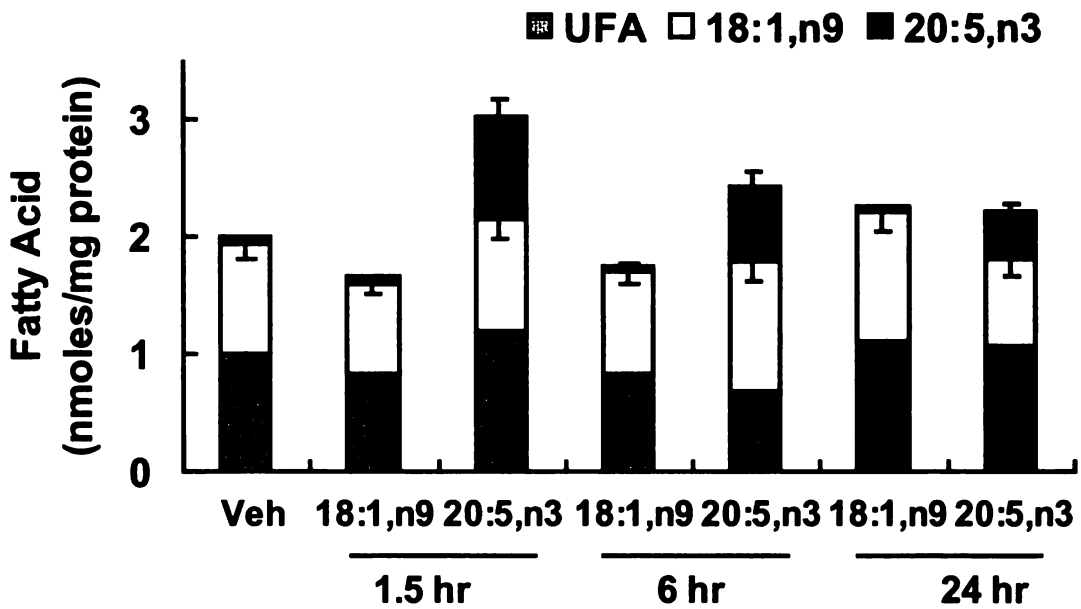

UFA
18:1,n9
20:5,n3
3
2
1
0
Fatty Acid
(nmoles/mg protein)
Veh
18:1,n9
20:5,n3
18:1,n9
20:5,n3
18:1,n9
20:5,n3
1.5 hr
6 hr
24 hr

Figure 2.4: **Time course study of ^{14}C fatty acid metabolism in primary hepatocytes.** Primary hepatocytes were prepared and treated with ^{14}C-18:1n-9 and ^{14}C-20:5n-3 (at 250 μM, 1.7 Ci/mole). At the times indicated, the cells were harvested for protein determination and lipid extraction. [A.] The lipid extracts were fractionated by TLC and developed in hexane: diethyl ether: acetic acid (90:30:1). After separation, the TLC plates were dried and exposed to a phosphorimaging screen, and the levels of radioactivity were quantified. ^{14}C-Labeled fatty acids were distributed to triacylglycerol (TAG), diacylglycerol (DAG), polar lipids (PL), and cholesterol esters (CE). The results are expressed as nmol of ^{14}C-fatty acid/mg protein; means $\pm$ S.D. from triplicate cell culture plates at each time point. [B.] Effect of 18:1n-9 and 20:5n-3 treatment on total unsaturated fatty acids in the NEFA fraction of primary hepatocytes. NEFA were quantified by first fractionating total lipids on aminopropyl columns followed by RP-HPLC. This graph illustrates changes in 18:1n-9 (white bars), 20:5n-3 (black bars), and other unsaturated fatty acids (UFA, gray bars) following treatment of cells with 18:1n-9 or 20:5n-3. The unsaturated fatty acids include 18:2n-6, 18:3n-3, 18: 3n-6, 20:4n-6, 22:4n-6, and 22:6n-3. Veh, vehicle. [144]

These studies provided time course information on the fatty acid metabolism in rat primary hepatocytes. Most of exogenous fatty acids were taken up by cells within 1.5 hours. The majority of these fatty acids were assimilated into the complex lipids. The NEFA mass is about 2 nmol/mg protein. The composition of NEFA pool was not affected by 18:1,n9 treatment, while the total mass of NEFA was increased by 20:5,n-3 treatment and the 20:5,n-3 amount in NEFA was highest by 1.5 hours treatment. The 20:5,n-3 impact on complex lipid content and NEFA pool composition starts within 1.5 hours (Fig. 2.4), which precedes the glucose-mediated accumulation of L-PK mRNA.

Having established the time course for the response of L-PK gene to glucose, fatty acid and WY14,643, I next examined the response of nuclear ChREBP, Mlx and HNF-4α protein abundance and L-PK promoter composition to the above challenges.

2.3.2 Glucose and PUFA Regulation of L-PK Transcription

Dietary regulation of rat hepatic nuclear content of ChREBP, Mlx and HNF-4α

L-PK gene transcription is induced by insulin-stimulated glucose metabolism and inhibited by n-3 PUFA and PPARα agonist [2]. The schematic in Fig.1.1 illustrates the regulatory factors controlling L-PK gene transcription, namely ChREBP, Mlx and HNF-4. I first examined hepatic nuclear ChREBP, Mlx and HNF-4 nuclear abundance in fasted and refed rats (Fig. 2.5). L-PK mRNA as well as ChREBP nuclear abundance are low in fasted rat livers. Refeeding fasted rats for 4 hours is

sufficient to induce hepatic L-PK mRNA nearly 6-fold and ChREBP and Mlx nuclear abundance nearly 4-fold and 2-fold. Nuclear level of HNF-4α remain unaffected by fasting and refeeding.

I next examined the effect of meal feeding rats a high carbohydrate diet supplemented with olive oil or fish oil for 7 days. Dietary effects on L-PK mRNA were compared to changes in hepatic abundance of ChREBP, Mlx and HNF-4 (Fig. 2.6). Changes in dietary fat composition had no effect on hepatic nuclear or cytoplasmic ChREBP or HNF-4α. Moreover, these diets had no effect on hepatic ChREBP mRNA or HNF4 mRNA (Fig. 2.7). However, the high carbohydrate diet supplemented with fish oil lowered Mlx nuclear protein levels by 50% (Fig. 2.6), without affecting Mlx mRNA abundance (Fig. 2.7). Cytoplasmic Mlx levels were higher in fish oil-fed animals when compared to olive oil fed animals (Fig. 2.6), suggesting fish oil treatment may act at a post-translational level to retain Mlx in the cytoplasm.

The above studies showed that ChREBP is responding to the fast/refeed, which lead to the increased L-PK mRNA abundance, while Mlx is responding to the fish oil diet. These results provide a possible explanation for inhibited L-PK mRNA accumulation. HNF-4 nuclear abundance is not affected by either n-3 PUFA or WY14,643. However, HNF-4 is required for nutrient control of L-PK.

Regulation of ChREBP and Mlx nuclear abundance in rat primary hepatocytes.

To see if ChREBP, Mlx and HNF-4 protein abundance change correlates with the L-PK mRNA response to glucose and 20:5,n-3, rat primary hepatocytes were used

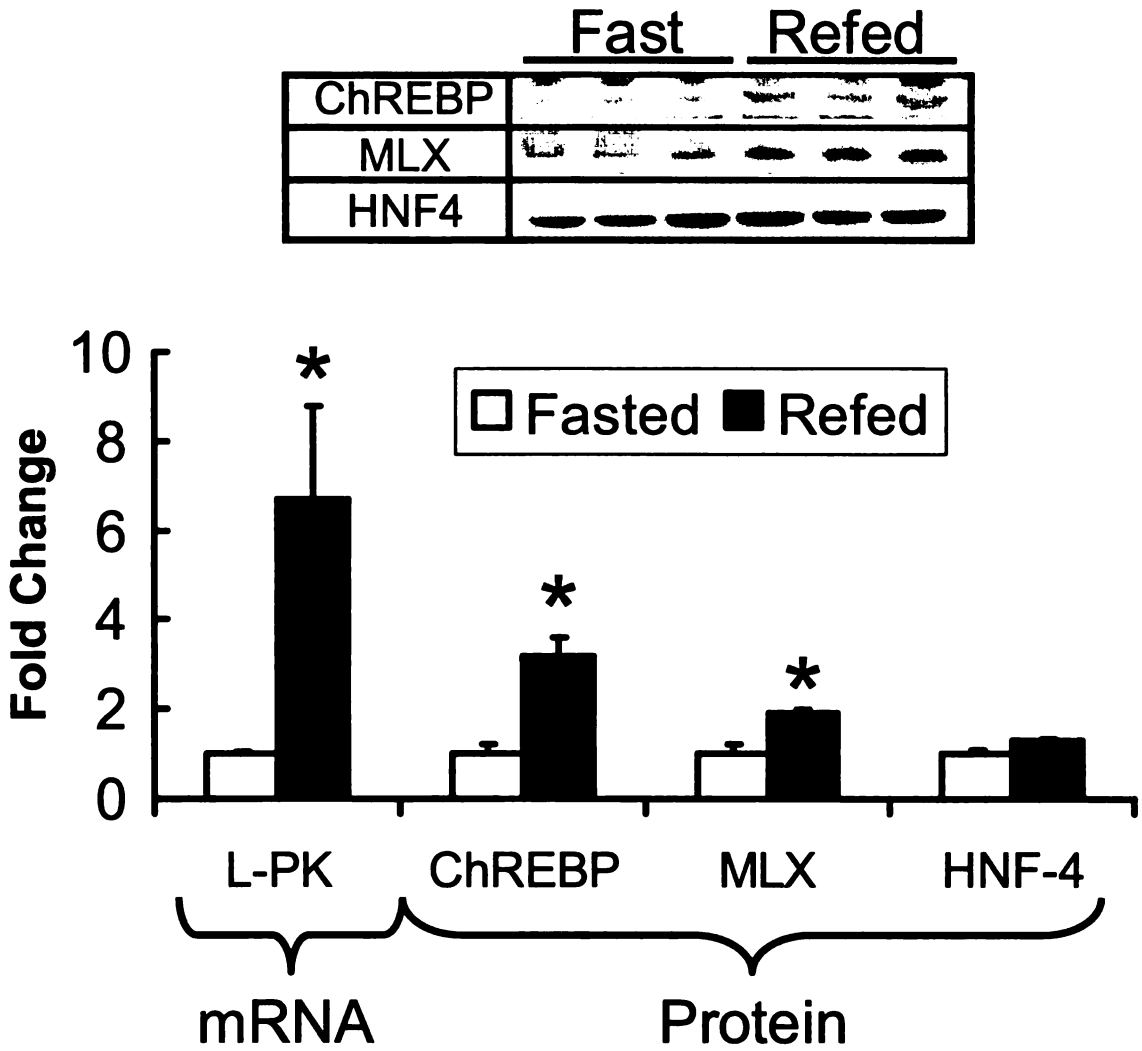


Figure 2.5: **Effect of fasting and refeeding on hepatic content of ChREBP, Mlx and HNF-4.** Rats were meal fed a high carbohydrate diet supplemented with olive oil for 7 days. Olive oil-fed rats were fasted overnight (white bars) or meal fed (black bars) the olive oil diet for 4 hours. Fasted rats were euthanized at 8 AM; fed rats were euthanized 2 hours after completion of the meal, i.e., at 2 PM. Hepatic RNA and nuclear protein extracts were prepared for qRT-PCR and Western Blot. Upper Panel: Western Blot of hepatic ChREBP, Mlx and HNF-4 abundance in liver nuclei derived from 3 separate rats. Lower Panel: L-PK mRNA was measured by qRT-PCR and quantified abundance of nuclear ChREBP, Mlx and HNF-4 measured by Western Blot. Quantified results are expressed as Fold Change from the fasted level, mean $\pm$ SD, N=3. $\star$, $p<0.05$

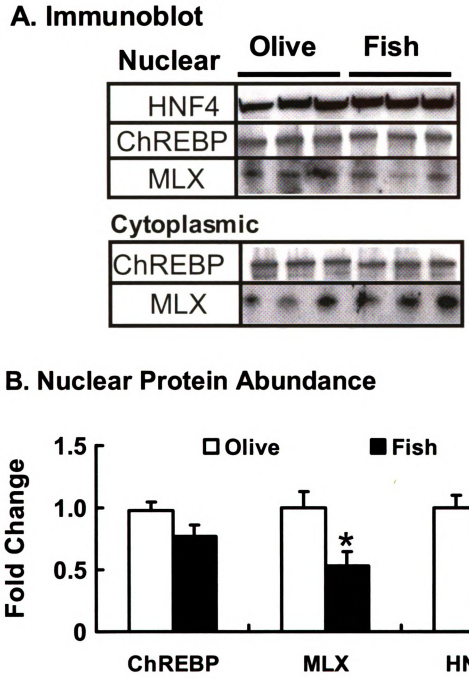


Figure 2.6: **Effect of high carbohydrate diet supplemented with olive oil or fish oil on hepatic content of ChREBP, Mlx and HNF-4.** Rats were fed high carbohydrate diet supplemented with olive oil or fish oil for 7 days. Hepatic nuclear protein extracts were prepared for immuno blotting. Panel A: Immunoblot of hepatic ChREBP, Mlx and HNF-4 in liver nuclei derived from 3 separate rats. Panel B: Quantified abundance of nuclear ChREBP, Mlx and HNF-4 measured by immunoblot. Results are expressed as Fold Change from the olive oil level, mean $\pm$ SD, N=3. $\star$, $p<0.05$

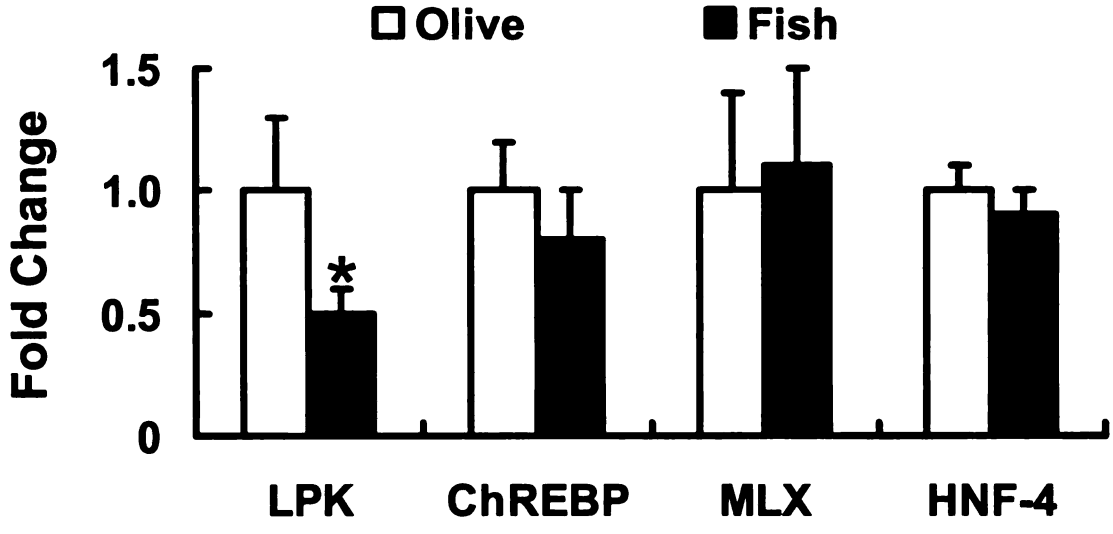


Figure 2.7: **Effect of high carbohydrate diet supplemented with olive oil or fish oil on mRNA of ChREBP, Mlx and HNF-4.** Rats were fed high carbohydrate diet supplemented with olive oil or fish oil for 7 days(Materials and Methods). Hepatic RNA were extracted and L-PK, ChREBP, Mlx and HNF-4 mRNA abundance were measured by qRT-PCR. Quantified results are expressed as Fold Change from the olive oil level, mean $\pm$ SD, N=3. $\star$, $p<0.05$

to examine the time course for glucose and fatty acid control of ChREBP and Mlx nuclear abundance (Fig. 2.8). Primary rat hepatocytes maintained in Williams E medium containing lactate (10 mM) and insulin (1 μM) overnight, were switched to Williams E medium containing glucose (25 mM) and insulin (1 μM) and 20:5,n-3 at 250 μM. Cells were harvested at the time points indicated to examine hepatocyte nuclear content of ChREBP and Mlx (Fig. 2.8).

As shown in Fig. 2.3, glucose had no significant effect on L-PK mRNA abundance at 1.5 hours after initiating glucose stimulation. By 6 hours of initiating treatment, L-PK mRNA was induced 12-fold by glucose. After 24 hours of glucose treatment L-PK mRNA was induced 20 to 25-fold by glucose. 20:5,n-3 significantly attenuated the glucose induction of L-PK mRNA at 6 hours by about 50%. By 24 hours, 20:5,n-3 had suppressed the glucose-stimulated accumulation of L-PK mRNA by 60%.

Both ChREBP and Mlx proteins were detected in nuclei of hepatocytes maintained in lactate containing medium (Fig. 2.8). Switching cells to glucose induced a robust increase in nuclear ChREBP with no change in nuclear Mlx (Fig. 2.8). By 1.5 hours, glucose had induced ChREBP nearly 5-fold; by 24 hours, ChREBP was induced 12-fold. 20:5,n-3 modestly inhibited glucose-stimulated increase in ChREBP nuclear abundance at 6 hours. Moreover, 20:5,n-3 transiently suppressed nuclear Mlx level by 70% at 1.5 hours; by 6 hours Mlx levels had nearly returned to pre-treatment levels. The effect of 20:5,n-3 on Mlx levels in hepatocytes correlates with the suppressive effect of n-3 PUFA on Mlx in liver (Fig. 2.6).

The suppression of nuclear Mlx happens at 1.5 hours after the 20:5,n-3 treatment. This is the point that 20:5,n-3 and 18:1,n9 had the biggest difference in fatty acid assimilation into hepatocytes. This is also the moment when the 20:5,n-3 abundance in the NEFA pool was the highest during the 24 hours 20:5,n-3 treatment. The difference in L-PK mRNA accumulation happens after this time point.

Regulation of L-PK promoter composition

Now we know that glucose and 20:5,n-3 can regulate ChREBP and Mlx nuclear protein abundance. We expect that ChREBP/Mlx on the L-PK promoter will also be regulated by glucose and 20:5,n-3. The same time course treatment was performed on rat primary hepatocytes to examine the L-PK promoter composition using the ChIP assay. In primary hepatocytes maintained in Williams E + insulin + lactate, when L-PK transcription rate [2] and L-PK mRNA are low (Fig. 2.3), little RNA polymerase II (RNA Pol II), acetylated histone-H4 (Ac-H4) and acetylated histone H3 (Ac-H3) is bound to the L-PK promoter (Fig. 2.9). However, both ChREBP and HNF-4 are readily detected on the L-PK promoter.

Switching primary hepatocytes from lactate to glucose-containing medium induced no change in RNA Pol II, Ac-H4, but increased Ac-H3 on the L-PK promoter at 1.5 hours. By 6 hours, RNA Pol II on the L-PK promoter increased to a maximum (3-fold) above basal values. However, by 24 hours RNA Pol II levels on the L-PK promoter returned to near pretreatment levels (Fig. 2.9). Ac-H4 on the L-PK promoter increased significantly (6-fold) by 6 hours and remained elevated (10-fold) after 24 hours of glucose treatment (Fig. 2.9). Ac-H3 abundance on the L-PK promoter was

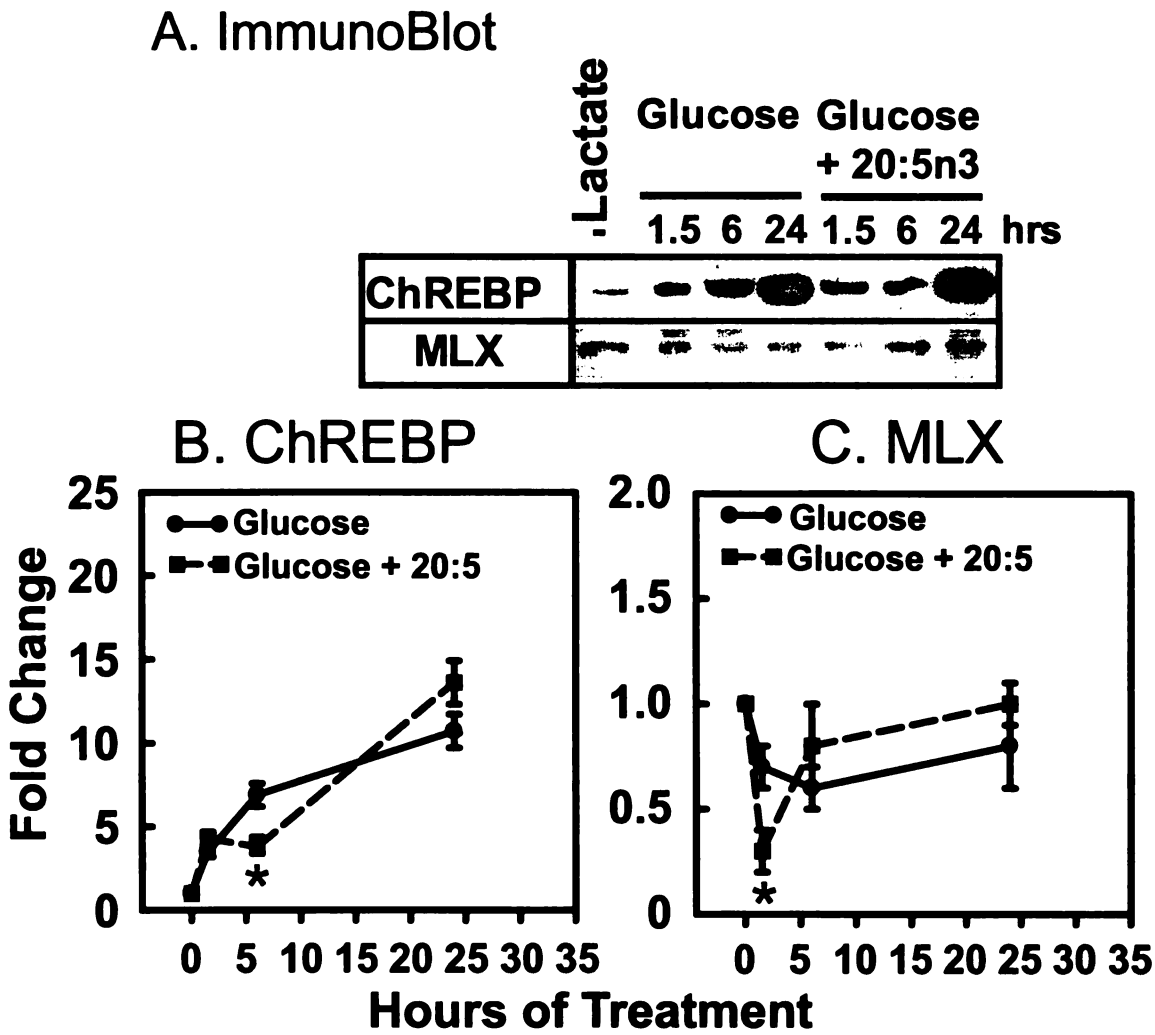


Figure 2.8: **Effect of glucose and 20:5,n-3 on the nuclear abundance of ChREBP and Mlx in rat primary hepatocytes.** Rat primary hepatocytes were maintained in lactate-containing Williams E + insulin then switched to Williams E + glucose + insulin without or with 20:5,n-3 (250 μM). Cells were harvested and nuclear proteins were extracted at the times indicated for immunoblotting [A]. Immunoblots were quantified for ChREBP [B] and Mlx [C] nuclear abundance. Results are expressed as Fold Change from lactate (0-hour) treated cells; mean $\pm$ SD, N=3. $\star$, $p<0.05$

similarly affected. The effects of glucose on Ac-H3 and Ac-H4 on the L-PK promoter were specific, since the acetylation status of these histones did not change the tyrosine aminotransferase (TAT) promoter. In contrast to RNA Pol II, Ac-H3 and Ac-H4, the interaction of ChREBP and HNF-4α with the L-PK promoter was not significantly affected by glucose treatment.

Treating cells with glucose + 20:5,n-3 inhibits glucose-stimulated L-PK gene transcription [2] and L-PK mRNA accumulation (Fig. 2.3). As expected, 20:5,n-3 attenuated recruitment of RNA Pol II to the promoter and the abundance of Ac-H3 and Ac-H4 on the L-PK promoter (Fig. 2.9, 2.10 A,B). Interestingly, 20:5,n-3 treatment had no suppressive effect on ChREBP abundance on the L-PK promoter until 24 hours after initiating treatment. By this time, both ChREBP and RNA Pol II are displaced from the L-PK promoter (Fig. 2.9, 2.10 A,C).

Negative controls were performed to examine the specificity for the ChIP assay. β-actin expression is not regulated by glucose or fatty acid. A β-actin-specific primer set (+2383 bp to +3091 bp) gave no signal with ChREBP ChIP. The S14 ChoRE is located between -1.4 and -1.35 kb. PCR amplification of S14 ChoRE gave bands, but PCR amplification of S14 promoter, which is -290 to +19 bp , did not pick up any products. These control studies gave evidence of good specificity for the ChIP assay, indicating that the consistent binding of ChREBP on the L-PK promoter is not due to the background or non-specific IP of the chromatin.

20:5,n-3 promoted a rapid (at 1.5 hours), but transient decline in HNF-4α (about 60%) on the L-PK promoter (Fig. 2.9,2.10D). The effect of 20:5,n-3 on HNF-4α in-

teraction with the L-PK promoter was specific. HNF-4 interaction with the TAT promoter remained unaffected by glucose or 20:5,n-3 (Fig. 2.9).

To this point, the following pattern has emerged. By 1.5 hours, 20:5,n-3 caused the biggest difference in complexed lipid content in hepatocytes, compared to 18:1,n9 treatment. At 1.5 hours, 20:5,n-3 abundance in the NEFA pool was the highest during the 24 hours 20:5,n-3 treatment; the suppression of nuclear Mlx happens at 1.5 hours after the 20:5,n-3 treatment. The transient decrease in HNF-4 binding to L-PK promoter was at 1.5 hours. The inhibition of L-PK mRNA accumulation was after this time point. Effects of glucose or fatty acid didn't show up until 6 hours after initiating treatment. It appears that changes in Mlx nuclear abundance correlates well with changes in the cellular non-esterified 20:5,n-3.

2.3.3 WY14,643 Regulation of L-PK Transcription

WY14,643 diet regulation of rat hepatic nuclear content of ChREBP, Mlx and HNF-4α

WY14,643, a PPARα agonist, suppresses glucose induced L-PK transcription, targeting the HNF-4 binding site on the L-PK promoter. This suppressive effect requires the presence of PPARα[2]. When I examine the effect of meal feeding rats a high carbohydrate diet supplemented with olive oil + WY14,643 compared to olive oil alone, I found that WY14,643 had no effect on hepatic nuclear content of ChREBP , Mlx or HNF-4α (Fig. 2.11). Moreover, these diets had no effect on hepatic mRNA of ChREBP , Mlx or HNF4 (Fig. 2.12).

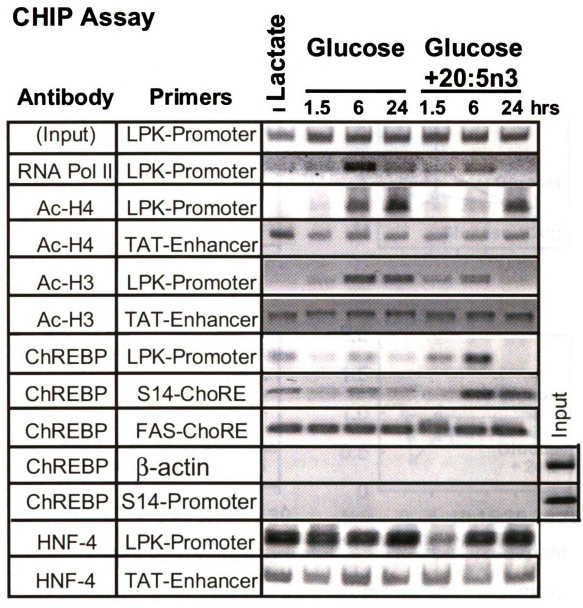


Figure 2.9: **Effect of glucose and 20:5,n-3 on L-PK promoter composition.** The CHIP assay was used to examine the composition of the rat L-PK, tyrosine aminotransferase (TAT), fatty acid synthase (FAS) and S14 promoters as described in Materials and Methods. β-actin coding region and S14 proximal promoter were used as negative control. Rat primary hepatocytes were treated as described in Fig. 2.8. Figure shows representative PCR products from the Input DNA and DNA immunoprecipitated with RNA Pol II, Ac-H3, Ac-H4, ChREBP or HNF-4α antibodies.

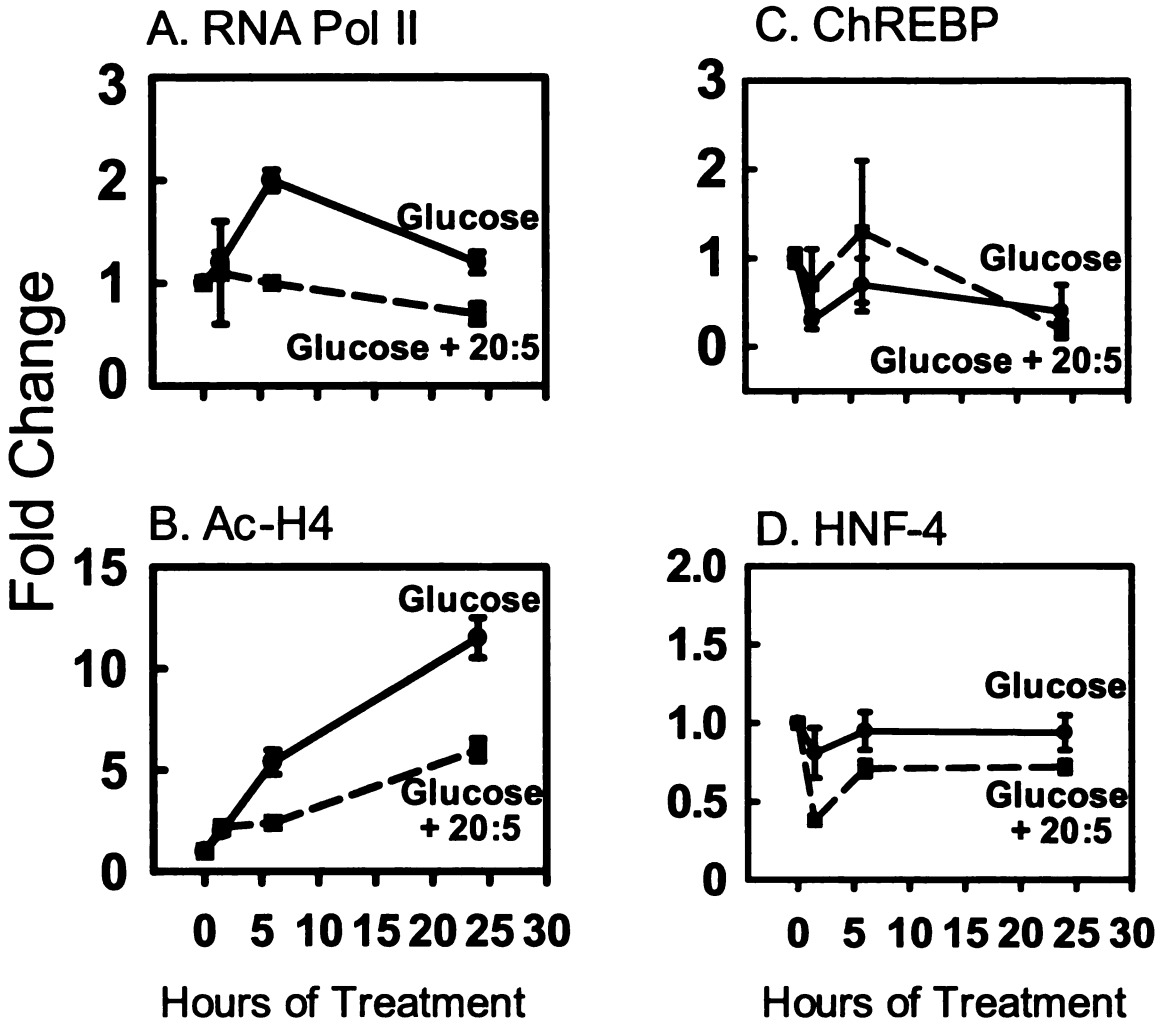


Figure 2.10: **Quantified results of glucose and 20:5,n-3 effect on L-PK promoter composition.** Results of the CHIP assay (2.9) were quantified. RNA Pol II[A], Ac-H4[B], ChREBP[C] or HNF-4α[D] binding to L-PK promoter were expressed as fold change to lactate treated samples; mean $\pm$ SD for 3 independent studies. Glucose-treated cells (Filled circles, solid line); Glucose + 20:5-treated cells (filled box, dashed line).

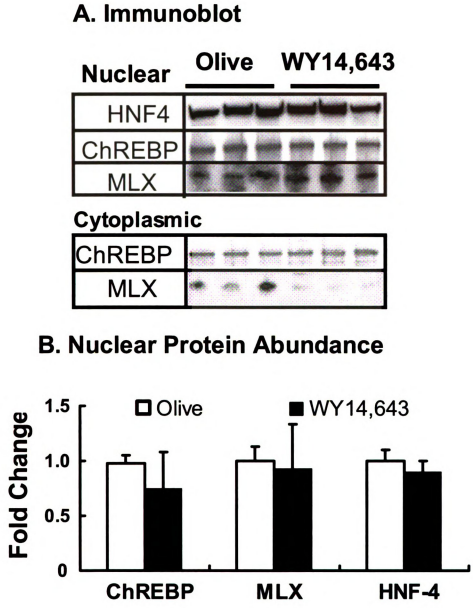


Figure 2.11: **Effect of high carbohydrate diet supplemented with olive oil or olive oil + WY14,643 on hepatic content of ChREBP, Mlx and HNF-4.** Rats were fed high carbohydrate diet supplemented with olive oil or olive oil + WY14,643 for 7 days. Hepatic nuclear protein extracts were prepared for immuno blotting. Panel A: Immunoblot of hepatic ChREBP, Mlx and HNF-4 in liver nuclei derived from 3 separate rats. Panel B: Quantified abundance of nuclear ChREBP, Mlx and HNF-4 measured by immunoblot. Quantified results are expressed as Fold Change from the olive oil level, mean $\pm$ SD, N=3.

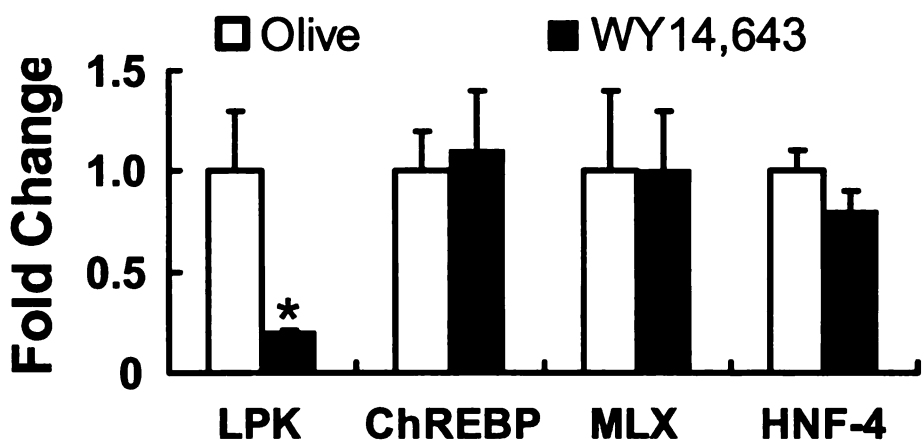


Figure 2.12: **Effect of high carbohydrate diet supplemented with olive oil or olive oil + WY14,643 on mRNA of ChREBP, Mlx and HNF-4.** Rats were fed high carbohydrate diet supplemented with olive oil or olive oil + WY14,643 for 7 days. Hepatic RNA were extracted and L-PK, ChREBP, Mlx and HNF-4 mRNA abundance were measured by qRT-PCR. Results are expressed as Fold Change from the olive oil level, mean ± SD, N=3. $\star$, $p<0.05$

WY14,643 effect on ChREBP and Mlx nuclear abundance in rat primary hepatocytes.

Time course studies were performed in rat primary hepatocytes to examine glucose and WY14,643 control of L-PK mRNA (Fig. 2.3), and ChREBP and Mlx nuclear abundance (Fig. 2.13). Cells were maintained in Williams E medium containing lactate (10 mM) and insulin (1 μM) overnight, then switched to Williams E medium containing glucose (25 mM), insulin (1 μM), without or with WY14,643 (100 μM). Cells were harvested at various times as indicated to examine hepatocyte abundance of L-PK mRNA (Fig. 2.3) and hepatocyte nuclear content of ChREBP and Mlx (Fig. 2.13).

WY14,643 significantly attenuated the glucose induction of L-PK mRNA at 6 hours by 50% (Fig. 2.3). By 24 hours, WY14,643 had suppressed the glucose-stimulated accumulation of L-PK mRNA by 80%. However, WY14,643 had no effect on nuclear ChREBP or Mlx levels (Fig. 2.13).

WY14,643 effect on L-PK promoter composition.

WY14,643 inhibits the glucose-mediated accumulation of L-PK mRNA but not nuclear ChREBP or Mlx protein. Treatment of cells with glucose + WY14,643 completely suppressed all acetylation of histone H4 on the L-PK promoter (Fig. 2.14 A,B), but had no effect on the interaction of HNF-4α with either the L-PK or TAT promoters (Fig. 2.14 A,C). Although RNA Pol II abundance on the L-PK promoter was not examined, previous studies established that WY14,643 strongly suppressed

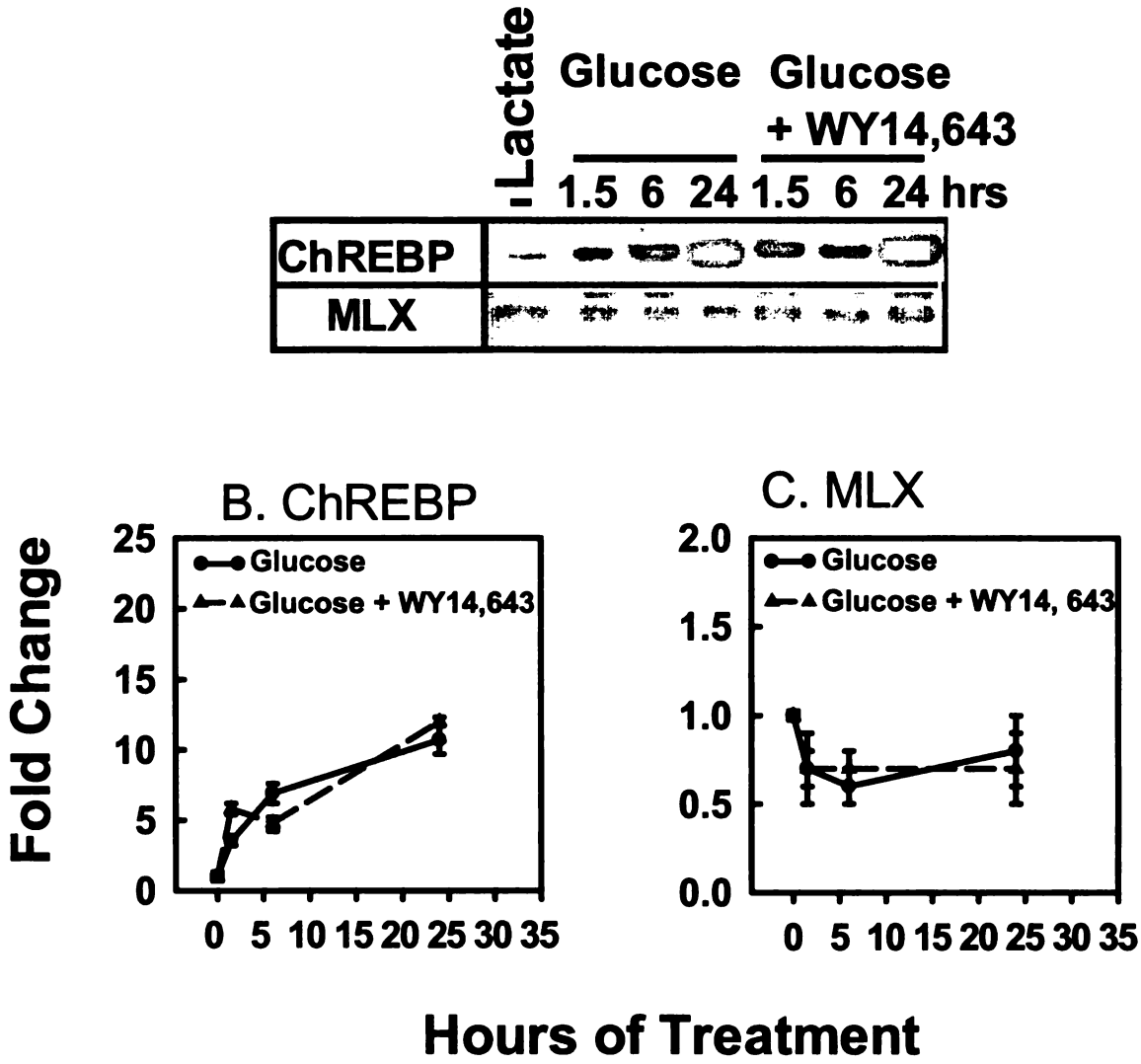


Figure 2.13: **Effect of WY14,643 on the nuclear abundance of ChREBP and Mlx in rat primary hepatocytes.** Rat primary hepatocytes were maintained in lactate-containing Williams E + insulin or switched to Williams E + glucose + insulin without or with WY14,643 (100 μM). Cells were harvested and nuclear proteins were extracted at the times indicated for immunoblotting [A]. Immunoblots were quantified for ChREBP [B] and Mlx [C] nuclear abundance. Results are expressed as Fold Change from lactate (0-hour) treated cells; mean ± SD, N=3.

L-PK gene transcription [2]. Such a profound effect of WY14,643 on L-PK histone H4 acetylation suggest that WY14,643/PPARα may affect acetyl transferase or deacetylase activity on histone H4.

2.4 Discussion

L-PK plays a central role in hepatic carbohydrate and lipid metabolism. Insulin-stimulated glucose metabolism induces L-PK gene expression, L-PK enzyme activity, and the flow of glucose metabolites toward fatty acid synthesis and storage. The above studies present new information to explain how glucose, n-3 PUFA, and WY14,643 regulate hepatic L-PK gene transcription through effects on ChREBP and Mlx nuclear abundance and L-PK promoter composition.

In vivo feeding studies confirmed previous studies showing that hepatic L-PK mRNA is induced by refeeding fasted animals, and this induction is inhibited by supplementation of diets with fish oil or WY14,643 (Fig. 2.1). Liver lipid analysis revealed that feeding rats fish oil led to significant increase in n-3 PUFA (20:5,n-3 and 22:6,n-3) in total hepatic lipid (Fig. 2.2).

Fatty acid treatment of hepatocytes showed that at 1.5 hours, the assimilation of 20:5,n-3 into complex lipid is 2-fold more than 18:1,n9. This difference diminished as time goes on (Fig. 2.4 A). The 20:5,n-3 in NEFA pool was highest also at 1.5 hours after fatty acid treatment (Fig. 2.4 B). Following the change in hepatocyte lipid content, the differences in L-PK mRNA accumulation show up by 6 hours of

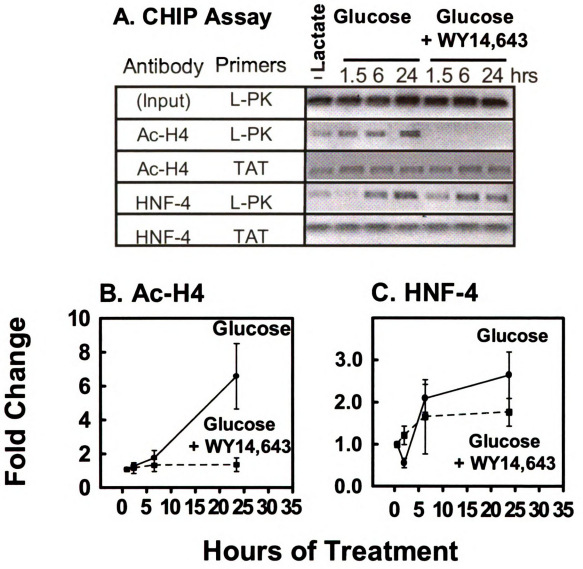


Figure 2.14: **Effect of WY14,643 on L-PK promoter composition.** CHIP assay was used to examine the composition of the rat L-PK and tyrosine aminotransferase (TAT) as described in Materials and Methods. Rat primary hepatocytes were treated as described in Fig. 2.13. Panel [A] shows representative PCR products from the Input DNA and DNA immunoprecipitated with Ac-H4, or HNF-4α antibodies. Quantified Ac-H4[B] or HNF-4α[C] binding to L-PK promoter were expressed as fold change to lactate treated samples. Glucose-treated cells (Filled circles, solid line); Glucose + WY14,643-treated cells (filled box, dashed line).

glucose, 20:5,n-3 and WY14,643 treatments, as shown in the time course studies (Fig. 2.3).

In the feeding studies, fasting and refeeding animals regulates the nuclear abundance of ChREBP in livers (Fig. 2.5), while n-3 PUFA containing diets regulate Mlx nuclear abundance in livers (Fig. 2.6). Hepatic nuclear HNF-4α was not affected by either n-3 PUFA or WY14,643. WY14,643 has no apparent effect on the nuclear abundance or mRNA levels for ChREBP, Mlx or HNF-4α (Fig. 2.11). The n-3 PUFA control of ChREBP and Mlx nuclear abundance involve post-translational mechanisms because mRNA levels of these transcription factors were not affected (Fig. 2.7). In the rat primary hepatocytes, glucose stimulated accumulation of ChREBP in nuclei (1.5 hours) precedes the glucose-stimulated rise in L-PK mRNA (6 hours). 20:5,n-3 transiently inhibited glucose stimulated nuclear ChREBP (6 hours), and suppresses Mlx nuclear abundance (at 1.5 hours). WY14,643 did not affect ChREBP, Mlx or HNF-4α abudance.

The ChIP assay with rat primary hepatocytes showed glucose induction, 20:5,n-3 and WY14,643 suppression of histone H3 and H4 acetylation as well as RNA Pol II recruitment to the L-PK promoter. These effects agree with the regulation of L-PK transcription. However, only 20:5,n-3 caused a transient decrease of HNF-4 at 1.5 hours but not ChREBP binding on the L-PK promoter. Neither glucose nor WY14,643 affected the binding of these two proteins to the L-PK promoter. The interaction of ChREBP and HNF-4α with the L-PK promoter is independent of the presence of RNA Pol II, acetylated histones H3 and H4, or the transcriptional status of the L-PK gene. We suspect that the change in complex lipid and NEFA

composition within 1.5 hours leads to relocalization of Mlx, in turn altered L-PK promoter composition and gene transcription. The fact that ChREBP/Mlx is required for glucose induction of L-PK transcription while ChREBP binding to the L-PK promoter was not affected by glucose indicates the presence of factors other than ChREBP on the L-PK promoter are also involved in glucose-mediated induction of L-PK gene expression.

The dissociation between glucose-stimulated ChREBP nuclear abundance and the lack of significant change in ChREBP occupancy on the L-PK promoter have at least two explanations. First, our measure of ChREBP and HNF-4α binding to the L-PK promoter is an artifact. Our control studies, however, validated the ChIP assay for ChREBP and HNF-4α. Second, the ChIP assay when applied to the measure of endogenous proteins on chromatin does not reveal an exchange of one protein for another; it only detects the presence or absence of a protein on chromatin. During the 90-min lag preceding RNA Pol II recruitment to the L-PK promoter, glucose stimulated a robust (5-fold) translocation of ChREBP to the nucleus. This translocation is likely stimulated by glucose metabolism and the generation of xylulose 5-phosphate, a PP2A activator. Dephosphorylated ChREBP moves to the nucleus [68, 26]. The influx of nascent ChREBP into the nucleus, coupled with its heterodimerization with Mlx, likely remodels the L-PK promoter with nascent ChREBP/Mlx heterodimers and HNF-4α homodimers. Binding of these nascent proteins to the L-PK promoter may induce recruitment of RNA pol II to the promoter and the acetylation of histones H3 and H4. Unfortunately, the differences in ChREBP abundance on the L-PK promoter over this time course did not reach significance.

Glucose-stimulated exchange of proteins on the L-PK promoter may not fully explain why L-PK promoter activity is low despite the presence of ChREBP on the promoter. It is equally possible that ChREBP heterodimerization with other basic helix-loop-helix proteins yields a complex that fails to recruit RNA pol II and the associated co-regulators required for gene activation. Although ChREBP binding to ChoRE requires Mlx [5], a role for other basic helix-loop-helix proteins forming heterodimer partners with ChREBP has not been excluded.

WY14,643 also strongly inhibits glucose-stimulated L-PK gene transcription [2], the acetylation of histone H4 on the L-PK promoter and the accumulation of L-PK mRNA. Like 20:5,n-3, WY14,643 only modestly affects glucose-stimulated ChREBP translocation into the nucleus. WY14,643 had no effect on hepatic ChREBP nuclear abundance *in vivo*. WY14,643 also had no effect on nuclear Mlx levels in liver or primary hepatocytes or the interaction of HNF-4α with the L-PK promoter.

The outcome of these studies indicate that 20:5,n-3 promotes transient changes in ChREBP and Mlx nuclear abundance and HNF-4α- LPK promoter interaction. Such transient effects of n-3 PUFA are not unusual. n-3 PUFA effects on PPARα-regulated transcripts and SREBP-1c mRNA closely parallel changes in intracellular nonesterified 20:5,n-3 [144, 61]. 20:5,n-3 is a minor fatty acid in both nonesterified and esterified lipid fractions of liver and hepatocytes [61]. At any time point examined in this study, 95% of all intracellular 20:5,n-3 is esterified. Intracellular nonesterified 20:5,n-3 is highest at 1.5 and 6 h and lowest at 24 h. The transient effects of 20:5,n-3 on ChREBP, Mlx, and HNF-4α may be sufficient to delay the onset of glucose-mediated induction of L-PK gene transcription.

During the course of our studies, Dentin et al. [71] reported that PUFA interfered with ChREBP translocation to the nucleus in mouse liver and hepatocytes. Our in vivo and primary hepatocytes studies, however, show no effect of n-3 PUFA on ChREBP nuclear abundance in vivo and only a transient effect in primary hepatocytes. Several factors can account for the different outcomes reported by Dentin et al. [71] and herein. First, rat primary hepatocytes were purified through Percoll (Amersham Biosciences), whereas Dentin et al. [71] used unfractionated mouse hepatocytes. Rats were meal-fed high carbohydrate diets supplemented with olive or fish oil for 7 days. Animals were euthanized 2 h after completion of the last meal. Dentin et al. [71] maintained mice on a high carbohydrate fat-free diet and switched the animals to a high carbohydrate diet supplemented with fatty acids; animals were euthanized 18 h later. Finally, Dentin et al. [71] did not examine hepatic Mlx levels. Animal species or experimental design may account for these differences. Our results indicate that PUFA control of ChREBP is not the sole mechanism for fatty acid regulation of L-PK. Mlx has emerged as a target for n-3 PUFA control.

Chapter 3

Possible Mechanisms Involved in the Regulation of L-PK Transcription

3.1 Introduction

ChREBP, Mlx and HNF-4α are key factors in the glucose and n-3 PUFA regulation of L-PK gene transcription. The nuclear abundance of ChREBP and Mlx is regulated by glucose and/or n-3 PUFA, while nuclear HNF-4 is not. The CHIP analysis, however, failed to provide clues as to how glucose, 20:5,n-3 or WY14,643 control RNA Pol II interaction with the L-PK promoter. More subtle mechanisms are likely involved. This chapter examines 7 mechanisms as possible explanations for the nutrient control of L-PK gene expression.

3.2 Materials And Methods

Animals and Primary Hepatocytes: 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. Feeding Studies were carried out as described in Chapter 2.

Primary Hepatocytes: Rat primary hepatocytes were prepared from male Spraque-Dawley rats fed Teklad chow *ad lib* as described in Chapter 2 Materials And Methods.

Recombinant Adenovirus: Recombinant adenovirus expressing GFP, ChREBP and Mlx-flag were obtained from H. Towle, University of Minnesota, Minneapolis, MN[28]. Adenovirus was prepared from Hek293 cell lysates. Virus-containing lysates were titered using the Adeno-X Rapid Titer Kit, Clontech. Confluent primary hepatocytes were infected (5-10 plaque forming units/cell). After infection, cells were either treated for 24 hours for RNA extraction or transfected with L-PK-Luc overnight followed by 24 hours of treatment for luciferase assay.

Reporter Plasmids: LPK-Luc was constructed by excising the -196 to +12 bp region from the LPKCAT[20] with BamHI and XhoI, subcloning into TOPOII (Invitrogen, Carlsbad, CA). After amplification, the TOPO-PK plasmid was digested with XhoI and SacI to release the L-PK promoter; the fragment was ligated into pGL3-basic vector (Promega, Madison, WI). phRG-Luc expressing Renilla luciferase was obtained from Promega (Madison, WS); phRG-Luc serves as an internal control for transfection efficiency.

The reporter plasmid TK-MH100X4-LUC contains four copies of the Gal4-binding element upstream of the thymidine kinase promoter driving luciferase expression. The pM-HNF4α1-LBD expression vector was constructed by PCR amplification of the E region, i.e. the ligand binding domain (LBD), of HNF-4α1 (forward primerAATTCATCAGCACGCGGAGGTCAACTAC; reverse primer GGGGATCCCGGCAGACCCCAAGCAGCATCTC) using pBS-HNF-4α1 (obtained from F. Sladek, University of California, Riverside, CA) as template. The HNF-4α1 LBD was ligated inframe with the Gal4 DNA binding domain of the pM vector (Clontech). Transcription of the Gal4-HNF4α fusion protein is driven by the SV40 early promoter.

Transfection & Fatty Acid Treatments, Luciferase Assay: Primary hepatocytes were plated in 6-well collagen-coated plates, transfected and treated as described in Chapter 2 Materials And Methods.

RNA Extraction & qRT-PCR: RNA was extracted from rat liver or primary hepatocytes with Trizol (Invitrogen, Carlsbad, CA). The synthesis of cDNA and real-time PCR with specific primers were described in chapter 2 Materials And Methods.

Nuclear Protein Extraction & Western Blot: Hepatic total, cytoplasmic and nuclear proteins were extracted and applied to western blot as described in Chapter 2 Materials And Methods.

Lipid Hydroperoxide Assay: Primary hepatocytes were treated in Williams E medium + 10 mM lactate + 1 μM insulin or switched to Williams E containing 25 mM glucose + 1 μM insulin in the absence or presence of 250 μM 18:1,n-9, 20:5,n-3

or 100 μM WY14,643, 100 μM H_2O_2 (Sigma). Cells were harvested at 1.5 hours for lipid hydroperoxide assay using the LPA kit (Cayman, MI).

Measurement of Reactive Oxygen Species: Rat primary hepatocytes were incubated in Lactate-containing medium overnight then changed into glucose medium in absence or presence of 250 μM 18:1,N9, 20:5,n-3, 100 μM WY14,643, or 100 μM H_2O_2. After 3 hours treatment, medium was discarded and cells were washed with prewarmed 1xPBS (37^oC). Then 10 μM H2DCF(Molecular Probes) in 1x PBS were added on the cells and incubated at 37^oC for 1 hour. The plates were read at 492-495 excitation and 517-527 emission using a Bio-Tek FL600 Microplate Fluorescence Reader.

Immunoprecipitation (IP): Rat liver nuclear protein was extracted as described in Chapter 2. In presence of protease inhibitors cocktail (Sigma), 800 μg of nuclear protein was diluted in 1 ml of IP buffer for each IP reactions. The nuclear protein was incubated with ubiquitin antibody (Cellsignaling, Inc) at 4^oC overnight. Then 50 μl protein A/agarose beads (invitrogen) were added and incubate at room temperature for 1 hour. Beads were spun down and washed with IP washing buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7Mm Tris-Cl PH 8.1, 167mM NaCl) for 3 times. Proteins were eluted with NuPAGE LDS Sample Buffer (4x) (Invitrogen) and loaded to SDS-PAGE. Separated proteins were transferred and detected with antibodies for SREBP1 (Santa Cruz), ChREBP (Novus Biologicals) or Mlx (Santa Cruz) as described in Western blot.

Affymetrix Array: RNA Samples: Rat primary hepatocytes were infected with 10 pfu/cell Ad-GFP or Ad-Mlx as described in Chapter 3. After incubation in

Lactate-containing medium for 24 hours, the cells were switched to glucose-containing medium and incubated for another 24 hours. Cells were then harvested for RNA extraction with Trizol.

cRNA labeling and hybridization: RNA from 3 experiments were pooled for reverse transcription and cRNA labeling. The reverse transcription used Invitrogen Superscript Choice System. cDNA were used as template for cRNA synthesis with BioArray High Yield RNA Transcript Labeling Kit from Enzo Life Sciences, Inc (Farmingdale, NY). Biotin-labeled cRNA were examined with Agilent chips (Agilent Technologies, Inc, Palo Alto, CA) for quality. 15 μg of each cRNA were submitted to MSU Research Technology Support Facility for hybridization with GeneChip Rat Genome 230 2.0 Array chips (Affymetrix, Inc, Santa Clara, CA).

Data Analysis: The preliminary analysis were performed with the GCOS software from Affymetrix to get the Cell files. The Cell files were analyzed using the Genesifter (www.genesifter.com) for ontology and clustering information.

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

3.3 Results

3.3.1 Mitogen Activated Protein Kinases Do Not Regulate L-PK Transcription

The mitogen-activated protein kinases (MAPKs) are a major serine/threonine kinase family. Three important members of this family are the extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). MAPKs activate numerous protein kinases, nuclear proteins, and transcription factors, leading to downstream signal transduction [145].

A few studies have suggested involvement of MAPK in the PUFA regulation network. N-3 PUFA like DHA, induce p38 MAPK phosphorylation [146, 147] and ERK phosphorylation [61]. The ERK pathway is involved in HNF-4 target gene expression by affecting HNF-4 expression and transactivation activity [148, 149]. Erk is reported to affect Apo CIII (a fatty acid regulated HNF-4 target gene) transcription by phosphorylation of HNF-4.

To examine if MAPK is also involved in the L-PK transcription, the interaction of MAPK inhibitors with fatty acid on L-PK promoter activity was examined. When challenging rat primary hepatocytes with 25 mM glucose with combination of fatty acids and/or MAPK inhibitors, neither ERK pathway inhibitor PD98059 nor p38 pathway inhibitor SB202190 changed the 20:5 inhibition of L-PK promoter activity (Fig. 3.1). The p38 inhibitor SB202190 lowered L-PK promoter activity when cells are treated with 25mM glucose, however this was not a significant difference according

to student t-test. These observations excluded the Erk and p38 pathways from the fatty acid regulation of L-PK gene transcription.

3.3.2 The Role of AMP Activated Protein Kinase (AMPK) in The Control of L-PK Gene Expression

Another kinase that might be involved in the control of L-PK transcription is AMPK. Two groups have reported that fatty acid activation of AMPK may account for fatty acid effects on gene expression [26, 150]. Once in cells, fatty acids are rapidly assimilated into complex lipids. A key event in this metabolic scheme is the conversion of fatty acids to fatty acyl-CoA, a reaction that requires ATP. Exogenous 20:5,n-3 is rapidly converted to 20:5-CoA in hepatocytes and rapidly assimilated into complex lipids, predominantly as triglycerides [144]. The fatty acid activation consumes ATP and might induce AMPK by increasing the cells requirement for energy.

AMPK is an established regulator of L-PK gene expression [26, 151]. Both ChREBP and HNF-4α are potential targets of AMPK. AMPK phosphorylation of ChREBP inhibits ChREBP translocation to the nucleus. AMPK phosphorylation of HNF-4α impairs HNF-4α dimer stability and DNA binding [102]. Two groups have reported that fatty acids activate AMPK phosphorylation [26, 150]. Since 20:5,n-3 and WY14,643 transiently antagonized (at 6 hours) glucose-mediated ChREBP translocation (Fig. 2.8,2.13)and 20:5,n-3 transiently antagonized HNF-4α interaction with the L-PK promoter (at 1.5 hours) (Fig. 2.9), I examined the effect of n-3 PUFA and WY14,643 on AMPK phosphorylation, an index of AMPK activity.

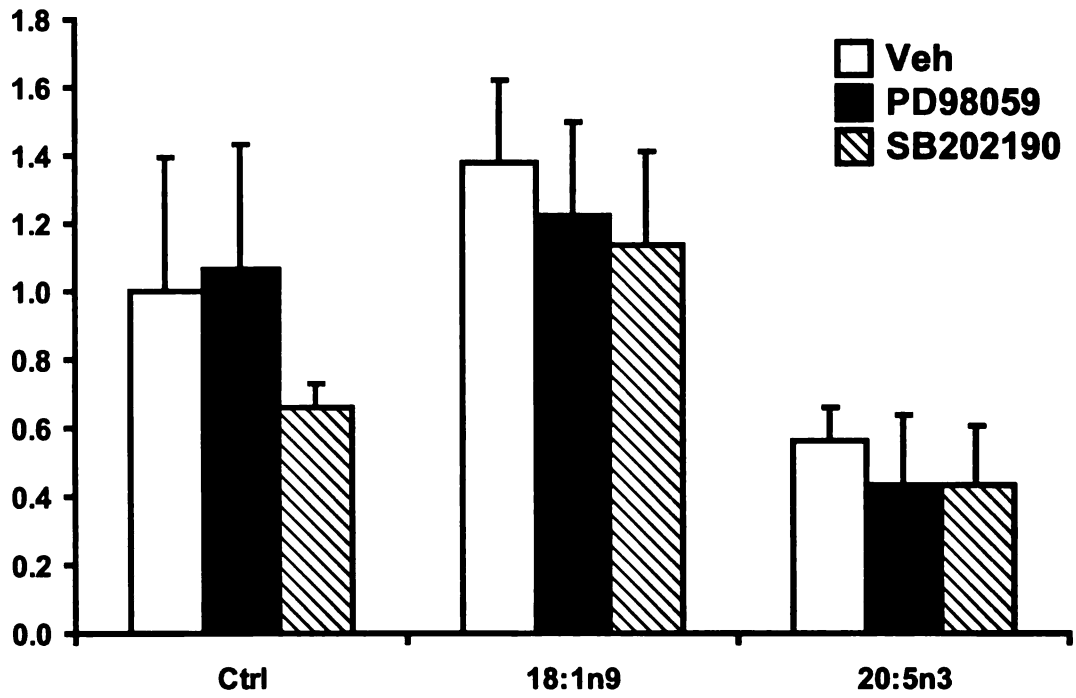


Figure 3.1: **Effect of MAPK inhibitors on L-PK promoter activity.** Primary rat hepatocytes were transfected with L-PK-CAT reporter plasmid. All cells were transfected while in Williams E medium containing lactate + 1μM insulin. Cells were switched to Williams E medium + glucose + insulin in the absence and presence of 250 μM 18:1,n-9 or 20:5,n-3, with or without 1 μM PD98059 or 1 μM SB202190. Cells were harvested 24 hours later for the CAT assay. Results are expressed as Fold Change of CAT UNIT (CAT activity/μg protein). Mean $\pm$ SD, N=6.

Metformin and 5-amino-4-imidazolecarboxamide ribotide (AICAR), two AMPK activators, strongly suppressed L-PK mRNA and L-PK promoter activity in rat primary hepatocytes (Fig. 3.2). Feeding rats n-3 PUFA or WY14,643 supplemented diets did not significantly induce phospho-AMPK (pAMPK, active form) in liver (*in vivo*)(Fig 3.3). In time course studies, glucose, 20:5,n-3 and WY14,643 did not significantly induce AMPK phosphorylation (pAMPK)(Fig. 3.4). The transient effects of n-3 PUFA and WY14,643 on ChREBP and Mlx nuclear abundance or HNF-4α interaction with the L-PK promoter can not be explained by activation of AMPK.

3.3.3 Oxidative Stress Is Not The Mechanism by Which N-3 PUFA Suppress L-PK Transcription

PUFA are susceptible to lipid peroxidation, which will increase lipid hydroperoxide levels in plasma, liver and kidney, and enhance the oxidative stress [152, 153]. Cellular oxidative stress has been related to glycolysis enzyme activity and gene expression [154, 127]. To determine if n-3 PUFA inhibits L-PK transcription by changing cellular oxidative status, antioxidant effects on L-PK promoter activity were examined.

First, lipid peroxide levels were determined in cells treated with glucose in absence or presence of 18:1,n9, 20:5,n-3, or WY14,643. 20:5,n-3 increased lipid peroxide level about 2-fold in primary hepatocytes (Fig. 3.5). To determine if 20:5,n-3 can increase cell oxidative stress, cellular reactive oxygen species (ROS) were measured in primary hepatocytes treated with glucose, fatty acids and WY14,643. Neither fatty

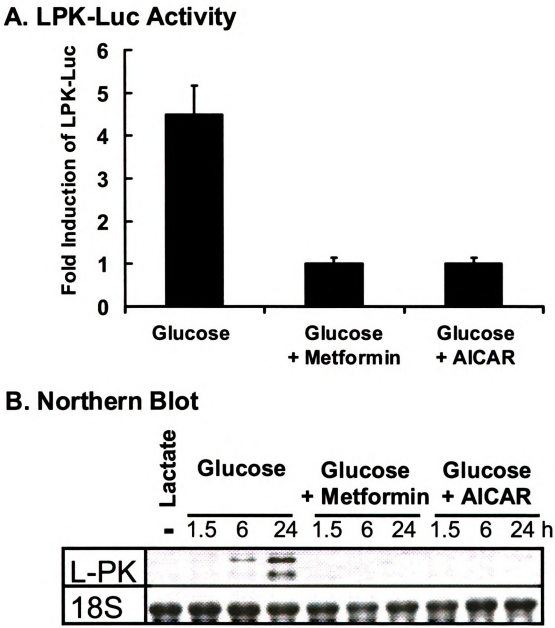


Figure 3.2: **AMPK activators suppress L-PK expression.** [A.]Rat primary hepatocytes were transfected with L -PK-Luc and phRG-Luc (Renilla Luc) while maintained in Williams E + lactate + insulin. Cells were switched to Williams E + glucose + insulin in the absence or presence of the AMPK activators, metformin (2 mM) or AICAR (2 mM). 24 hours later cells were harvested for luciferase activity. Results are expressed as fold induction of L-PK-Luc activity, mean ± SD, N=6. [B]. Non-transfected primary hepatocytes were treated as above with glucose in the absence or presence of metformin (2 mM) and AICAR (2 mM) for the times indicated. Cells were harvested for RNA extraction and northern analysis for L -PK and 18S RNA. Results are representative of 2 separate experiments.

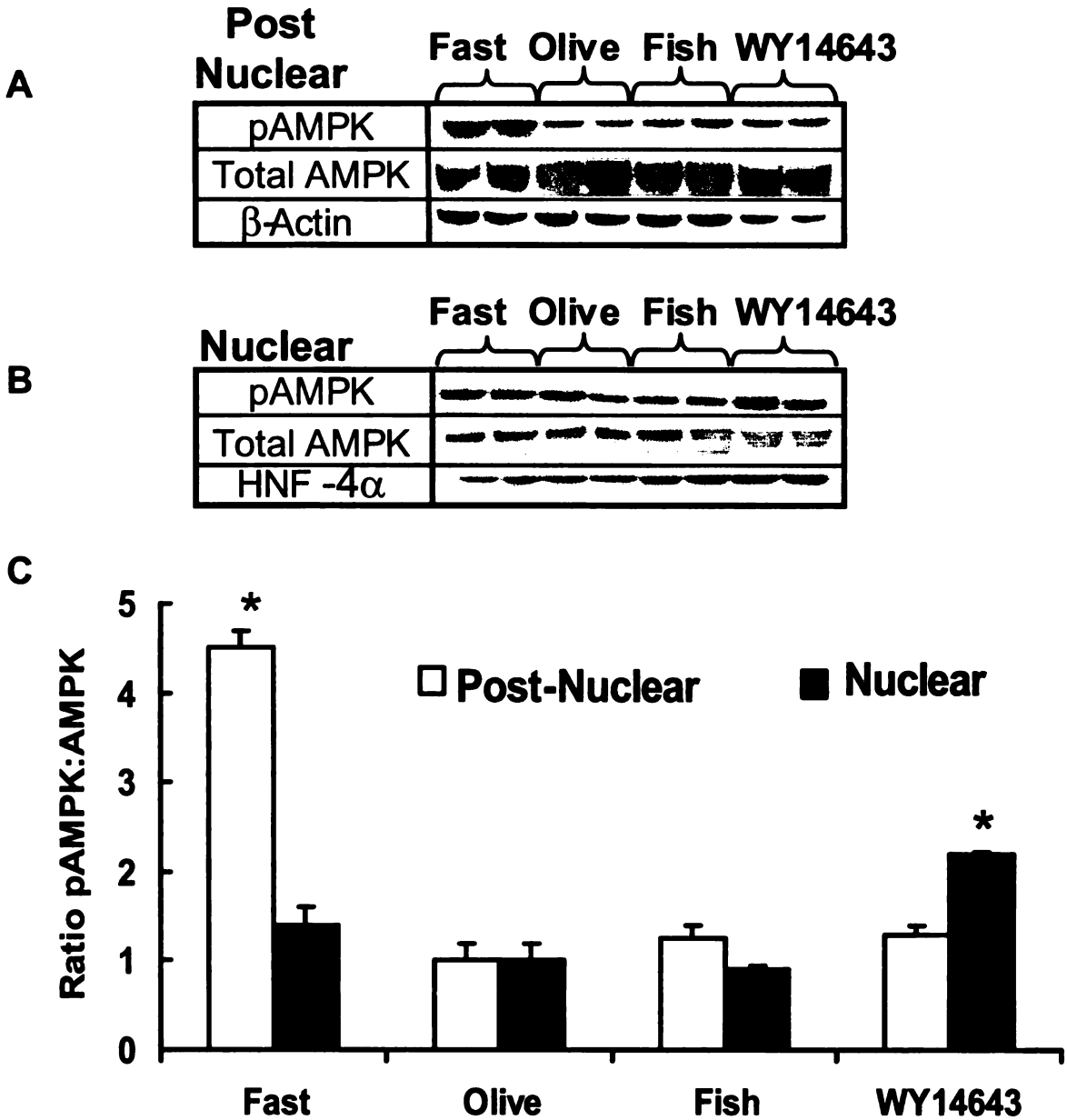


Figure 3.3: **Dietary fat does not affect AMPK phosphorylation in rat liver.** Rat liver extracts were prepared from animals meal-fed high carbohydrate diets supplemented with olive oil, fish oil or olive oil + WY14,643 for 7 days (Materials and Methods). Duplicate samples for each treatment were fractionated for immunoblotting with antibodies against total AMPK and phospho-AMPK (pAMPK). [A.]Cytosolic abundance of pAMPK, total AMPK and β-actin. [B.] Nuclear abundance of pAMPK, total AMPK and HNF-4 as control. [C.] quantified results of [A.] and [B.].

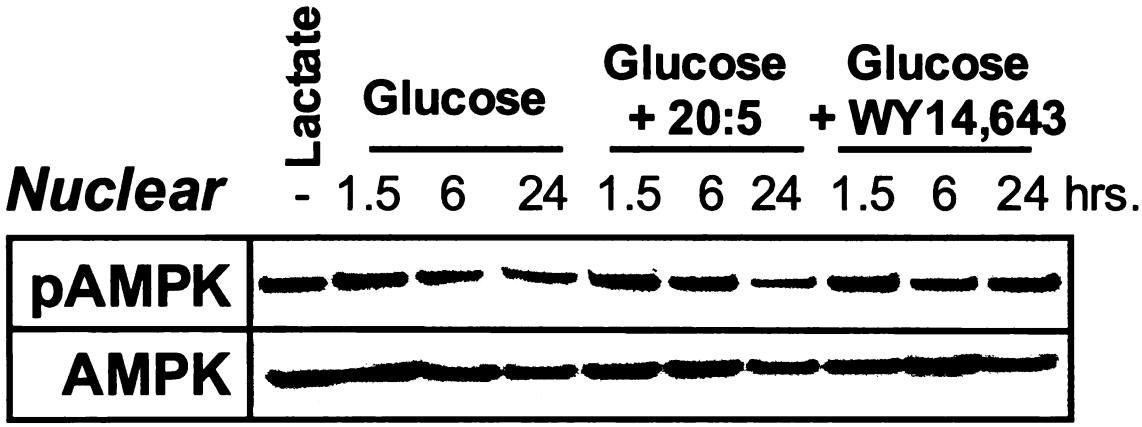


Figure 3.4: **20:5,n-3 and WY14,643 do not affect AMPK phosphorylation in primary hepatocytes.** Rat Primary hepatocytes were maintained in Williams E medium containing lactate + insulin or switched to Williams E medium containing glucose + insulin supplemented with 20:5,n-3 or WY14,643 as described before. Cells were harvested at the times indicated for isolation of nuclear proteins. Proteins were fractionated for immunoblotting with antibodies against total AMPK and phospho-AMPK (pAMPK).

acids nor WY14,643 changed cellular Reactive Oxygen Species (Fig. 3.6). Only the positive control, H_2O_2, increased cellular ROS by 2-fold.

Next, the effect of lipid peroxidation and oxidative stress on L-PK promoter activity was examined by transient transfection of L-PK-LUC into primary hepatocytes. The ion chelator desferrioxamine (DFX) is an inhibitor of ion-dependent lipid peroxidation, while Vitamin E is a lipid antioxidant [155]. Treating cells with Vit E or DFX did not change L-PK promoter activity (Fig. 3.7 black bars). However, they partially eliminated 20:5,n-3 inhibition of L-PK promoter activity. H_2O_2 also eliminated 20:5,n-3 inhibition of L-PK promoter activity (Fig. 3.7 open bars). The reason might be that fatty acids were oxidized by H_2O_2. The changing of fatty acids structure by oxidation will eliminate its effect on gene expression.

In summary, 20:5,n-3 can induce lipid peroxide accumulation in hepatocytes. Peroxidation inhibitors and antioxidants can also partially reverse the 20:5,n-3 inhibition of L-PK promoter activity. However, the cellular oxidative stress was not affected by treatment of 20:5,n-3. These observations disputed the hypothesis that 20:5,n-3 inhibition of L-PK transcription is by increasing cell oxidative stress. The effects observed with DFX and VitE may be through other unknown pathways.

3.3.4 N-3 PUFA Does Not Regulate HNF-4α Transactivation Activity

The above three studies examined cell signaling and metabolic pathways that might be involved in PUFA control of L-PK transcription. Now I focus on the factors that

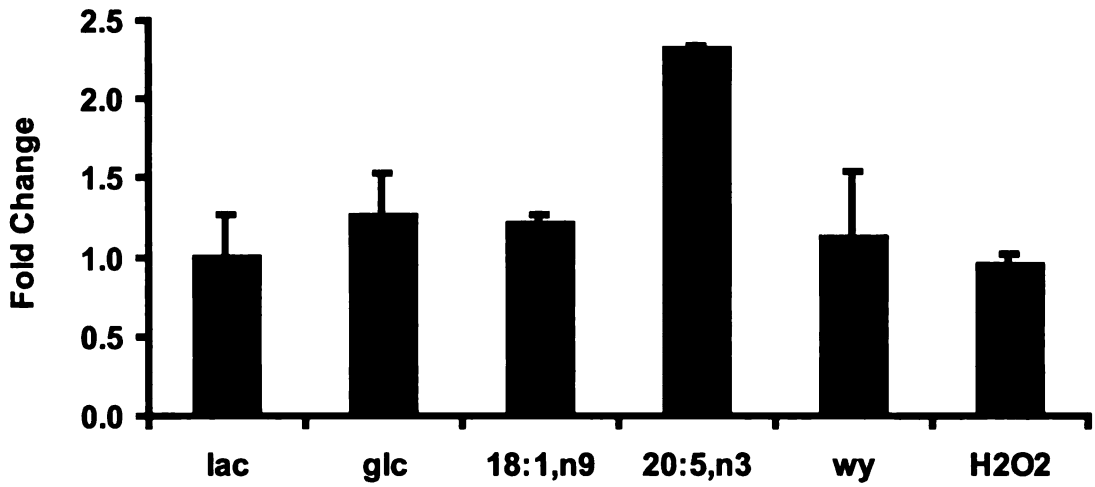


Figure 3.5: **Fatty acids and WY14,643 effect on cellular lipid hydroperoxide level.** Rat primary hepatocytes were maintained in Williams E medium + 10 mM lactate + 1 μM insulin or switched to Williams E containing 25 mM glucose + 1 μM insulin in the absence or presence of 250 μM 18:1,n-9, 20:5,n-3 or 100 μM WY14,643. Cells were harvested after 3 hours for lipid hydroperoxide assay (Cayman, MI). Results are expressed as fold change to lactate treated cells. mean $\pm$ SD, n=2.

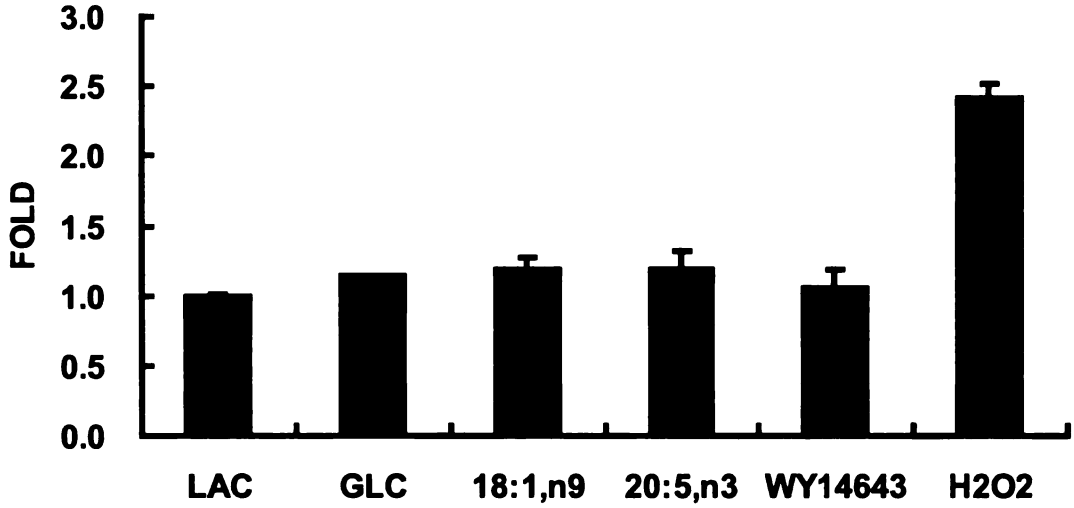


Figure 3.6: **Fatty acids and WY14,643 effect on cellular ROS.** Rat primary hepatocytes were incubated in Lactate-containing medium overnight then changed into glucose medium in absence or presence of 250 μM 18:1,N9, 20:5,n-3, 100 μM WY14,643, or 100 μM H_2O_2. After 3 hours treatment, medium was discarded and cells were washed with prewarmed 1xPBS. Then 10 μM H2DCF in 1x PBS was added on the cells and incubated at 37^{o}C for 1 hour. The plates were read at 492-495 excitation and 517-527 emission using a Bio-Tek FL600 Microplate Fluorescence Reader. Results are expressed as fold change to lactate treated cells. mean $\pm$ SD, n=2.

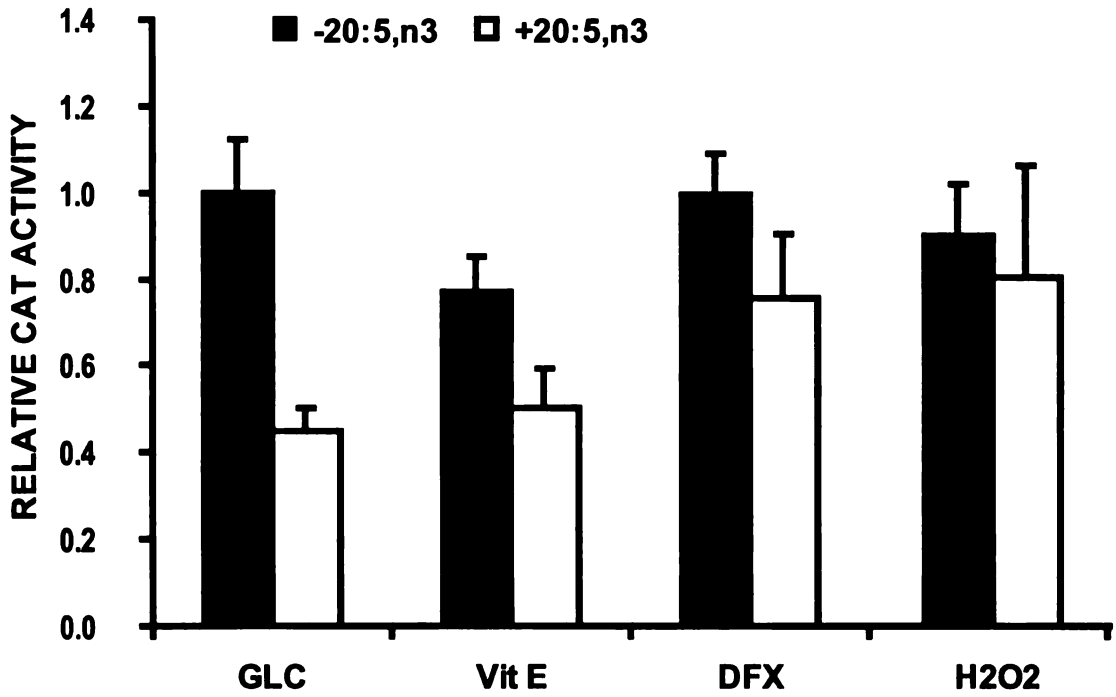


Figure 3.7: **Antioxidant effects on L-PK promoter activity.** Rat primary hepatocytes were transfected with L-PK-CAT plasmid overnight then treated with 25mM glucose, glucose + 250 μM 20:5,n-3, in absence or presence of Vitamin E (VitE), DFX and H_2O_2. After 24 hours of treatment, cells were harvested for CAT assay. Results are expressed as fold change in CAT unit (dpm/μg protein). Mean $\pm$ SD, n=6.

bind the L-PK promoter. First is HNF-4α. Treatment of primary hepatocytes with exogenous fatty acids leads to the rapid uptake of fatty acids, conversion to fatty acyl-CoA and assimilation of fatty acids into complex lipids [144]. HNF-4α and -4γ bind fatty acids and fatty acyl CoA in vitro [93, 92, 156]. HNF-4α is implicated as a target of fatty acid regulation because the DR-1 element, which binds HNF-4α, is required for the n-3 PUFA and WY14,643 suppression of L-PK gene transcription [2]. Moreover, 20:5,n-3, but not WY14,643 transiently antagonized HNF-4α interaction with the L-PK promoter (Fig. 2.9). A transfection approach was used to determine if HNF-4α1 transactivation was affected by either fatty acids or WY14,643. This study compared the effects of fatty acids (18:1,n-9 and 20:5,n-3) and WY14,643 on two reporter systems: 1) L-PK-Luc and 2) the Gal4-responsive reporter plasmid (TKMH100x4) activated by the Gal4-HNF-4α1 expression vector (Fig. 3.8). The L-PK-luciferase reporter gene contains L-PK promoter elements extending to -197 bp. Treatment of transfected hepatocytes with glucose (Vehicle) or glucose + 18:1,n-9 stimulated L-PK-Luc promoter activity 5 to 6 fold. Treatment with glucose + 20:5,n-3 or glucose + WY14,643 attenuated this response by about 80%.

Transactivation of HNF-4α1 used an expression vector containing the HNF-4α1 full length protein or HNF-4α1 ligand binding domain (E-region) fused to the Gal4-DNA binding domain to create the pM-HNF-4α1-LBD and pM-HNF-4α1-full expression vector. The TKMH100x4-Luc reporter plasmid contains Gal4 regulatory elements that bind the Gal4- HNF-4α1 fusion proteins. Co-transfection of pM-HNF-4α1 or pM-HNF-4α1-LBD with MHTK100x4-Luc induces luciferase activity 7 and 6-fold (Fig. 3.8). Transfection of hepatocytes with pM-VP16 + MHTK100x4-Luc

induced Luc activity 25-fold, while transfection of hepatocytes with the pM vector (contains only the Gal4-DBD) + MHTK100x4-Luc had no effect on luciferase activity (not shown). Primary hepatocytes transfected with pM-HNF-4α1 or pM-HNF-4 a1-LBD plus THMH100x4-Luc and treated with glucose + 18:1,n-9 or glucose + 20:5,n-3 had no effect on luciferase activity (Fig. 3.8). In contrast, WY14,643 treatment of transfected hepatocytes suppressed luciferase activity by 75%. While co-transfection of pM-VP16 + TKMH100x4-Luc induced luciferase activity 25-fold, glucose, 20:5,n-3 or WY14,643 treatment had no effect on this response. Thus, the inhibitory effect of WY14,643 on HNF-4α1 is specific and not due to a generalize effect on gene transcription or luciferase activity. These results indicate that WY14,643 interferes with HNF-4α1 transactivation. Fatty acids (18:1,n-9 or 20:5,n-3), however, have no effect on HNF- 4α1 transactivation.

3.3.5 Role of ChREBP and Mlx in n-3 PUFA control of L-PK transcription

20:5,n-3, but not WY14,643, transiently regulates HNF-4 interaction with the L-PK promoter in primary hepatocytes (Fig. 2.9). Since this effect was not due to a direct effect of 20:5,n-3 on HNF-4α1 transactivation or an indirect effect through AMPK phosphorylation, other factors must be involved. 20:5,n-3, but not WY14,643, induced a transient reduction in Mlx nuclear abundance (Fig. 2.8, 2.13). Insulin-induced glucose metabolism promotes the translocation of ChREBP from the cytosol to the nucleus (Fig. 2.8) [4, 27, 26, 68]. ChREBP and Mlx form an obli-

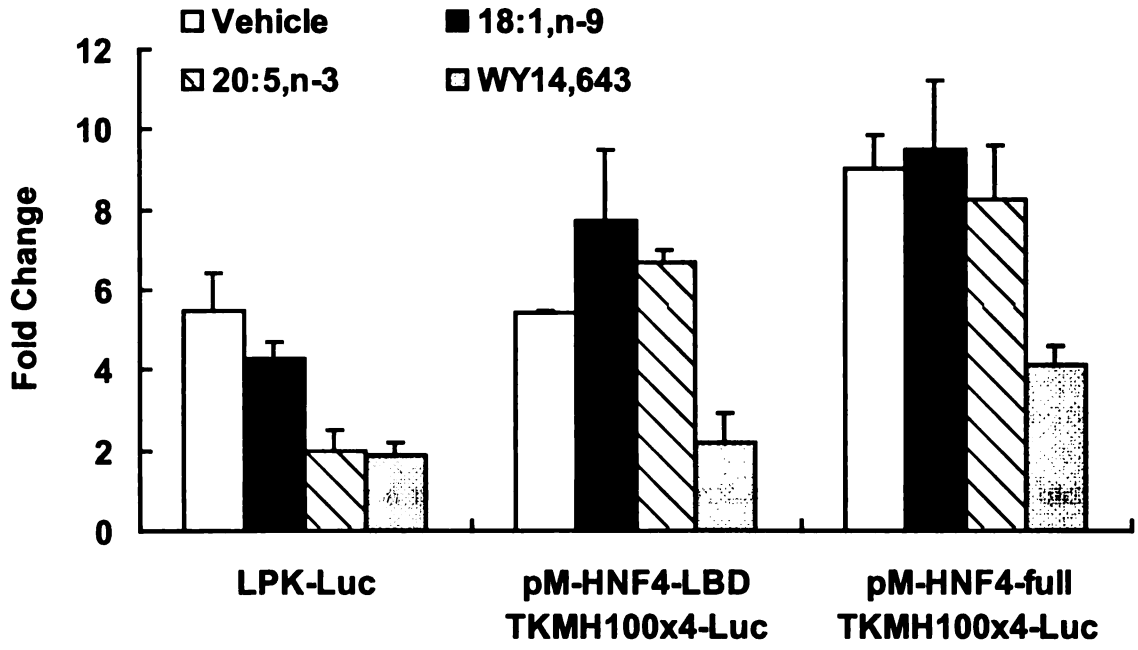


Figure 3.8: **Fatty acid effect on HNF-4 transactivation activity.** Primary rat hepatocytes were transfected with L-PK-Luc reporter plasmid or co-transfected with the TKMH100X4-Luc reporter plasmid plus the pM-HNF4α1-LBD or pM-HNF4α1-full plasmid. All transfections included phRG-Luc as an internal control. (Materials and Methods). All cells were transfected while in Williams E medium containing lactate + 1μM insulin. Cells were switched to Williams E medium + glucose + insulin in the absence and presence of 250 μM 18:1,n-9, 20:5,n-3 or 100 μM WY14,643. Cells were harvested 24 hours later for the luciferase assay. Results are expressed as-Fold Induction of RLA (Relative Luciferase Activity). L-PK-Luc activity was induced by glucose, while TKMH100x4-Luc activity was induced by co-transfection of pM-HNF-4α-LBD. Lactate and glucose did not affect TKMH100x4-Luc activity in the presence or absence of pM-HNF-4α-LBD. Mean $\pm$ SD, N=6.

gated heterodimer that binds the two E-boxes in the L-PK promoter (Fig. 1.1)[5]. ChREBP/Mlx binding to the L-PK promoter may regulate HNF-4 binding to the promoter. N-3 PUFA suppression of Mlx nuclear abundance *in vivo* (Fig. 2.6) and in primary hepatocytes (Fig. 2.8) may account for the fatty acid-mediated suppression of HNF-4-L-PK promoter interaction and glucose-stimulated L-PK gene transcription.

To test this possibility, primary hepatocytes were infected with recombinant adenoviruses expressing green fluorescent protein (GFP), ChREBP or Mlx (Fig. 3.9). In control (GFP) infected cells, glucose induced L-PK-Luc activity 5-fold, while 20:5,n-3 and WY14,643 inhibited the glucose response over 50%.

Infection of cells with a recombinant adenovirus expressing ChREBP induced L-PK-Luc activity 4-fold in hepatocytes exposed to lactate. Changing the culture medium to glucose further induced L-PK-Luc activity 4-fold. Changing the culture medium to glucose supplemented with 20:5,n-3 or WY14,643 suppressed L-PK-Luc activity by 70% and 40%, respectively. ChREBP was not able to eliminate 20:5,n-3 inhibitory effect, but partially recovered WY14,643 inhibition of L-PK promoter activity.

L-PK-Luc activity in lactate-treated hepatocytes infected with a recombinant adenovirus expressing Mlx was induced 3-fold. Glucose treatment induced L-PK-Luc activity another 5-fold. Switching cells to glucose + 20:5,n-3 induced L-PK-Luc activity 8-fold. However, overexpressed Mlx did not abrogate the WY14,643 suppression of L-PK-Luc activity.

Supporting the role of Mlx in the regulation of L-PK transcription, anti-Flag antibody was able to pull down L-PK promoter in Ad-Flag-Mlx hepatocytes in ChIP assay (Fig. 3.10), indicating Mlx binds to the L-PK promoter. Since overexpression of Mlx eliminated 20:5,n-3 inhibition of L-PK expression, I do not expect to see changes in Mlx binding to L-PK promoter with this overexpression system.

These findings indicate that the 20:5,n-3 suppression of L-PK promoter activity can be abrogated by overexpressed Mlx, but not overexpressed ChREBP. This observation coupled with the fact that 20:5,n-3 regulates Mlx nuclear abundance *in vivo* (Fig. 2.6) and in primary hepatocytes (Fig. 2.8) suggest that Mlx is a target of n-3 PUFA regulation.

3.3.6 Regulation of Mlx Nuclear Abundance

Observations in Chapter 2 revealed that nuclear Mlx protein abundance is regulated by PUFA, both *in vivo* and in primary hepatocytes. In addition, Mlx overexpression in primary hepatocytes totally eliminated the 20:5,n-3 inhibition of L-PK promoter activity (Fig. 3.9). These results suggest that Mlx is a new target for the fatty acid regulation. In this section, two possible mechanisms involved in fatty acid regulation of Mlx nuclear abundance were examined.

The n-3 PUFA control of Mlx nuclear abundance is through post-translational mechanisms, because the Mlx mRNA was not affected by fish oil diet in rat livers. The best studied post-translational regulation of Mlx is its exportation from nucleus. This process requires the binding of CRM1 and is dependant on 14-3-3 proteins [76].

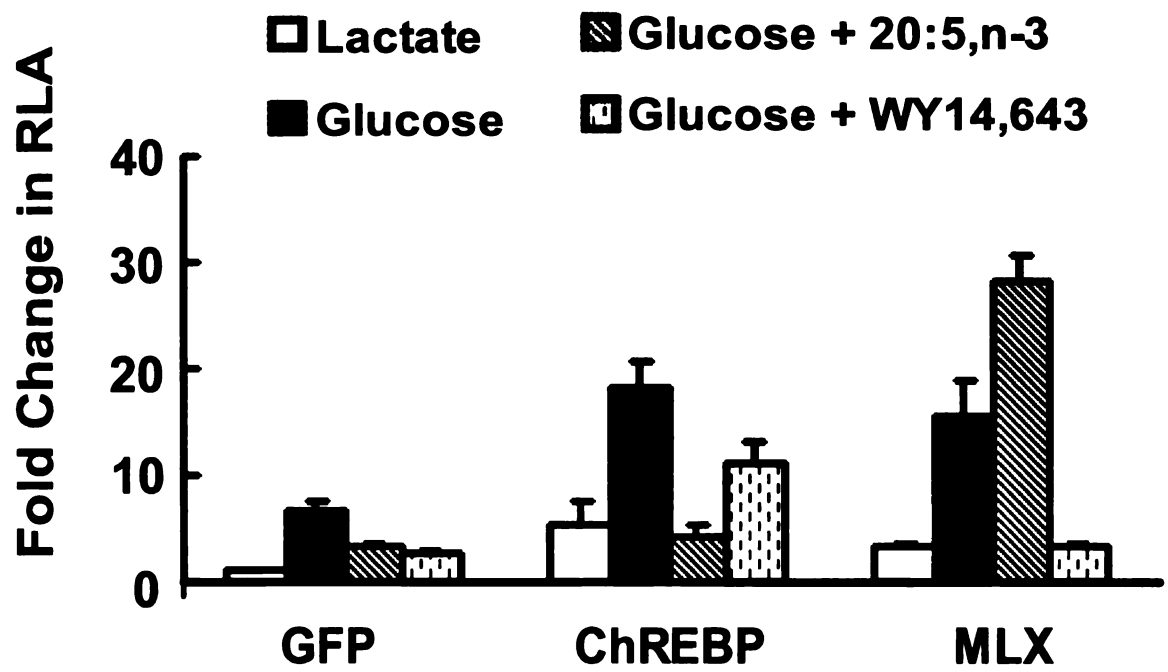


Figure 3.9: **Effect of ChREBP and Mlx overexpression on L-PK promoter activity.** Rat primary hepatocytes were infected with recombinant adenovirus expressing green fluorescent protein (GFP), ChREBP-GFP or Mlx-GFP while in Williams E medium containing lactate and insulin. 24 hours after infection, cells were transfected with LPK-Luc and phRG-Luc overnight. The next day cells were maintained in Williams E containing lactate + insulin or switched to Williams E + glucose + insulin without or with 20:5,n-3 or WY14,643. Cells were harvested 24 hours later for luciferase activity. Results are expressed as Fold Change in RLA (Relative Luciferase Activity). mean ± SD, n=3.

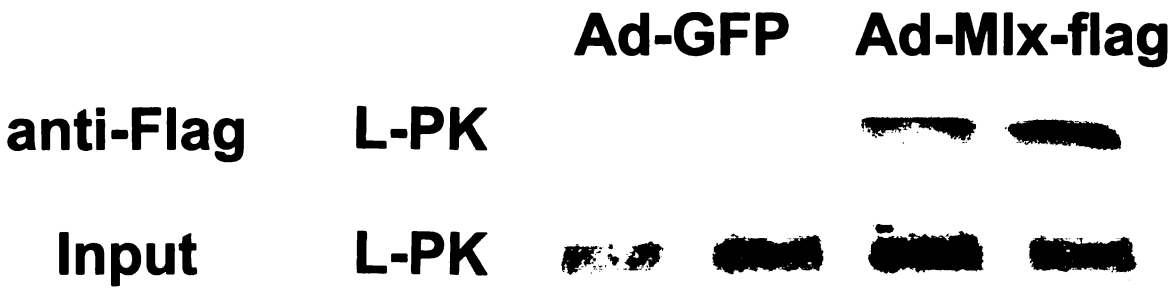


Figure 3.10: **Mlx binds to L-PK promoter.** Rat primary hepatocytes were infected with recombinant adenovirus expressing green fluorescent protein (GFP), or Flag-Mlx-GFP while in Williams E medium containing lactate and insulin. 24 hours after infection, cells changed into Williams E + glucose + insulin medium. Cells were harvested 24 hours later for ChIP assay.

Leptomycin B has been shown to inhibit this process and lead to accumulation of Mlx in nucleus [75]. When I treat rat primary hepatocytes with leptomycin B, although leptomycin B modestly increased nuclear Mlx abundance, it did not eliminate the 20:5,n-3 inhibition of nuclear Mlx protein level (Fig. 3.11 A). Neither did leptomycin B treatment affect the 20:5,n-3 inhibition of L-PK promoter activity (Fig. 3.11 B). So the n-3 PUFA regulation of Mlx nuclear abundance is through mechanisms other than the protein exportation from nucleus.

Another possible mechanism for controlling of nuclear protein abundance is through 26S proteasome mediated degradation. Ubiquitination of transcription factors and their 26 S proteasomal degradation has emerged as an important mechanism controlling promoter composition [157, 158, 159]. 26S proteasomal degradation is one of the mechanisms that regulate hepatic transcription factors nuclear content, such as SREBP1c [61]. To evaluate the role of 26S proteasome in the control of nuclear Mlx abundance and L-PK transcription, rat primary hepatocytes were treated with MG132, a 26 S proteasome inhibitor. The MG132 treatment attenuated glucose activation of L-PK gene transcription. In addition, MG132 potentiated 20:5,n-3 inhibition of L-PK mRNA accumulation (Fig. 3.12). SDS-PAGE and Western Blot showed that treating primary hepatocytes with MG132 for 1.5 hours decreased nuclear Mlx abundance, as well as 20:5,n-3 did. The combination of MG132 and 20:5,n-3 treatment essentially eliminated all nuclear Mlx. This explains why MG132 inhibited L-PK transcription and suggests 26S proteasome is involved in the control of Mlx nuclear content.

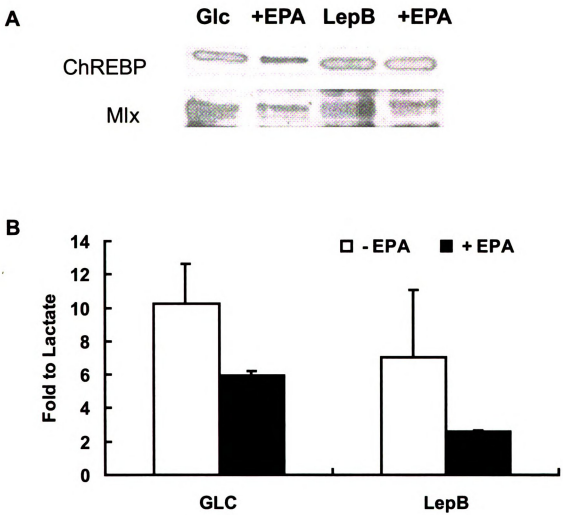


Figure 3.11: **Leptomycin B does not impact 20:5,n-3 inhibition of nMlx or L-PK promoter activity.** [A.] Rat primary hepatocytes were incubated in Lactate-containing media overnight, then changed into glucose+insulin+dex,with 250 μM 20:5,n-3, leptomycin B (1 nM) or their combination for 24 hours. Nuclear protein was extracted from cells and applied to SDS-PAGE and western blot. Figure shows the representative of 2 independent studies. [B.] Rat primary hepatocytes were transfected with L-PK-LUC overnight in lactate media, then treated with glucose+insulin+dex,with 250 μM 20:5,n-3, leptomycin B (1 nM) or their combination for 24 hours. Cells were harvested for luciferase assay. mean $\pm$ SD, n=6

To decide if Mlx is really ubiquitinated, rat liver nuclear proteins were immunoprecipiated with anti-ubiquitin antibody and applied to Western Blot. While the blot with SREBP1 gave strong signals in the ubiquitin-IP sample (Fig. 3.14 lane "Ub"), no Mlx or ChREBP was detected (Fig. 3.14 lane "Ub"). The outcome of these studies does not support the notion that Mlx or ChREBP is ubiquitinated.

3.4 Discussion

In order to understand how n-3 PUFA controls L-PK promoter composition and activity, 7 mechanisms that might be involved in PUFA control of L-PK transcription were studied.

The first two mechanisms I studied were protein kinase pathways. Insulin induces both the p38 and Erk pathways [145]. Erk phosphorylation is known to be induced by 22:6,n-3 in primary hepatocytes and regulate nSREBP abundance [61]. However, Erk or p38 inhibitors had no impact on 20:5,n-3 regulation of L-PK expression. The AMPK pathway is closely regulated by cellular energy status (AMP/ATP ratio). Fatty acids have been reported to induce AMPK phosphorylation [26, 150]. I and others [71, 160] found no evidence for fatty acid effects on AMPK phosphorylation in primary hepatocytes. Although feeding the WY14,643 supplemented diet induced nuclear pAMPK in rat livers, this was not seen during the 24 hours treatment of primary hepatocytes. The *in vivo* effect of WY14,643 on pAMPK might be due to the hepatomegaly caused by PPARα activation [161].

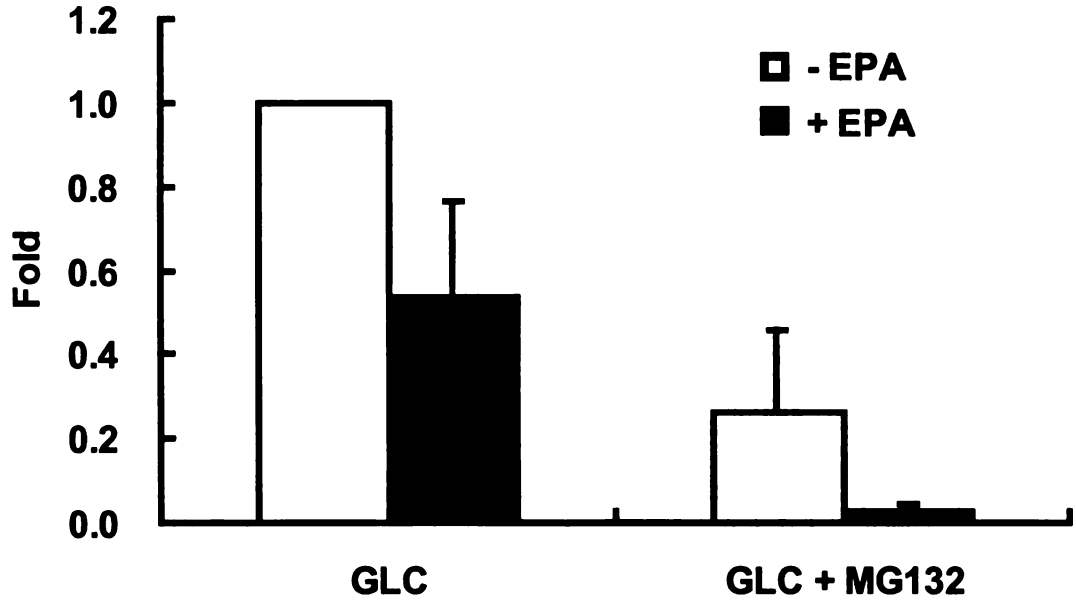


Figure 3.12: **26S proteasome inhibitor suppress L-PK expression.** Rat primary hepatocytes were incubated in Lactate-containing media overnight, then changed into glucose+insulin+dex,with 250 μM 20:5,n-3, the 26S proteasome inhibitor MG132 (10 μM) or their combination for 24 hours. Cells were harvested in Trizol for total RNA. q-RT PCR were used to measure the relative abudance of L-PK mRNA. Results are expressed as L-PK mRNA fold change to glucose treated samples. mean $\pm$ SD, n=3.

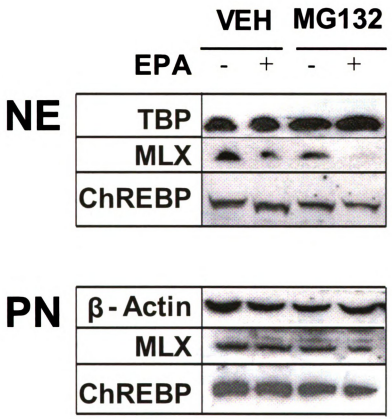


Figure 3.13: **The 26S proteasome inhibitor effect on Mlx nuclear abundance.** Rat primary hepatocytes were incubated in Lactate-containing media overnight, then changed into glucose+insulin+dex,with 250 μM 20:5,n-3, the 26S proteasome inhibitor MG132 (10 μM) or their combination for 1.5 hours. Nuclear protein (NE) and cytosolic protein (PN) were then extracted from the cells and analyzed by SDS-PAGE and Western Blot for ChREBP and Mlx. TATA binding protein (TBP) serves as nuclear protein control and β-actin as cytosolic protein control. The figure shows a representative of 2 independent studies.

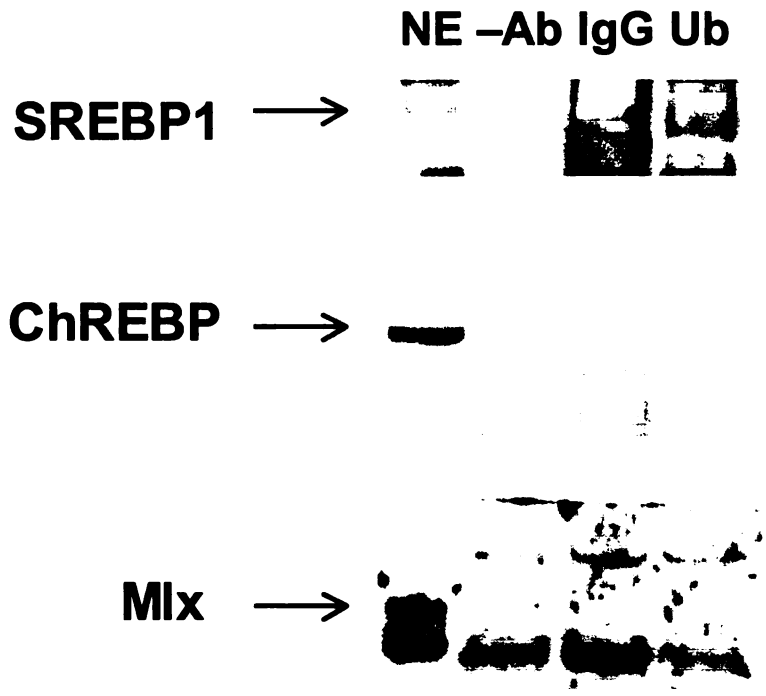


Figure 3.14: **Ubiquitination of transcription factors in rat liver nucleus.** Rat liver nuclear protein was extracted as described in materials and methods. 800 μg of nuclear protein was diluted in 1 ml of IP buffer for each IP reactions. The nuclear protein was incubated with anti-ubiquitin antibody (Ub), normal mouse IgG (IgG) or without any antibody (-Ab) at 4^{o}C overnight and pulled down by protein A/agarose. The samples were eluted and applied to SDS-PAGE and Western Blot. Rat liver nuclear protein (NE) was run together as positive control.

Another mechanism examined was cellular oxidative stress. Although PUFA such as 20:5,n-3 and 22:6,n-3 are more susceptible to peroxidation [152, 153], I did not see any difference in cellular ROS when I treat primary hepatocytes with different fatty acids. Thus the cellular oxidative stress is likely not involved in the PUFA regulation of L-PK gene transcriptional.

When we look back on the factors binding to L-PK promoter, there was no surprise that PUFA did not regulate HNF-4 transactivation activity. HNF-4 is abundantly expressed in liver and targets over 1/3 of hepatic genes [162]. L-PK is one of the few HNF-4 regulated genes affected by fatty acids. Although HNF-4α interaction with the L-PK promoter is transiently regulated by 20:5,n-3, this event could be independent of direct effects of fatty acids on HNF-4α , per se. This is supported by the absence of any effect of fatty acids on HNF-4α1 transactivation or HNF-4α interaction with other HNF-4-regulated promoters (TAT) and the fact that other HNF-4-regulated genes (TAT and PepCk) are not affected by n-3 PUFA [1, 3]. This indicated that PUFA control of gene transcription is not through a general effect on HNF-4 transactivation activity, but through other gene-specific mechanisms.

Binding upstream of HNF-4 on the L-PK promoter is ChREBP, a potential target for fatty acid regulation. My studies provided no support for PUFA regulation of its expression or nuclear abundance. Moreover, ChREBP overexpression did not abrogate the PUFA inhibition of L-PK transcription. The previously report that fatty acids control nuclear ChREBP abundance was dependant on the regulation of AMPK activity [26], was not seen by us or by Dentin et. al [71]. On the other hand, the Mlx overexpression study provided evidence for Mlx participating the PUFA regulation of

L-PK expression. Together with the PUFA effect on Mlx nuclear abundance, these observations suggested that Mlx is a novel target for PUFA regulation.

Contrary to PUFA, WY14,643 significantly antagonized HNF-4α1 transactivation. In addition, overexpressed ChREBP, but not overexpressed Mlx, partially reversed the WY14,643 suppression of L-PK promoter activity and L-PK mRNA. HNF-4α regulates many hepatic genes, but only a few are inhibited by strong PPARα agonists (WY14,643) like L-PK [2], apolipoprotein CIII [163], and Cyp7A [164]. A likely explanation for this effect is that PPARα interferes with co-regulator interaction with HNF-4α on the L-PK promoter. A test of this hypothesis will first require identifying the co-regulators that interact with the L-PK promoter. Clearly, obvious differences exist in the n-3 PUFA and WY14,643 control of L-PK transcription. These two regulators control L-PK gene transcription through different pathways.

Finally, to better understand n-3 PUFA regulation of Mlx, more studies were performed to identify the mechanism of n-3 PUFA suppress of nuclear Mlx level. n-3 PUFA suppresses SREBP-1 nuclear abundance by multiple mechanisms, including enhanced 26 S proteasomal degradation [3, 61]. I was not able to detect ubiquitination of Mlx. However, my studies with 26S proteasome inhibitor MG132 suggested that 26S proteasome is involved in the nuclear Mlx abundance regulation. There are several explanations for this observation. One is that ubiquitinated Mlx is rapidly degraded so that I could not detect it. Ubiquitin-IP of cellular proteins from MG132 treated primary hepatocytes gave similar results as above (not shown), which disputed this possibility. Another explanation is that Mlx is multi-ubiquitinated. In Fig. 3.14, the blot can detect the ligation of one or two ubiquitin to Mlx. It is possible that

more than three ubiquitin are linked to Mlx protein. More studies will be needed to test the ubiquitination status of Mlx. Clearly 26S proteasome inhibitors do not stabilize nuclear Mlx but accentuate the 20:5,n-3 mediated disappearance from nuclei. A possible explanation for this effect is that the regulation by 26S proteasome is not through direct ubiquitination and degradation of Mlx. Instead, the 26S proteasome might control the cellular abundance of other factors involved in the Mlx nuclear translocation. Mlx and MondoA export from nucleus is inhibited by Leptomycin B, resulting in their accumulation in nucleus. However, treating hepatocytes with Leptomycin B resulted in modestly increased Mlx abundance, but it did not change the 20:5,n-3 regulation of nuclear Mlx abundance or L-PK promoter activity. As such, nuclear Mlx abundance is not regulated at the level of export from nucleus. On the contrary, I think it is through a nuclear import mechanism. A hypothetical model for the Mlx translocation in a 26S proteasome dependent manner is illustrated in Fig 3.15.

ChREBP, the Mlx heterodimer partner, translocates into nucleus by induction of glucose. Metabolism of glucose through the pentose pathway is required for glucose- regulated gene transcription [165]. Glucose-derived metabolites, i.e. xylulose 5-phosphate, induce dephosphorylation of ChREBP by activating the phosphatase PP2A [27, 68]. Dephosphorylated ChREBP moves to the nucleus where ChREBP/Mlx heterodimers bind ChoREs in target genes [140, 69]. Phosphorylation of ChREBP by AMPK or PKA inhibits ChREBP nuclear translocation, thus suppressing transcription of L-PK and other glucose-regulated genes [27, 26]. Mlx movement into the nucleus is not known to be controlled by glucose. However, recent

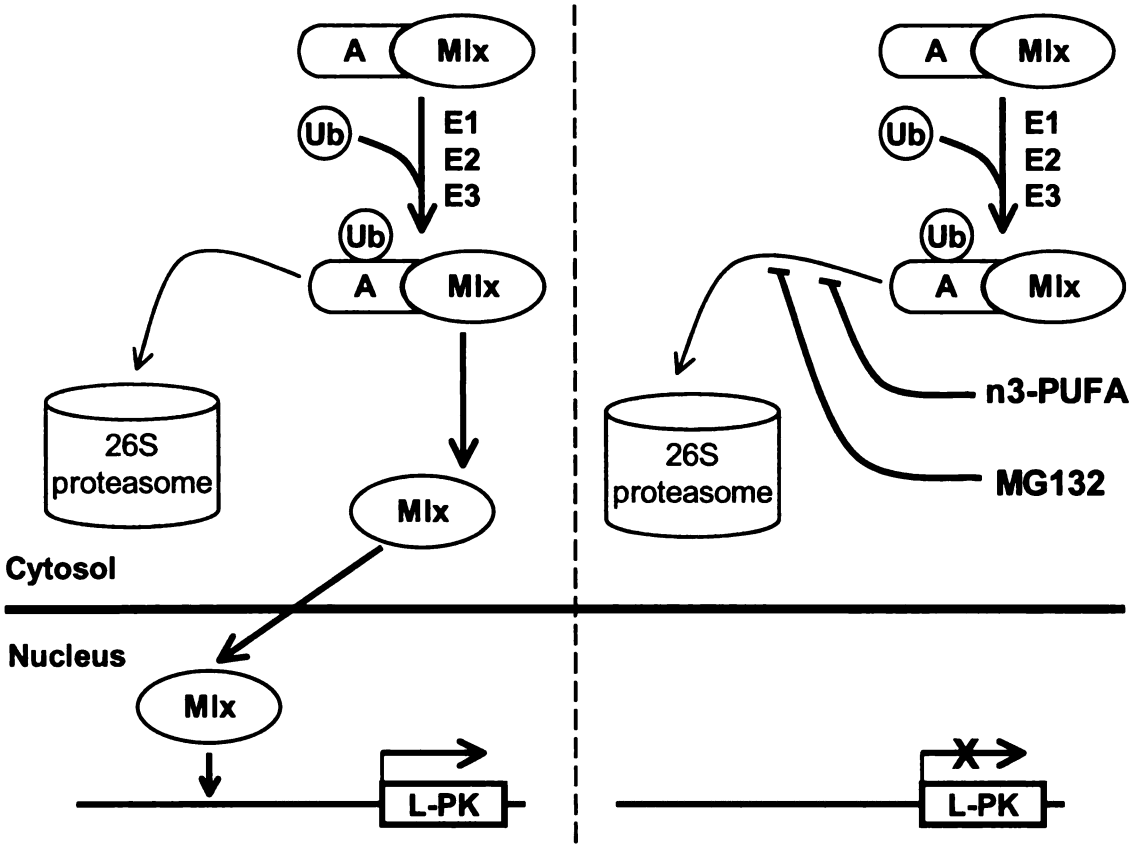


Figure 3.15: **A Model of Mlx translocation.** The diagram illustrates a hypothetical model for the Mlx nuclear translocation in a 26S proteasome dependent manner. A protein "A" acts as the Mlx partner in cytosol. Its binding with Mlx prevents Mlx from getting into nucleus. Under regular conditions (left), A is ubiquitinated and subject to 26S proteasome degradation, which releases the Mlx to enter the nucleus, dimerize with ChREBP (not shown in figure) and promote its target genes transcription, such as L-PK. In the presence of inhibitors such as n-3 PUFA and MG132 (right), A is not degraded and keeps binding to Mlx. This prevents Mlx from entering nucleus. Transcription of Mlx targets, such as L-PK, is inhibited. To examine this hypothesis, the "A" protein which serves as the Mlx partner in cytosol need to be identified first.

reports indicated the Mlx mitochondria localization is regulated by cell energy status by colocalization with MondoA in muscle cells [77]. This gives a hint for the ability of MondoA/Mlx to sense cell energy status for relocalization.

In summary, the studies in this chapter excluded MAPK, AMPK, oxidative stress and HNF-4α transactivation as mechanisms for n-3 PUFA inhibition of L-PK transcription. On the contrary, they confirmed the involvement of Mlx in n-3 PUFA regulation of L-PK transcription. 26 S proteasome pathway, but not the nuclear export pathway, is involved in the n-3 PUFA suppression of Mlx nuclear abundance.

Chapter 4

The Role of ChREBP/Mlx in Chronic Diseases

4.1 Introduction

Observations in Chapter 2 and 3 revealed that nuclear Mlx protein abundance is regulated by PUFA, both *in vivo* and in primary hepatocytes. In addition, Mlx overexpression in primary hepatocytes totally eliminated the 20:5,n-3 inhibition of L-PK promoter activity (Chapter 3). These results suggested that Mlx is a new target for the fatty acid regulation. In addition, the ChREBP/Mlx heterodimer is required for glucose activation of glycolytic and lipogenic genes transcription [5, 32, 23, 34, 22, 21, 25].

To further establish the role of ChREBP/Mlx in the fatty acid regulatory network, three chronic disease models were used to determine the correlation of ChREBP/Mlx and glucose and lipid metabolism: Streptozotocin-induced diabetes,

high fat diet induced glucose intolerance, and leptin deficiency induced obesity. Hepatic nuclear abundance of ChREBP, Mlx and HNF-4 proteins were measured in comparison to L-PK mRNA.

4.2 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 Diabetic Rats: Male Spraque-Dawley rats (200-250g) (Charles River Laboratories, Kalamazoo, MI) were maintained on Harlan- Teklad laboratory chow (#8640) and water *ad lib.* Rats were injected with streptozotocin (7.5 mg/100 g.BW) and 3ml of 25% glucose. Three weeks later, blood glucose was measured on control animals (without streptozotocin treatment) or diabetic animals (streptozotocin treated). Blood glucose was measured using a glucose meter. Control and diabetic rats were euthanized for liver protein and total RNA extraction.

High Fat Fed Mice: 2-month-old Male C57BL6 mice (Jackson Laboratories, Bar Harbor Maine) were on diets containing low fat (#D12450B) or high fat (#D19492) (Research Diets, Inc.) *ad lib* for 10 weeks. Mice were euthanized for liver protein and total RNA extraction.

Lean and Obese Mice: Male lean and obese C57BL7 mice (Jackson Laboratories, Bar Harbor, Maine) were maintained on Harlan-Teklad laboratory chow (#8640) diet and water *ad lib.* Livers were obtained from Dale Romsos and Kate Claycomb,

Department of Food Science and Human Nutrition, Michigan State University, East Lansing.

Nuclear Protein Extraction & Western Blot: Hepatic total, cytoplasmic and nuclear proteins were extracted and applied to western blot as described in Chapter 2 Materials And Methods.

RNA Extraction & qRT-PCR: Hepatic RNA were extracted and applied to qRT-PCR as described in Chapter 2 Materials And Methods.

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

4.3 Results

4.3.1 Streptozotocin-Induced Type I Diabetes

Streptozotocin induces diabetes by destroying insulin secreting pancreatic β cells. Due to the lack of insulin, diabetic rats have a significant increase in blood glucose (378 $\pm$ 21 mg/dl), when compared to control animals (77.9 $\pm$ 5.2 mg/dl) [166].

In spite of their high blood glucose level, hepatic L-PK mRNA was suppressed by 50% in diabetic animals; the gluconeogenic gene PepCK was induced 3-fold [166].

Hepatic nuclear Mlx protein was reduced by 70%, while no significant change in ChREBP or HNF-4α nuclear abundance was observed (Fig. 4.1).

The decrease in L-PK mRNA might due to the absence of insulin and decreased glucose metabolism. According to this observation, Mlx might be under the control of insulin dependant glucose metabolism. These results phenomenon support the importance of Mlx in the control of L-PK gene expression.

4.3.2 High Fat Diet Induced Glucose Intolerance

High fat diets induce glucose intolerance, insulin resistance, fatty liver and altered hepatic metabolism [167, 168]. The difference between low and high fat diets is in the calories from fat. The low fat diet contains 10% of the calories as fat, while the high fat diet contains 60% of the calories as fat. The animals on low fat diet have lower body weight (~29 g) and normal blood glucose and insulin levels (121 mg/dl and 0.5 ng/ml). The high fat diet increased mice body weight to ~44 g, with elevated blood glucose (152 mg/dl) and insulin (3.9 ng/ml) [166]. When compared to the low fat fed group, high fat fed animals were glucose intolerant and have fatty livers [167].

In high fat diet mice livers, nuclear abundance of Mlx were suppressed 40%, accordingly, hepatic L-PK mRNA decreased about 40%, while ChREBP and HNF-4α nuclear abundance remained unchanged (Fig. 4.2).

The decrease of hepatic L-PK mRNA in mice on high fat diet is probably due to less carbohydrate uptake and excess amount of fat uptake, which leads to a weakened glucose signal. Since carbohydrate alone is not affecting Mlx nuclear level (Fig. 2.6), the decrease of nuclear Mlx abundance suggests that lipid is interfering with certain signaling pathways that control Mlx nuclear localization.

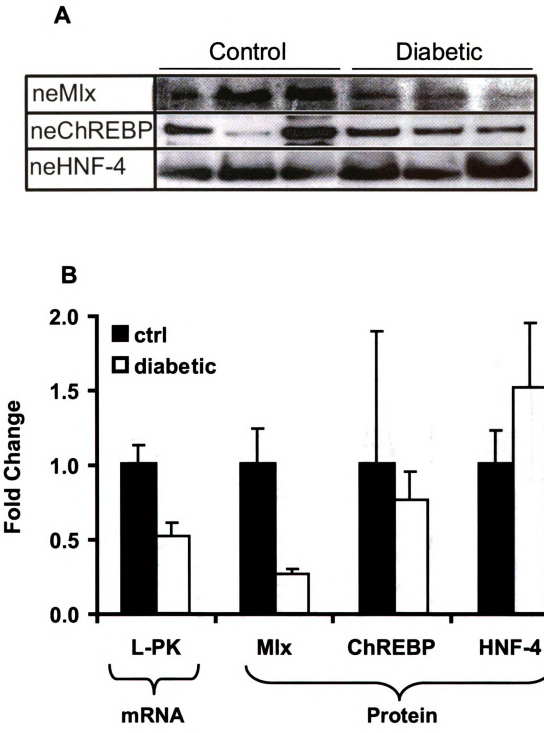


Figure 4.1: **L-PK gene expression in Streptozotocin-induced-diabetes rats.** Normal or STZ treated rats were kept for 3 weeks before sacrifice. Livers were taken and total RNA and nuclear proteins were extracted for qRT-PCR of L-PK mRNA and western blot of Mlx, ChREBP and HNF-4. Panel [A] shows picture of western blot. Panel [B] gives quantified results of L-PK mRNA and nuclear abundance of Mlx, ChREBP and HNF-4. Results were expressed as fold change to control (ctrl) animals. n=3.

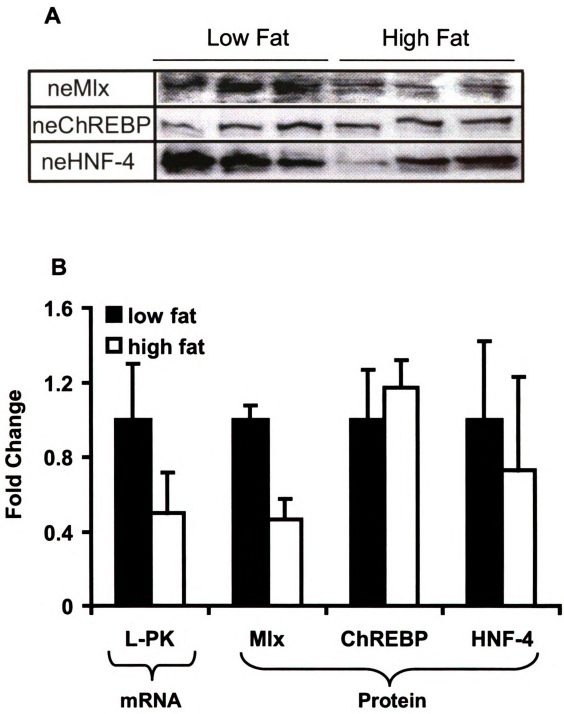


Figure 4.2: **L-PK gene expression in mice on high fat diet.** Livers from mice on low or high fat diet were taken and total RNA and nuclear proteins were extracted for qRT-PCR of L-PK mRNA and western blot of Mlx, ChREBP and HNF-4. Panel [A] shows picture of western blot. Panel [B] gives quantified results of L-PK mRNA and nuclear abundance of Mlx, ChREBP and HNF-4. Results were expressed as fold change to animals on low fat diet. n=3

4.3.3 Leptin-Deficient Obesity

Leptin is produced by adipose tissue in response to changes in caloric intake. Defective leptin expression in C57BL/6J-Lep$^{ob/ob}$ mice leads to hyperphagia, hyperinsulinemia, insulin resistance and obesity [169]. When compared to the lean (Lep$^{ob/+}$) littermates, obese animals are heavier and have elevated blood levels of glucose and insulin. The livers of obese mice are massively engorged with lipid, predominantly as neutral lipid [166].

In these leptin-deficient obese mice, there was a 6-fold increase in L-PK mRNA and 2-fold increase in Mlx nuclear abundance, while no change in ChREBP or HNF-4α nuclear abundance was observed (Fig. 4.3). The hyperphagia caused by leptin-deficient obesity requires a higher *de novo* lipogenesis, which boosted glycolysis to provide substrates for lipid synthesis. Thus, L-PK is induced in these obese animals. Nuclear Mlx is also increased. Thus a change in Mlx correlates with a change in L-PK mRNA.

4.3.4 Mlx Target Genes

The above chronic disease models indicate that change in Mlx and not ChREBP and HNF-4 nuclear abundance correlate well with changes in L-PK expression. Changes in nuclear Mlx abundance clearly affect expression of glucose-regulated genes. However, other Mlx target genes are unknown. In an effort to identify other Mlx target genes, I used a micro array approach. Affymetrix Array was performed to identify the Mlx controlled genes (Appendix A). RNAs from Ad-GFP and Ad-Mlx infected primary

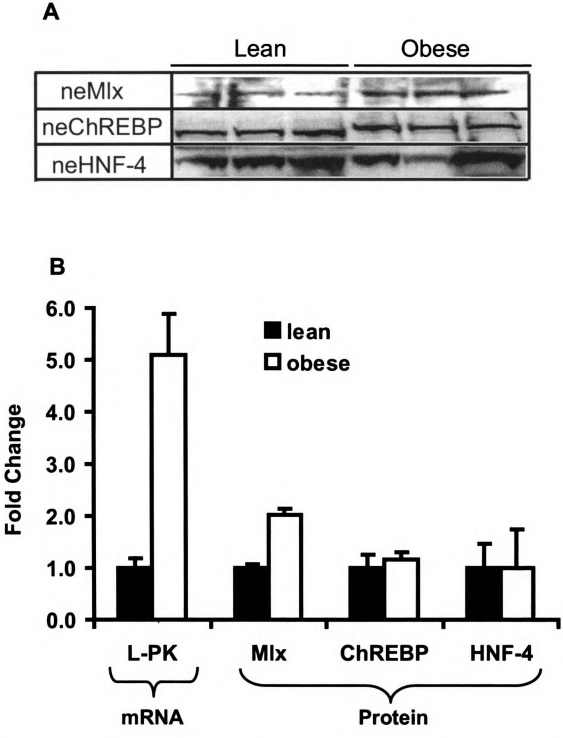


Figure 4.3: **L-PK gene expression in leptin deficient ($lep^{-/-}$) mice.** Livers from lean or obese mice were taken and total RNA and nuclear proteins were extracted for qRT-PCR of L-PK mRNA and western blot of Mlx, ChREBP and HNF-4. Panel [A] shows picture of western blot. Panel [B] gives quantified results of L-PK mRNA and nuclear abundance of Mlx, ChREBP and HNF-4. Results were expressed as fold change to lean animals. n=3.

hepatocytes were analyzed. Genes that are responsive to Mlx overexpression are listed in Appendix A. It is not surprising to see carbohydrate, lipid and protein metabolic genes in the list. In addition, genes involved in cell cycle, G-protein coupled receptors were also regulated by Mlx. Among them, one group of genes draw special interest. The mRNA of nuclear receptor PPARα and its target genes including cytochrome P450-4A (Cyp4A), cytosolic thioesterase (CTE), fatty acid elongase 5 (elolv5), fatty acid elongase 6 (elolv6) and fatty acid desaturase 9 ($\triangle$ 9) were all down-regulated by the Mlx overexpression in hepatocytes. q-RT-PCR were used to confirm the responses (Fig. 4.4). These findings suggested a new role for Mlx in the regulation of lipid metabolism: involvement in the PPARα-regulated peroxisome oxidation and fatty acid elongation and desaturation.

4.4 Discussion

As the key regulators in glucose-responsive genes transcription, ChREBP can regulate lipogenic genes expression. Recent reports showed that the lack of ChREBP reduced the gain of body weight in leptin-deficient obese animals [170]. My studies showed that hepatic nuclear Mlx, but not ChREBP or HNF-4α abundance, is regulated in three chronic metabolic disease animal models.

In the STZ-induced type I diabetic rats, hepatic L-PK mRNA were suppressed by 50%, which correlates with a 70% decline in hepatic nuclear Mlx protein. In livers from high fat diet fed mice, nuclear abundance of Mlx was suppressed 40%. Accordingly, hepatic L-PK mRNA decreased about 40%. In mice with leptin deficiency, there

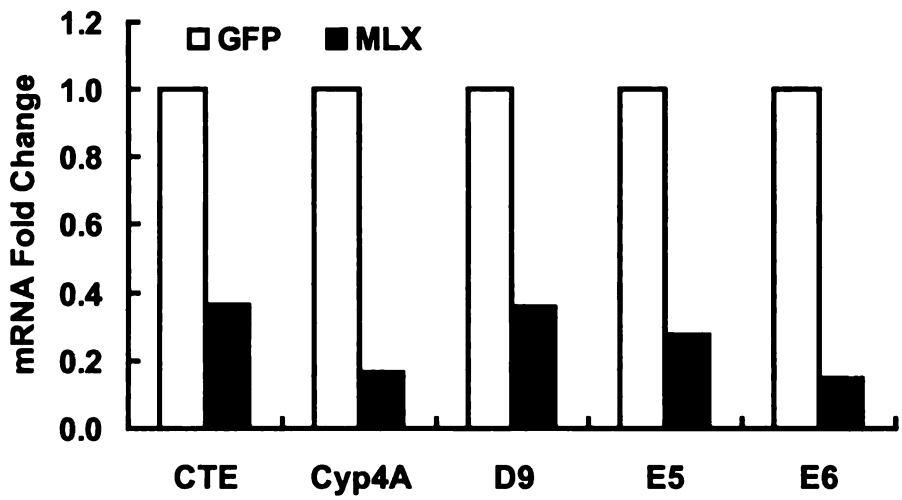


Figure 4.4: **qRT-PCR analysis of Mlx effects on PPARα target genes.** Rat primary hepatocytes were infected with 10pfu/cell Ad-GFP or Ad-Mlx for 24 hours in lactate-containing medium followed by 24 hours of incubation in glucose-containing medium and RNA was extracted from the cells. RNAs from 3 experiments were pooled for qRT-PCR. Results was expressed as fold change of Ad-Mlx infected samples (black bars) compared to Ad-GFP infected samples (white bars). CTE, cytosolic thioesterase; Cyp4A, cytochrome P450-4A; D9, fatty acid desaturase 9; E5, fatty acid elongase 5; E6, fatty acid elongase 6

was a 6-fold increase in L-PK mRNA and 2-fold increase in Mlx nuclear abundance. All of these studies indicate that the regulation of hepatic L-PK mRNA abundance is tightly linked to nuclear Mlx abundance, but not ChREBP or HNF-4α nuclear abundance.

In these animals, gluconeogenesis gene PEPCK, lipogenic gene fatty acid synthase (FAS) and fatty acid elongase and desaturase expression were also affected [166]. Some of these genes are targets of SREBP1c and PPARα, but the regulated Mlx protein level suggested its possible participation in these genes regulation. In fact, Adenovirus overespression of dominant-negative Mlx in primary hepatocytes abrogated the glucose induction of lipogenesis genes such as FAS[28].

These three animal models provided two pieces of information: First, Mlx nuclear protein level is changed in metabolic disorders. Second, in these carbohydrate and lipid metabolic disorders, the expression of glycolytic gene L-PK was affected, in a way that correlate with the Mlx nuclear protein level. Although the mechanisms by which hepatic nuclear Mlx abundance was regulated are not clear, these studies strongly supported the role of Mlx in the control of carbohydrate and lipid metabolism, specifically in the regulation of L-PK gene transcription.

The Affymetrix Array data provide an insight into the Mlx regulatory network. It suggested the potential role of Mlx in the PPARα regulation of hepatic genes. More detailed analysis and investigation will be needed to study the Mlx targets.

Chapter 5

Conclusions and Future Directions

L-PK plays a central role in hepatic carbohydrate and lipid metabolism. Insulin-stimulated glucose metabolism induces L-PK gene expression, L-PK enzyme activity, and the flow of glucose metabolites toward fatty acid synthesis and storage. The inhibitory effect of dietary n-3 PUFA on L-PK gene transcription is part of a larger regulatory network that shifts hepatic metabolism away from lipid synthesis and storage and toward fatty acid oxidation [3]. Glucose and n-3 PUFA manage these metabolic pathways by controlling the activity or nuclear abundance of key transcription factors, including ChREBP, Mlx, SREBP-1, and PPARα. WY14,643 and n-3 PUFA activate PPARα by direct binding [171]. n-3 PUFA suppresses SREBP-1 nuclear abundance by multiple mechanisms, including enhanced 26 S proteasomal degradation [3, 61]. Neither PPARα/RXRα nor SREBP-1 binds the L-PK promoter; neither transcription factor regulates L-PK expression directly [2, 143, 172].

This study presents new information to explain how glucose, n-3 PUFA, and WY14,643 regulate hepatic L-PK gene transcription through effects on ChREBP and Mlx nuclear abundance and L-PK promoter composition.

Fasting and refeeding rats or treating rat primary hepatocytes with glucose induced a robust accumulation of ChREBP in hepatic nuclei and the induction of L-PK mRNA (Fig. 2.1,2.8). Glucose treatment of rat primary hepatocytes stimulates ChREBP accumulation in nuclei, recruitment of RNA pol II to the L-PK promoter, the acetylation of histones H3 and H4 (Fig. 2.9), and increased L-PK promoter activity.

20:5,n-3 transiently suppressed ChREBP and Mlx nuclear abundance at 6 and 1.5 h, but WY14,643 had no effect on these proteins nuclear abundance. 20:5,n-3 and WY14,643 suppressed L-PK promoter activity and inhibited RNA Pol II recruitment to the L-PK promoter and the acetylation of histone H3 and H4 on the LPK promoter (Figs. 2.9,2.14). HNF-4α interaction with the L-PK promoter was transiently suppressed by 20:5,n-3 but not WY14,643.

Overexpression of ChREBP in rat hepatocytes stimulates L-PK gene expression but does not abrogate 20:5,n-3 suppression of L-PK expression. In contrast, overexpression of Mlx fully abrogates 20:5,n-3 suppression of L-PK gene expression. This finding identifies Mlx as a novel target for n-3 PUFA regulation.

WY14,643 inhibits HNF-4α transactivation but n-3 PUFA does not. The WY14,643 inhibition of L-PK transcription was partially reversed by ChREBP overexpression, but not Mlx overexpression. The different responses caused by n-3 PUFA and WY14,643 indicate that they use different mechanisms to regulate L-PK transcription.

The transient changes in ChREBP and Mlx nuclear abundance and HNF-4α-LPK promoter interaction closely parallel changes in intracellular nonesterified 20:5,n-3 [144, 61]. 20:5,n-3 is a minor fatty acid in both nonesterified and esterified lipid fractions of liver and hepatocytes [61]. At any time point examined in this study, 95% of all intracellular 20:5,n-3 is esterified. Intracellular nonesterified 20:5,n-3 is highest at 1.5 and lowest at 24 h. The transient effects of 20:5,n-3 on ChREBP, Mlx, and HNF-4α may be sufficient to delay the onset of glucose-mediated induction of L-PK gene transcription.

As a novel target for n-3 PUFA control, Mlx nuclear protein abundance is inhibited by n-3 PUFA. The 26 S proteasome inhibitor, MG132, suppresed nuclear Mlx protein and blocked glucose-activated L-PK gene transcription. With combined treatment of MG132 and 20:5,n-3, the 26 S proteasome inhibitor potentiated the inhibition of both Mlx nuclear protein level and L-PK promoter activity by 20:5,n-3 . This implicates the 26 S proteasome in this regulatory scheme. The suppressive effect on Mlx nuclear abundance without effects on Mlx expression implicates mechanisms that control Mlx nuclear import or export. Another heterodimer partner of Mlx is MondoA. The subcellular distribution of MondoA/Mlx heterodimers is determined by CRM1-dependent nuclear export signals in MondoA that interact with 14-3-3 family members. Leptomycin B, a nuclear export inhibitor, promotes MondoA/Mlx accumulation in nuclei [76]. Leptomycin B, however, did not prevent the n-3 PUFA suppression of Mlx abundance, neither did it impact PUFA inhibition of *L*-PK promoter activity (Fig. 3.11). I speculate that n-3 PUFA transiently inhibits

Mlx import into the nucleus. The mechanism underlying this process requires further analysis.

In summary, glucose stimulates ChREBP translocation into hepatocyte nuclei, recruitment of RNA pol II to the L-PK promoter, and increased histone H3 and H4 acetylation on the L-PK promoter without major changes in promoter-bound ChREBP or HNF-4α. 20:5,n-3 interferes with this process by transiently suppressing nuclear Mlx and ChREBP nuclear abundance. Mlx is an obligate heterodimer partner of ChREBP that is required for ChREBP binding to the L-PK ChoRE. Overexpressed Mlx eliminates the 20:5,n-3 but not the WY14,643 control of L-PK gene expression. Overexpressed ChREBP relieves the WY14,643 suppression of L-PK promoter activity but has no effect on n-3 PUFA inhibition of L-PK promoter activity. WY14,643 inhibits HNF-4αtransactivation but n-3 PUFA does not. These studies have identified Mlx as a key target for n-3 PUFA control of L-PK gene expression. n-3 PUFA and WY14,643 clearly use distinct mechanisms to inhibit glucose-activated L-PK gene transcription.

These studies have provided clear evidence to judge our hypothesis: The ChREBP /Mlx heterodimer is responsive to glucose induction and in turn stimulates L-PK gene transcription. Mlx is the target for n-3 PUFA inhibition of L-PK gene transcription. Although n-3 PUFA does not directly target HNF-4, it changes the L-PK promoter composition, including the binding of HNF-4 to the promoter. The PPARα agonist WY14,643 and n-3 PUFA use different mechanisms to regulate L-PK gene transcription.

Based on the above information, a revised view of L-PK promoter is shown in Fig. 5.1. Previous linker-scanning analysis indicated that the DR-1 element was required for both WY14,643 and n-3 PUFA control of L-PK gene transcription [20, 2] (Fig. 1.1). This interpretation, however, is confounded by the fact that both n-3 PUFA and WY14,643 only inhibit glucose-activated L-PK expression and not basal L-PK expression. HNF-4α transactivation activity is not regulated by n-3 PUFA (Fig. 3.8), but transiently affects HNF-4α interaction with the L-PK promoter (Fig. 2.9). In contrast, WY14,643 interferes with HNF-4 transactivation (Fig. 3.8) but has no effect on HNF-4α interaction with the L-PK promoter (Fig. 2.14). A likely explanation for the WY14,643 effect that ligand-activated PPARα interferes with co-activator recruitment to the L-PK promoter. The robust inhibition of histone H4 acetylation on the L-PK promoter supports this concept (Fig. 2.14). N-3 PUFA only transiently affects HNF-4α interaction with the L-PK promoter (Fig. 2.9). The novel identified n-3 PUFA target for regulation of L-PK transcription is Mlx. N-3 PUFA decreases nuclear Mlx abundance and overecpression of Mlx in hepaotcytes reverse the 20:5,n-3 inhibition of L-PK promoter activity. In revision of the original model (Fig. 1.1), the PUFA cis-regulatory targets should include the ChREBP/MLX and HNF-4α-binding sites (Fig. 5.1).

With the clarification of previously existing puzzles, along comes the questions. Mlx has emerged as a novel player in the n-3 PUFA regulatory network. Although my studies provided preliminary information on the n-3 PUFA control of its nuclear protein level, more work is needed to understand its regulation of target genes. First is the Mlx nuclear transportation. 26S proteasome is involved in this process, possi-

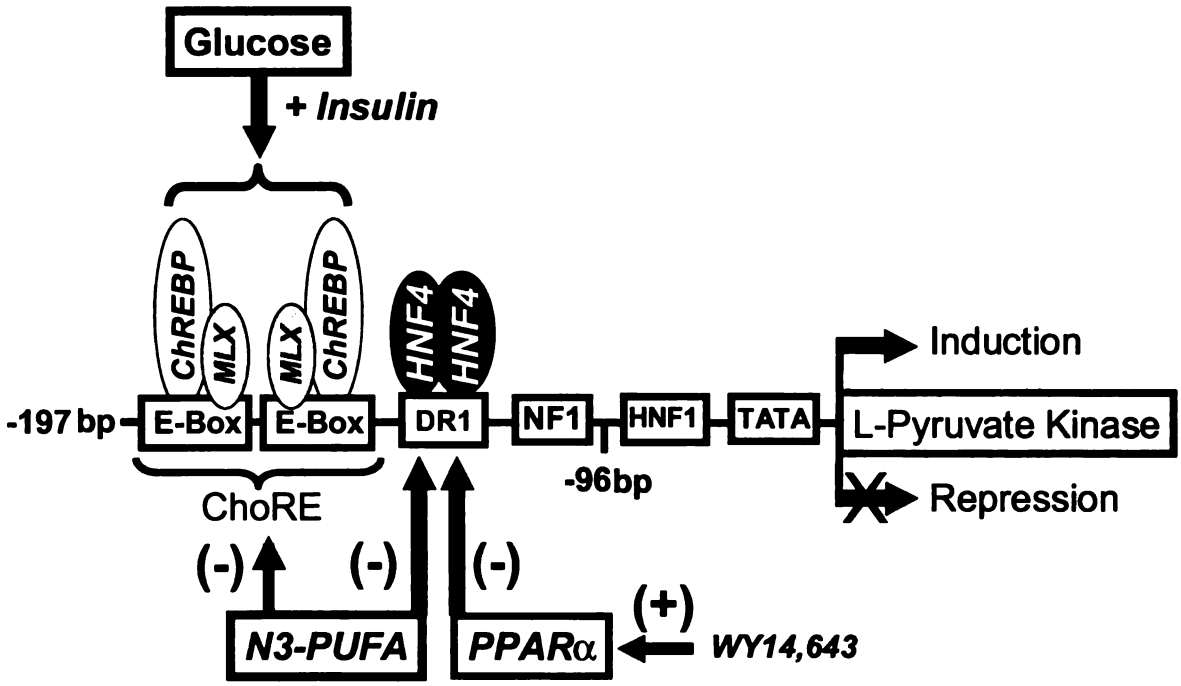


Figure 5.1: **Revised view of the L-PK promoter.** Glucose targets on ChoRE that binds ChREBP/Mlx heterodimers to induce L-PK transcription. The n-3 PUFA targets both the ChoRE and HNF-4α-binding site to inhibit L-PK transcription. PPARα agonist WY14,643 targets the HNF-4α-binding site and inhibit L-PK transcription.

bly through certain nuclear transporters. Other signaling pathways may affect this process as well. Second is whether Mlx has other heterodimer partners. Mlx can form heterodimers with Myc family members and Mondo family members. Except for the role of MondoB (ChREBP) in glucose regulation and MondoA in nuclear transportation, little is known about how these partners participate in the Mlx regulation of target genes. Identification of Mlx partners will provide new information for the Mlx functions. A hypothetical model for the Mlx translocation in a 26S proteasome dependent manner is illustrated in Fig 3.15. Finally, how does Mlx interact with target gene promoters? I was able to confirm that overexpressed flag-Mlx binds to the L-PK promoter. However, I could not address whether Mlx binding to the L-PK promoter was regulated. Development of Mlx antibody applicable in ChIP assay will help to resolve this missing piece, and contribute to the studies of other Mlx target genes as well.

With over 60% of the U.S. adult population considered overweight or obese, and 7% of the population (adult and children) diagnosed with diabetes, the dietary impact on human health has caught more and more attention in scientific research. A major aim is to find methods to reduce the progression of metabolic chronic diseases caused by unbalanced food intake, namely, excess amount of saturated fat and carbohydrate in the diet. As an exception from dietary fat, n-3 PUFA inhibits *de novo* lipogenesis and helps to reduce plasma lipid. The glycolytic enzyme L-PK is one of the genes in the regulatory process. This thesis has studied the dietary carbohydrate and fatty acids regulation the L-PK gene transcription. Although several transcription factors have been identified in the glucose and PUFA regulation of lipid metabolic enzymes

expression, the mechanism of L-PK regulation is unique and not well understood. My studies provide new information on the carbohydrate and PUFA regulation of L-PK, which contributes to the understanding of PUFA regulatory network. In addition, a new member of PUFA regulatory network, Mlx, is identified. Hopefully, these studies will help in the effort to improve human health.

Appendix A

Publications

Journal Articles

1. **J. Xu**, B. Christian, and D. B. Jump. Regulation of rat hepatic L-Pyruvate kinase promoter composition and activity by glucose, N-3 polyunsaturated fatty acids and peroxisome proliferator activated receptor-alpha agonist. *J Biol Chem. 2006 Jul 7; 281 (27): 18351-62. Epub 2006 Apr 27.*

2. Y. Wang, D. Botolin, **J. Xu**, B. Christian, E. Mitchell, B. Jayaprakasam, M. Nair, J. Peters, J. Busik, L. K. Olson, and D. B. Jump. Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J Lipid Res. 2006 Jun 21 [Epub ahead of print]*

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

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

5. A. Pawar*, **J. Xu***, E. Jerks, D. J. Mangelsdorf, D. B. Jump. Fatty acid regulation of liver X receptors (LXR) and peroxisome proliferator-activated receptor alpha (PPARalpha) in HEK293 cells. *J Biol Chem. 2002 Oct 18; 277(42): 39243-50*
* Equal-contribution first authors

Conference Abstracts

1. **J. Xu**, B. Christian and D. B. Jump. Polyunsaturated fatty acids (PUFA) regulate hepatic nuclear factor-4 (HNF4) binding to the rat hepatic L-Pyruvate kinase (LPK) promoter. *EB 2006 meeting, San Francisco, California*

2. Y. Wang, D. Botolin, **J. Xu**, B. Christian and D. B. Jump. Regulation of rat hepatic elongase and desaturase expression. *EB 2006 meeting, San Francisco, California*

3. D. B. Jump, Y. Wang, D. Botolin, **J. Xu**, O. Demeure, J. Busik, W. Chen and B. Christian. Gene regulation in response to polyunsaturated fatty acids. *46th ICBL, 2005, Corsica, France*

4. D. Z. Ye, K. Linning, **J. Xu**, D. B. Jump, and L. K. Olson. Poly (ADP-ribose) polymerase (PARP) inhibitors prevent pathologic changes in gene transcrip-

tion in INS-1 cells exposed to high glucose. *Diabetes 52 (supplement 1) A367-A368, 2003*

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