ROLES OF DIETARY FACTORS AND HEPATIC GENES IN THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE

A Dissertation in
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by
Lei Hao

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The dissertation of Lei Hao was reviewed and approved* by the following:

A. Catharine Ross  
Professor and Occupant of Dorothy Foehr Huck Chair in Nutrition  
Dissertation Advisor  
Chair of Committee  

Michael H. Green  
Professor of Nutrition  

Gary J. Fosmire  
Emeritus Associate Professor of Nutrition  

Joshua D. Lambert  
Associate Professor of Food Science  

Gordon Jensen  
Professor of Nutrition  
Head of the Department of Nutritional Sciences  

*Signatures are on file in the Graduate School
ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterized by pathological accumulation of lipid in the liver in the absence of significant alcohol consumption. NAFLD is associated with insulin resistance and increased risk of cardiovascular diseases. NAFLD begins with lipid accumulation in liver and may progress to necroinflammatory steatohepatitis, advance fibrosis or cirrhosis, and eventually hepatocellular carcinoma. While hepatic accumulation can be simplified as an imbalance between the pathways of lipid input and output, the nutritional and hormonal regulation of hepatic lipid metabolism is quite complicated and has been the subject of research for several decades. We hypothesize that dietary factors, physical activity and hepatic genes are all involved in the development of NAFLD. Therefore, a series of studies was performed to investigate the effect of dietary factors, including simple carbohydrate, unsaturated fat, and retinoids on the development of NAFLD at the intact animal, cellular, and molecular levels.

Dietary factors, including macronutrients and micronutrients, are involved in the development of NAFLD. In chapter 3, we aimed to investigate the roles of high simple carbohydrate diet, unsaturated fat and retinal supplementation on hepatic steatosis in mice. Mice were assigned to diets groups that included normal chow diet (NC), high carbohydrate only diet (HCD), high carbohydrate diet plus supplementation with lipid emulsion (HCD+4% LE or 13.5% LE), high carbohydrate diet with supplementation with retinal (HCD+RAL) and high carbohydrate diet with both LE and RAL (HCD+LE+RAL). Mice were maintained on experimental treatments for 4 or 5 weeks. The main finding was that the HCD only diet, without supplementation, induced hepatic steatosis as demonstrated by histology and biochemical TG quantification, while supplementation with LE reduced lipid accumulation; however, retinal had no effect on hepatic steatosis. Gene expression analysis revealed that HCD only diet induced hepatic steatosis via...
stimulation of three key lipogenic genes, namely sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). By contrast, supplementation with LE suppressed SREBP-1c, ACC and FAS. In addition, the results showed that hepatic inflammation was initiated, because inflammation markers, chemokine (C-C motif) ligand 2 (CCL2) and serum amyloid P component (APCS) were significantly induced in liver by the HCD only diet. In total, the results indicated that reducing the percentage of carbohydrate with unsaturated fat supplementation can provide a beneficial preventative effect in experimental NAFLD.

Vitamin A (retinol) is critical for many developmental and biochemical processes, such as cell differentiation, embryonic development, reproduction, vision and immune function. Recently, the roles of vitamin A and other retinoids in lipid and energy metabolism have received increasing attention. In chapter 4, we aimed to clarify the roles of retinoids, including retinal, retinoic acid and β-carotene in the development of hepatocellular steatosis in liver cells, using HepG2 cells as a model. Hepatocellular steatosis was induced by either increasing the glucose concentration of the medium (high glucose) or adding oleic acid to the cultures of HepG2 cells. The results showed that none of those retinoids reduced lipid accumulation in HepG2 cells; conversely, gene expression data showed that retinoic acid stimulated expression of the lipogenic genes, FAS and SREBP1c in a dose dependent manner. The results indicated that retinoids did not have beneficial effects on treatment of hepatocellular steatosis induced by high glucose or oleic acid in vitro in HepG2 cells, and in fact, retinoic acid may contribute to lipid anabolism. However, we successfully established a hepatocellular steatosis model induced by high glucose, which provides a useful and convenient tool to study the pathogenesis of NAFLD in vitro.
Human epidemiologic studies suggest that genetic factors are also involved in the development of NAFLD. More specifically, several independent genome-wide association studies have found a strong association between NAFLD with a non-synonymous variant of patatin-like phospholipase domain-containing 3 (PNPLA3) encoding an isoleucine to methionine substitution at position 148 (I148M). However, the biological function of PNPLA3 is still under debate. In chapter 5, we aimed to explore the biological function of NAFLD associated gene, PNPLA3 (also known as adiponutrin). Firstly, we studied hepatic PNPLA3 expression in our mouse hepatic steatosis model induced by the high-carbohydrate diet (samples came from studies in chapter 3). The results showed that PNPLA3 gene expression was increased ~22-fold in the high-carbohydrate diet group compared to the control group. However, lipid emulsion completely reversed PNPLA3 induction by the high carbohydrate diet. Interestingly, the expression pattern of several lipogenic genes, including SREBP1c, FAS and ACC, followed that of PNPLA3. Secondly, we studied hepatic PNPLA3 expression in a mouse model of hepatic steatosis induced by a high fat diet (samples came from another independent study). In this study, mice were assigned to normal chow (NC ~10% fat), high fat diet (HF ~60% fat), and high fat with voluntary exercise (HFE) for 16 weeks. Hepatic PNPLA3 gene expression was not changed in mice fed high fat diet although they developed hepatic steatosis; however, voluntary exercise dramatically decreased hepatic PNPLA3 gene expression compared to normal chow and high fat diet-fed mice. Thirdly, we investigated nutritional and hormonal regulation of PNPLA3 in liver cells in vitro. In HepG2 cells that were cycled between 5.5 and 25 mM glucose (to mimic starvation and refeeding conditions), PNPLA3 was significantly decreased ~5-fold by glucose starvation and increased ~2.5-fold by glucose refeeding, while insulin and glucagon had no effect. Carbohydrate response element binding protein (ChREBP) expression correlated with
PNPLA3 expression. Unsaturated fatty acids, oleic acid, linoleic acid, EPA and DHA (400 nM each, 24 h), each significantly decreased PNPLA3 mRNA by ~50%, but linolenic acid did not. Lastly, we studied the effect of PNPLA3 knockdown on hepatocellular steatosis in HepG2 cells. The results showed that PNPLA3 knockdown did not affect total lipid content when HepG2 cells were incubated in normal medium or treated either with high glucose or oleic acids, although we observed that PNPLA3 knockdown increased FAS expression. Overall, studies in chapter 5 suggest that PNPLA3 plays a role in lipogenesis but not lipolysis.

Fibroblast growth factor 21 (FGF21), a novel member of the FGF family, is mainly expressed in liver and adipose tissue. Recently, studies have consistently shown that FGF21 beneficially affects carbohydrate and lipid metabolism. In addition, human studies observed increased serum FGF21 in patients with NAFLD. However, the physiological actions of FGF21 are still not fully understood. In chapter 6, we aimed to explore the potential role of FGF21 in hepatic lipid metabolism by evaluating how nutritional, hormonal signals and physical activity regulate FGF21 expression in mice and HepG2 cells. In in vivo studies, hepatic FGF21 gene expression was significantly increased in mice fed the high carbohydrate diet, but not in mice fed a high fat diet. By contrast, adipose FGF21 mRNA was not changed in mice fed the high carbohydrate diet. In addition, our studies revealed that hepatic FGF21 mRNA expression was suppressed in mice with voluntary access to a running wheel exercise for 16 weeks. However, short-term physical activity (1 week) had no effect on hepatic FGF21 expression, but suppressed muscle FGF21 expression. In in vitro studies, FGF21 mRNA was shown to be strongly induced in HepG2 cell incubated in medium with either high glucose or 2-deoxy-D-glucose. In addition, insulin significantly induced FGF21 expression, while glucagon had no effect. Collectively, these results
showed that FGF21 in liver is regulated by dietary carbohydrate, insulin, and physical activity, suggesting that FGF21 functions as a regulator in gluconeogenesis and lipogenesis in liver.

In summary, my dissertation work indicates that dietary factors are important players in the development of NAFLD, and that the hepatic enzyme (PNPLA3) and peptide hormone (FGF21) contribute to metabolic adaption of the liver to nutritional and physical activity signaling.
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LIST OF ABBREVIATIONS

APCS  serum amyloid P component
ACC   acetyl-CoA carboxylase
ADPN  adiponutrin
ALA   α-linolenic acid
ALD   alcoholic fatty liver disease
ALT   alanine aminotransferase
AST   aspartate aminotransferase
ATGL  adipose triglyceride lipase
ATRA  all-trans retinoic acid
CCL2  chemokine (C-C motif) ligand 2
ChREBP carbohydrate response element binding protein
CPT1  carnitine-palmitoyl transferase-1
CRABP cellular retinoic acid binding protein
CRBP  cellular retinol binding proteins
CYP26 cytochrome P450 family 26
DAG   diacylglycerol
DGAT  acyl-CoA:diacylglycerol acyltransferase
DHA   docosahexaenoic acid
DMEM  Dulbecco's Modified Eagle's medium
EPA   eicosapentaenoic acid
FAS   fatty acid synthase
FFA   free fatty acid
<table>
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<th>Acronym</th>
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<tr>
<td>FGF21</td>
<td>fibroblast growth factor 21</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>HCD</td>
<td>high carbohydrate diet</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HF</td>
<td>high fat diet</td>
</tr>
<tr>
<td>IR</td>
<td>insulin resistance</td>
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<tr>
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<td>ketogenic diet</td>
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<td>LA</td>
<td>linoleic acid</td>
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<tr>
<td>LE</td>
<td>lipid emulsion</td>
</tr>
<tr>
<td>LF</td>
<td>low fat diet</td>
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<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LPAAT</td>
<td>lysophosphatidic acid acyltransferase</td>
</tr>
<tr>
<td>LRAT</td>
<td>lecithin:retinol acyltransferase</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacylglycerol</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
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<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PNPLA3</td>
<td>patatin-like phospholipase domain-containing 3</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RAL</td>
<td>retinal</td>
</tr>
<tr>
<td>Acronym</td>
<td>Gene/Protein Name</td>
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<tr>
<td>RALDH</td>
<td>retinal dehydrogenase</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RBP4</td>
<td>retinol binding protein 4</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoic X receptors</td>
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<td>SNP</td>
<td>single-nucleotide polymorphism</td>
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<td>SREBP-1c</td>
<td>sterol regulatory element-binding protein-1c</td>
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<td>STRA6</td>
<td>stimulated by retinoic acid-6</td>
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<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>VAD</td>
<td>vitamin A deficient</td>
</tr>
<tr>
<td>VAS</td>
<td>vitamin A sufficient</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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ACKNOWLEDGEMENT

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CHAPTER 1

LITERATURE REVIEW
1. Non-alcoholic fatty liver disease

1.1 Definition of non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of liver diseases, ranging from simple hepatic steatosis to steatohepatitis (NASH), fibrosis and cirrhosis. Maxwell et al. (1) documented hepatic steatosis in the livers of obese patients undergoing biopsy before or after jejunoileal bypass surgery. Ludwig et al. (2) recognized alcoholic hepatitis-like lesions among over-weight or diabetic non-alcoholic drinkers and he coined the term non-alcoholic steatohepatitis. NAFLD is defined by a hepatic fat infiltration of >5% of hepatocytes, as assessed by liver biopsy or magnetic resonance spectroscopy, in the absence of significant alcohol intake, defined by two standard drinks (20g ethanol) daily for men and one standard drink (10 g ethanol) daily for women (3).

1.2 Prevalence

It has been reported that the prevalence of NAFLD is between 20-30% in the general population (4-6). However, the estimate of prevalence may vary depending on diagnostic method and populations. Based on liver biopsy and postmortem series, the estimated prevalence of NAFLD is between 15-39%, while an estimate derived from unexplained elevations in aspartate aminotransferase or alanine aminotransferase suggests a prevalence of 5.5% (4). Notably, serum aminotransferase can be normal in up to 75% of cases of NAFLD (4). Beyond different case definitions, gender and ethnicity affect the prevalence of NAFLD (7). It is clear that both male and female are affected by NAFLD, but some studies have revealed that NAFLD is more common in men than women in morbidly obese populations (8). A study also showed that
Hispanic populations have a higher prevalence of NAFLD than White and African-American populations (4).

NAFLD not only affects western populations but also has become a focus of concern in several Asian-Pacific countries, such as China, Korea, Japan and India (9, 10). Notably, in China the incidence of obesity has increased dramatically and it has been estimated that one-third of adults are overweight or obese and 10%-20% of adults are affected by metabolic syndrome (11). A study showed that prevalence of NAFLD based on ultrasound was 16.7% in India (12). The prevalence of NAFLD in Japan has more than doubled from ~13% in 1988-9 to ~30% in 2004 (13). Meanwhile, there has been a dramatic increase in obesity and type 2 diabetes due to lifestyle changes, as indicated by parallel rise in car sales and other criteria of affluence and inactivity in Japan (14). A study, conducted in Korea, showed that the prevalence of NAFLD, diagnosed with ultrasonography, is 25% (15). Taken together, the prevalence of NAFLD in the Asian-Pacific region is very similar to that described in Western country surveys, and NAFLD is an emerging public health issue worldwide.

1.3 Pathogenesis

1.3.1 2-hit hypothesis

Day and James (16) first proposed the original “2-hit” hypothesis when they studied the mechanism of liver injury in NAFLD/NASH. The 'first hit', hepatic triglyceride (TG) accumulation, or steatosis, increases the susceptibility of the liver to injury-mediated by 'second hits', such as inflammatory cytokines/adipokines, mitochondrial dysfunction and oxidative stress, which in turn promote the development to steatohepatitis and/or fibrosis. However, there is an increasing recognition of the fact that free fatty acids (FFA) can directly cause toxicity by
increasing oxidative stress and by activating inflammatory pathways. Furthermore, several studies suggest that hepatic TG per se is not toxic and may, in fact, even protect the liver from lipotoxicity caused by accumulation of FFA. Yamaguchi et al. (17) demonstrated that pharmacological inhibition of TG synthesis (mediated through the reduction in diglyceride acyltransferase activity) improved liver steatosis, but at a price of increased liver damage. Levels of hepatic fatty acids, cytochrome P450, and markers of lipid peroxidation and oxidant stress, as well as fibrosis, were all markedly increased in this study. Therefore, the emerging evidence has led to the formation of a new “2-hit” hypothesis, in which the accumulation of FFA, instead of TG, represents the first hit and is sufficient to induce liver damage (18). Moreover, various FAs have differential toxicity. Li et al. (19) found that exposure of murine or human hepatocytes to monounsaturated fatty acids resulted in lipid accumulation without changes in cell viability. In contrast, cells incubated with saturated fatty acids showed significantly decreased cell viability and increased caspase activation and apoptosis, with only minor lipid droplet accumulation. These studies also have indicated that accumulation of TG should not be used as a criterion for the evaluation of liver damage, since TG is a protective mechanism by preventing the toxic effects of FFA.

However, it is unlikely that a single mechanism explains the pathogenesis of NAFLD in all affected patients. More likely, NAFLD develops as a consequence of a ‘multi-hit’ process. The initial hit, which leads to the development of fatty liver, is the dysregulation of fatty acid metabolism. The initial hit renders hepatocytes susceptible to a variety of hits that follow, which finally lead from fatty liver to NASH or even cirrhosis. These subsequent hits, including adipokine alterations, lipid peroxidation and oxidative stress, endotoxemia, hepatic stellate cell
activation and mitochondrial dysfunction, stimulate an inflammatory and fibrogenic response in some patients with genetic susceptibility (20).

1.3.2 Hepatic fat accumulation

Mechanisms potentially contributing to hepatic steatosis include: increased FA delivery to liver from adipose TG, dietary FFA overflow to the liver, impaired hepatic FFA elimination through oxidation and impaired secretion in very low density lipoprotein (VLDL). Studies in humans and in rodent models have demonstrated that the mechanisms leading to the excessive accumulation of hepatic TG are mainly linked to increased delivery of FFA from peripheral expanded adipose tissue to the liver and enhanced de novo lipid synthesis via the lipogenic pathway in the liver itself, while lipid disposal via β-oxidation and VLDL export is only moderately affected (21). Under non-pathological conditions, insulin can suppress activity of hormone sensitive lipase, which hydrolyzes FFA release from adipose tissue, but under insulin resistance condition, insulin does not adequately inhibit HSL, and lipolysis in adipose tissue is enhanced, therefore FFA derived from peripheral fat stores contribute to hepatic TG accumulation. Under non-stimulated conditions, the contribution of de novo fatty acid synthesis to TG and VLDL is small in humans, estimated to be less than 5% in the postabsorptive state (22, 23). By contrast, Donnelly et al. (24) who used a stable isotope technique, demonstrated that 59% of hepatic TG derives from adipose tissue FFA spill-over, 26% from hepatic de novo lipogenesis and 15% from dietary fat in NAFLD. In theory, the assembly and secretion of VLDL TG could be impaired in NAFLD, but there is no strong evidence showing how VLDL secretion is related to steatosis, except that one study suggested that the G/T single nucleotide polymorphism in microsomal triglyceride transfer protein (MTP) is associated with NASH (25). The MTP plays a key role for the assembly and secretion of VLDL by transporting TG into the lumen of the endoplasmic reticulum. Namikawa
et al. (25) found that the incidence of G allele and G/G genotype was higher in NASH patients than in healthy control subjects. A previous study demonstrated that the G allele reduced MTP expression compared to the T allele, accordingly Namikawa et al. (25) argued that the low MTP production caused by the G allele results in failure of TG secretion from liver in NASH.

**1.3.3 Insulin resistance**

Insulin has many effects at both the cell signaling and gene expression levels, including its effects on carbohydrate, lipid and protein metabolism. Insulin resistance (IR) is defined as an inadequate response to the physiological effects of circulating insulin in specific target tissues, such as skeletal muscle, liver, and adipose tissue (26). IR can be central (hepatic) or peripheral (muscle, fat) depending upon the primary site of involvement. Peripheral IR impairs glucose uptake from blood into muscles (postabsorptive and fasting states), while hepatic IR manifests as unrestrained glucose production by liver under fasting conditions (27).

Although an association between NAFLD and IR is well accepted, it remains unclear whether IR causes the excessive accumulation of TG in liver, or IR is the consequence of fat accumulation in liver (21). Some investigators believe that hepatic lipid accumulation is a result, rather than cause, of peripheral IR in the development of NAFLD (28). In skeletal muscle, peripheral IR primarily affects a large portion of the total glucose uptake resulting in increased serum glucose levels (29), while in adipose tissue, peripheral IR induces an impaired anti-lipolytic action of insulin and increased release of FFA (30). The de novo synthesis of fatty acids is regulated by insulin and glucose. Insulin regulates hepatic lipogenesis by activation of the transcription of sterol regulatory element-binding protein-1c (SREBP1-c) (31). SREBP1-c can activate the transcription of many genes required for lipogenesis (32). For example, SREBP1-c can activate
acetyl-CoA carboxylase (ACC) that produces malonyl-CoA at the mitochondrial membrane. On one hand, the increase of malonyl-CoA leads to increased production of fatty acids; on the other hand, malonyl-CoA inhibits the shuttle enzyme carnitine-palmitoyl transferase-1 (CPT1), thereby reducing fatty acid oxidation in mitochondria. SREBP1-c also activates the transcription of fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) (32). In addition, lipogenesis is also regulated by a second transcription factor, the carbohydrate response element binding protein (ChREBP) (33). At low glucose concentration, ChREBP is an inactive phosphorylated cytosolic protein, but at high glucose concentration, ChREBP undergoes dephosphorylation and translocates from the cytosol into the nucleus, and as a result, ChREBP can bind to DNA to regulate lipogenic gene expression (34).

In summary, IR in adipose tissue and skeletal muscle increases the level of circulating free fatty acids and glucose. IR and glucose regulate lipogenesis respectively by activation of the transcription factors, SREBP-1c and ChREBP. Thus the increased lipogenesis combined with possibly impaired VLDL assembly and secretion leads to steatosis in patients with NAFLD. The mechanism of IR induced steatosis can be illustrated in Fig 1-1 (35).
FIGURE 1-1 The mechanism of insulin resistance induced steatosis (H Dancygier. 2010. Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases.)
Conversely, studies have shown that lipid intermediates can promote insulin resistance. Nagle et al. (36) demonstrated that excess diacylglycerol, produced by overexpression of glycerol-3-phosphate acyltransferase 1, activates protein kinase C, and phosphorylates insulin receptor substrate-1 on Ser307, which suppresses IRS-1 tyrosine phosphorylation and diminishes phosphatidylinositol 3-kinase activation, and finally insulin sensitivity is impaired in rats. In addition, other lipid intermediates, such as lysophosphatidic acid (LPA), and phosphatidic acid (PA), and ceramide, have also been implicated in the development of insulin resistance. However, a study by Monetti et al. (37) suggested diglyceride acyltransferase-mediated lipid accumulation in the liver is insufficient to cause insulin resistance. In this study, diglyceride acyltransferase 2 was overexpressed in mice, which had increased amounts of TG, diacylglycerol, ceramides, and unsaturated long-chain fatty acyl-CoAs in the liver. However, the mice had no abnormalities in plasma glucose and insulin levels, glucose and insulin tolerance, rates of glucose infusion and hepatic glucose production during hyperinsulinemic-euglycemic clamp studies, or activities of insulin-stimulated signaling proteins in the liver. This discrepancy might be explained by different species used in the above two studies. Notably two different enzymes, glycerol-3-phosphate acyltransferase 1/diglyceride acyltransferase 2 were overexpressed in their models in order to increase lipid intermediates, so it is likely that the composition of lipids was different in these two studies, which may also explain the discrepancy.

Steatosis is not necessarily associated with IR. This is illustrated by several examples: First, parenteral nutrition often results in fatty liver, but patients may not have IR. In this case, increased de novo hepatic synthesis of fatty acids and subsequent esterification into TGs is an important cause of steatosis (38). Second, pharmacological inhibition of glucose-6-phosphatase by S4048 resulted in massive steatosis within several hours, but plasma glucose and insulin
levels were decreased (39). Third, pharmacological inhibition of CPT1 induced acute hepatic steatosis, but hepatic insulin sensitivity was not reduced by the pharmacological inhibition of beta-oxidation (40).

1.3.4 Inflammation

Based on the two hit hypothesis, a ‘second hit’ triggers the necroinflammatory response characterizing NASH. Several studies have identified elevated serum and hepatic levels of tumor necrosis factor-α (TNF-α). TNF-α not only has pro-inflammatory effects but also promotes insulin resistance. Does simple steatosis have inflammation markers? A study by Haukeland et al. (41) showed that some general inflammation markers, such as CCL2/MCP-1, TNF-α, and interleukin (IL)-6, were elevated in both patients with NASH and simple steatosis. Comparing NASH with simple steatosis, levels of TNF-α and CCL2 were elevated and adiponectin was decreased. Therefore, this study supports the notion that NAFLD is characterized by a systemic low-grade inflammation. In this study, patients’ BMI, more than 28.9, could be a confounder since obesity per se is defined as a low-grade inflammatory state. Data from our lab showed that CCL2 and APCS mRNA were significantly increased in livers of mice with simple steatosis induced by a high simple carbohydrate, which suggests that inflammation is also involved in the pathogenesis of simple steatosis (42). This gives rise to a key question, what are the mechanisms responsible for elevated cytokine production in patients with NAFLD? It is believed that a surplus of FFA plays a key role in liver inflammation and injury. Several studies explored the mechanisms of FFA lipotoxicity that contribute to the development of NAFLD/NASH.

An in vitro study by Feldstein et al. (43) demonstrated that FFA treatment of liver cells resulted in the translocation of Bcl-2-associated X protein (Bax), a pro-apoptotic regulator, to lysosomes
and lysosomal destabilization with release of cathepsin B (Ctsb), a lysosomal cysteine protease, into the cytosol. Once in the cytosol, Ctsb triggered a cascade culminating in activation of nuclear factor κB (NF-κB), with TNF-α generation. TNF-α promotes TG accumulation and hepatic steatosis, perhaps in a paracrine or autocrine manner. TNF-α can promote insulin resistance by signaling IKK-β activation and c-jun-N-terminal kinase activation. TNF-α may also further promote lysosomal destabilization, resulting in a feed-forward, self-perpetuating pathway, further accentuating liver injury. Boden et al. (44) showed that infusion of lipid (which increased plasma FFA) activated the proinflammatory NF-κB pathway in rat liver and that the expression of inflammatory cytokines, such as TNF-α, IL-6, IL-1β were also elevated in liver tissue. Malhi et al. (45) found that free fatty acids caused JNK activation, which in turn resulted in hepatocyte lipoapoptosis.

Alkhouri et al. (46) argued that FFA may activate death receptors, such as Fas, DR5, trigger endoplasmic reticulum stress and result in mitochondria dysfunction. FFA lipotoxicity can be summarized in Fig 1-2.
FIGURE 1-2 Mechanism of free fatty acid induced inflammation
Contrary to other inflammatory mediators, adiponectin protects against the metabolic derangements that may result in type 2 diabetes, obesity, atherosclerosis and NAFLD. Several studies have reported a significant decrease in the serum levels of adiponectin in NAFLD patients (47-50). With respect to the expression of adiponectin in simple steatosis vs NASH, the data are not consistent (48, 51). Tokushige et al. (52) found that adiponectin SNPs were associated with the progression of liver fibrosis and insulin resistance in Japanese; by contrast, Wang et al. (53) failed to observe the association between adiponectin SNPs and NAFLD in a Chinese population although they found hypoadiponectinemia in NAFLD with metabolic syndrome. Xu et al. (54) found that delivery of recombinant adiponectin into NAFLD mice dramatically alleviated hepatomegaly and steatotic fatty liver and also significantly attenuated inflammation and the elevated levels of serum alanine aminotransferase. All these data indicate that adiponectin is involved in the pathogenesis of NAFLD and it could be promising target for the treatment of NAFLD.

1.3.5 Oxidative stress

An association between oxidative stress and NAFLD is well established in human and animal studies (55). Beta-oxidation within the normal liver takes place in the mitochondria, but in the context of NAFLD, this process can become overwhelmed as a result of increased FFA load, giving rise to reactive oxygen species (ROS). ROS induce oxidative stress, with subsequent activation of inflammatory pathways, and also mitochondrial damage (18). Cytochrome P450 2E1 (CYP2E1), a key enzyme to produce ROS, was increased in human and animal models of NASH and hepatocyte-specific CYP2E1 overexpression results in increased oxidative stress and nitrosative stress (56). Conversely, genistein, a strong antioxidant agent, significantly decreased
the plasma TNF-α level and remarkably prevented the emergence of NASH by improving the biochemical and histopathological abnormalities via attenuating oxidative stress (57).

1.3.6 Role of genetics in NAFLD

Accumulating evidence suggests that NAFLD may have a genetic component. Most of the studies in this field have been focused on the association between single nucleotide polymorphisms of some candidate genes and NAFLD. For example, several studies have suggested that TNF-α gene polymorphisms may play a role in the development of NAFLD. In a study of 102 patients with NAFLD, Tokushige et al. (58) reported that the frequency of two polymorphisms in the TNF-α promoter region was more common in patients with NASH as compared to patients with simple steatosis. Valenti et al. (59) also found that one of the polymorphisms in the TNF-α promoter was significantly higher in patients with NAFLD as compared to control patients without steatosis. The patatin-like phospholipase domain-containing protein 3, PNPLA3, belongs to the adiponutrin family (PNPLA1-5), which are genes mainly expressed in human liver and adipose tissue. The association of PNPLA3 with NAFLD will be discussed in more detail later.

In brief, variation of gene structure is not necessary to change the function of genes. It is still unknown how those polymorphisms are involved in the development of NAFLD. Future studies are needed to elucidate the pathophysiological mechanisms underlying genetic associations before they can be used to predict individual risk of steatosis, NASH and fibrosis and to develop genetic molecular therapeutic strategies.
1.3.7 Roles of dietary factors

It is clear that caloric excess is associated with obesity, but increased energy intake was not observed in patients with NAFLD. Is dietary composition associated with NAFLD? The following section will review the roles of macronutrients and micronutrients in the development of NAFLD. A Japanese study (60) found that there was no difference in total carbohydrate/energy intake between NAFLD patients and the general population, although there was an excess intake of carbohydrates in patients with NASH compared with patients with hepatic steatosis. Among carbohydrates, simple carbohydrate intake was higher in those with NASH. This study also showed that fat intake tended to be higher in patients with NAFLD compared with the general Japanese subjects. A study by Cortez-Pinto et al. (61) revealed that carbohydrate consumption was lower and total fat consumption was higher in patients with NASH compared with the general population. Conversely, Solga et al. (62) found that higher carbohydrate intake was associated with significantly higher odds of liver inflammation, while higher fat intake was associated with significantly lower odds of liver inflammation. However, the small sample size in NAFLD groups is a common limitation in above three studies. Recently, studies have examined the effect of different types of fat on NAFLD. Li et al. (19) demonstrated that exposure of murine or human hepatocytes to monounsaturated fatty acids (MUFAs) resulted in lipid accumulation without changes in cell viability. In contrast, cell incubation with saturated fatty acids significantly decreased cell viability and increased caspase activation and apoptosis. The authors argued that it is the nature rather than quantity of FFA that determines hepatic stress (46). In vivo studies, other groups found that monounsaturated fat (63) and n-3 fatty acids (64) alleviated intrahepatic lipid accumulation in animal models of NAFLD or patients.
Fructose, the sweetest of all naturally occurring carbohydrates, is widely used in the Western diet (e.g. soft drinks). Many studies have found that excessive consumption of fructose is associated with the development of metabolic syndrome and NAFLD (65-67). In a rat model, the histological evaluation showed that rats given a beverage containing 15% fructose had increased hepatic storage of lipids compared with the water control group (68).

A case control study by Ouyang et al. (69) showed that consumption of fructose in patients with NAFLD was nearly 2 to 3-fold higher than controls. In patients with NAFLD, hepatic expression of fructokinase (KHK), an important enzyme for fructose metabolism, and fatty acid synthase (FAS), an important enzyme for lipogenesis, were increased. The findings suggest that fructose may have a role in the pathogenesis of NAFLD in western countries as total fructose intake averages approximately 12% of total energy intake in the US population (70).

The potential rationale that fructose may cause liver injury can be briefly explained by the following mechanism: 1) Fructose is mainly metabolized in the liver as compared with glucose, which can be used in any tissue. The excessive acetyl-CoA produced from fructose provides substrates for triglycerides synthesis in liver, thereby fructose increases de novo lipogenesis. 2) Unlike glucokinase, the phosphorylation of fructose by fructokinase is not rate limited. The high activity of fructokinase in phosphorylating fructose to fructose-1-phosphate can lead to hepatic ATP depletion, which may cause hepatic ischemia. 3) Fructose-1-phosphate can activate mitogen-activated protein kinase kinase 7 (MKK7), which then stimulates the activation of mitogen-activated protein kinase 8. This kinase can trigger serine phosphorylation and subsequent inactivation of insulin receptor substrate-1 IRS-1, which results in insulin resistance implicated in the pathogenesis of NAFLD (68, 71).
Some studies reported plasma vitamin E and vitamin A levels in patients with NAFLD. Cankurtaran et al. (72) showed that 32% of NAFLD patients were vitamin E deficient and that a correlation was found between low vitamin E levels and hepatic steatosis. A study conducted in Brazil found that there was a significant correlation of low serum retinol and beta-carotene with NAFLD (73). In animal studies, the findings are controversial. Aguilar et al. (74) showed that abundant fat droplets were present in the cytoplasm of the hepatocytes in rats fed a vitamin A deficient diet for 3 months. By contrast, Oliveros et al. (75) found that vitamin A deficiency induced a hypolipidemic effect by decreasing serum triacylglycerol, cholesterol and HDL-cholesterol levels in rats. Also, liver fatty acid synthesis decreased, as was indicated by activity and mRNA expression of ACC. Unfortunately, liver fat content was not examined in the latter study, so these two studies may not be comparable.

In summary, the worldwide increased prevalence of NAFLD is paralleled to the global export of Western diet (71). The macronutrients and micronutrients of the diet may underlie the NAFLD epidemic, but the mechanisms by which they are involved in the development of NAFLD need to be fully elucidated.

1.3.8 Gut microbiota and NAFLD

Over the past years, studies have highlighted the role of gut microbiota in the development of obesity and metabolic disorders. One breakthrough in this area is that Backhed et al. (76) found that conventionally raised mice had over 40% more total body fat compared with those raised under germ-free conditions, even though the former ate 30% less diet than did the germ-free mice. Furthermore, they found that conventionalization of germ-free mice via colonization with a cecum-derived distal microbial community from normal mice resulted in a 60% increase in total
body fat and insulin resistance. These findings suggest that the gut microbiota are an important environmental factor that affects energy storage in the host. Furthermore, Ley et al. (77) found that ob/ob mice had a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes compared with their lean counterparts. A burning question is how diet changes the gut microbiota that may contribute to low grade inflammatory conditions, such as obesity and metabolic syndrome. Cani et al. (78) showed that mice fed a high-fat diet exhibited a significant increase in plasma lipopolysaccharide, which triggers the secretion of proinflammatory cytokines when it binds to the complex of CD14 and the toll like receptor 4 at the surface of innate immune cells. Several lines of evidence also suggest a role for the gut microbiota in the development of NAFLD. Cope et al. (79) found that gastrointestinal ethanol production was significantly higher in ob/ob mice, and that obese mice treated with neomycin decreased ethanol production level by 50%. Unfortunately, this study did not show liver histology after treatment with the antibiotics. A recent study showed that small intestinal bacterial overgrowth was associated with severe hepatic steatosis in morbid obese individuals (80). Gratz et al. (81) proposed a hypothesis that the change in gut microbiota in NAFLD leads to increased production of ethanol and lipopolysaccharides in the intestinal lumen, which increases hepatic oxidative stress.

1.3.9 Parenteral nutrition induced NAFLD

Parenteral nutrition (PN) is a lifesaving therapy for patients who have intestine failure or critically ill patients who cannot get adequate energy through enteral nutrition. However, patients on long term PN therapy may have liver injury including hepatic steatosis, cholestasis, and gallbladder (82). Hepatic steatosis is the most common liver injury among adult patients, and cholestasis often occurs with children patients (83). Many factors, such as fat free-PN, excess
calories input, excess lipid emulsion, contribute to the development of hepatic steatosis, the early stage of liver injury. Nishimura et al. (84) revealed that infusion of excessive glucose without lipid emulsion in TPN results in increased lipogenesis and decreased lipolysis in liver and as a consequence, fat free TPN leads to triacylglycerol accumulation as lipid droplets in the liver.

In short, multifactorial complex interactions between nutritional factors, lifestyle, gut microbiota and genetic determinants contribute the development of NAFLD. In the future, a comprehensive and individual based treatment strategy should be considered to combat this disease.

1.4 Treatment of NAFLD

At present, there is no consensus on the treatment of NAFLD. Derived from understanding of the pathogenesis of NAFLD, various treatment strategies have been developed. Lifestyle modification should include nutritional counseling with or without exercise. The pathophysiological basis for this approach is that weight reduction results in the loss of white adipose tissue, which decreases IR. Exercise can also improve muscle insulin sensitivity, which may improve the impact of IR on NAFLD (85). Many pharmacological therapies have been applied to the treatment of NAFLD in clinical trials. These clinical trials have provided encouraging but often mixed results. One main pharmacological therapy is insulin-sensitizing drugs, including metaformin and glitazones. Metaformin is an insulin-sensitizing biguanide that is widely used in the treatment of type 2 diabetes. Its major effects include reduction of hepatic glucose output, and increase of glucose uptake in skeletal muscle and adipocytes. In clinical trials, metaformin was reported to decrease aminotransferase levels, but did not significantly improve histological steatosis, necroinflammation or fibrosis (86). Glitazones, PPARγ agonists,
show promise for the treatment of NASH, but there is conflicting evidence of possible increased cardiovascular morbidity and mortality associated with their use.

1.5 Conclusions

NAFLD, the most common chronic liver disease in the Western world, is tightly associated with obesity and insulin resistance. The exact pathogenesis of NAFLD is not well defined although it is believed that both environmental and genetic factor contribute to its development. Despite a large number of drugs that have been tested to treat NAFLD, most studies have been small in size and of short duration, and results have been mixed. Therefore, a better understanding of the pathophysiological process that underlies the mechanism of NAFLD is urgently needed.

2. Retinoids and lipid metabolism

2.1 Vitamin A metabolism

Vitamin A is an essential micronutrient for human health. Vitamin A is indispensable for a variety of biological process, including vision, embryonic development, reproduction, and immune function. Vitamin A was first discovered about 100 years ago by two research groups in the US. As reviewed by Ross (87) and Wolf (88), going back to 1500 BC, Egyptian doctors already knew that the liver of ox, rich in vitamin A, can be used to treat night blindness although they had no idea about vitamin A at that time. Research on vitamin A is still very active. For instance, more than 48,000 citations for retinol and 31,000 for retinoic acid can be found in the Pubmed database. Vitamin A metabolism was extensively investigated in early studies. Recently, the roles of vitamin A in immune function and lipid metabolism have received great attention.
Vitamin A in foods is present in two forms: provitamin A, such as β-carotene, α-carotene and β-cryptoxanthin, which are found in plant food (e.g., carrots, papaya, squash) and preformed vitamin A, such as retinyl esters and retinol, which are mainly present in animal source food, such as liver, milk, cheese and butter. In the intestine, β-carotene is converted into retinal by β-carotene 15, 15'-dioxygenase and then subsequently retinal is reduced to the alcohol form, retinol by retinal reductase. Retinyl esters must be hydrolyzed by retinyl ester hydrolase to retinol before intestinal uptake. Within the enterocytes, retinol is re-esterified with fatty acids (e.g., palmitate and oleate) by lecithin:retinol acyltransferase (LRAT) to form retinyl esters. Retinyl ester is incorporated into lipid core of chylomicrons and transported to liver for storage.

In humans, under conditions of adequate vitamin A intake, 90% of the vitamin A is stored in liver (89, 90). It is believed that both stellate cells and hepatocytes are involved in retinoid metabolism in liver. Hepatocytes are responsible for taking up and processing of dietary retinoid into the liver; whereas, stellate cells are the cellular sites for retinoid storage in liver (91). When retinol is needed by extrahepatic tissues, the storage form retinyl ester within stellate cells is hydrolyzed to retinol and it is transferred back to hepatocytes (92). The molecular mechanism by which hepatocytes interact with stellate cells to regulate storage and trafficking of retinoid remains to be clearly established. In order to be transported to peripheral tissues, retinol must bind to retinol binding protein 4 (RBP4) produced by hepatocytes (93). Retinol binding protein 4, known as RBP4, is secreted by hepatocytes but also can be found in adipose tissue. In the circulation, the RBP4-retinol complex binds to transthyretin which prevents its loss through the kidney filtration. Although the main function of RBP4 is to transport retinol to peripheral tissues, accumulating evidence suggest that RBP4 contributes to insulin resistance and other components of metabolic syndrome (94-96), which will be discussed later.
The molecular mechanism by which retinol is absorbed by the cells was not clear until Sun et al. (97) identified the receptor for retinol binding protein, stimulated by retinoic acid-6, known as STRA6, in bovine retinal pigment epithelium cells. The discovery of Stra6 provided new insight into the role of retinol-RBP4-transthyretin complex transport system on vitamin A homeostasis. STRA6 is expressed in a variety of organs including brain, spleen, kidney, female genital tract, testis, heart and lung, but STRA6 is not present in intestine and liver, which are very important organs involved in uptake and storage of vitamin A. Just recently, Graham et al. (98) discovered a novel receptor for RBP4, named RBPR2, which regulates retinol uptake mainly in liver, intestine and adipose tissues. More interestingly, they also revealed that RBPP2 was induced in adipose tissue in obese mice, suggesting RBPP2 may modulate the effect of RBP4 on metabolic syndrome.

Within cells, retinol undergoes either esterification or a series of oxidations with the help of variety of chaperone proteins and enzymes. As for free fatty acid binding to the fatty-acid-binding proteins, retinoids need to bind to retinoïd-binding protein for further metabolism. To date, many types of retinoid-binding proteins have been identified to regulate vitamin A intracellular metabolism. It is believed that all-trans-retinol and all-trans-retinal are bound to cellular retinol binding proteins (CRBP I and CRBP II), whereas, all-trans-retinoic acid is associated with cellular retinoic acid binding proteins (CRABP I and CRABP II) (99). CRBPI plays an important role in the process of esterification of retinol to retinylester catalyzed by LRAT. In addition, retinol bound to CRBP I can be converted to retinoic acid through 2 steps of oxidation. In the first step, retinol is oxidized by retinol dehydrogenase (RDH) to form retinal, which is then converted to retinoic acid, the bioactive metabolite of vitamin A, by the action of retinal dehydrogenase (RALDH1,2,3 and 4) (100). It seems that CRBP II is also involved in
formation of retinylesters and oxidation of retinol. Similar to CRBPs, CRABPs regulate activity of enzymes which act on oxidation of retinoic acid (101). In addition, a growing body of information also suggests that CRABPs play a role in delivery of retinoic acid to nuclei of cells since both CRABP I and II were found in nuclei of cells. Controlling of RA concentration is critical for cell function since RA can regulate indirectly or indirectly more than 500 target genes (102). A cytochrome of the P450 gene family, CYP26, carries out the oxidation of retinoids including retinoic acid to form 4-oxo-RA, 4-hydroxy and 18-hydroxy metabolites, by which levels of RA are tightly controlled in response to excess or deficiency of intracellular retinoids. Of note, others and our studies showed that CYP26A1 and CYP26B1 gene expression is regulated by retinoic acid in vivo and in vitro (103-105).

2.2 Retinoid receptor and signaling

Vitamin A has a variety of biological functions in all vertebrates, regulating embryonic development, reproduction, cell proliferation and differentiation and immune function. All these biological functions of vitamin A mainly depend on the transcriptional activity of retinoic acid. Indeed, several lines of evidence suggest that the activity of RA can be through nongenomic process, such as retinolylation of protein. This review only focuses on the molecular mechanism of gene regulation by RA. It has been known that RA regulates biological processes through activation of two types of nuclear hormone receptors, namely retinoic acid receptors (RAR α, β and γ) and retinoic X receptors (RXR α, β and γ). All-trans-RA is able to bind with high affinity to retinoic acid receptors, whereas 9-cis-retinoic acid mainly activates RXR.

RARs and RXRs belong to a superfamily nuclear hormone receptors, which also include thyroid hormone receptors (TRs), vitamin D receptors (VDRs), peroxisome proliferator-activated
receptors (PPARs), liver X receptors (LXRs), farnesoid X receptor (FXR), and more. These nuclear hormone receptors share six conserved and well organized domains designated A–F. These domains are the ligand-independent activation function domain (AF1) located at the amino terminus of the retinoid receptors; and a conserved DNA-binding domain (C) in the center of receptors; a hinge region (D); and a multifunctional ligand-binding domain (LBD) in the carboxy terminus of receptors. In order to transduce retinoid signals, RAR and RXR need to pair with other to form dimers either as RAR/RXR complex or RXR/RXR complex, most as RAR/RXR heterodimers (106). Of note, RXRs interact not only with RARs but also with several other nuclear receptors, such as PPARs, LXRs, VDRs, FXRs and TRs. The signaling through PPAR/RXR regulates lipid metabolism, which will be discussed in more detail in other part of this thesis.

How is the retinoid signaling transduced to activate target genes? (Fig. 1-3). First step: in the absence of ligand, the heterodimer RAR/RXR binds to a specific DNA sequence on promoter region of target genes known as retinoid acid response element (RARE). RARE is composed of two direct repeats of a core hexameric motif, PuG(G/T)TCA, commonly separated by 1, 2 or 5 bp (referred to as DR1, DR2 and DR5 respectively). The heterodimer RAR/RXR bound to RARE recruits the corepressor NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic and thyroid hormone receptor). Then the corepressors recruit histone deacetylase (HDAC), which promotes chromatin condensation and repression of gene expression. Second step: in the presence of ligand, ligand-induced conformational changes in the receptors causes release of corepressors and recruitment of coactivators, which catalyze histone acetylation, methylation and phosphorylation on histone proteins, resulting in chromatin decondensation and enhancement of gene expression. Third step: once chromatin has been decondensed, the
RAR/RXR heterodimer is capable of recruiting the transcription machinery (e.g., RNA polymerase II) and mediators, thus initiating the transcription of target genes (107).
FIGURE 1-3 Model of retinoids signaling transduction (Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 2004;328:1-16.)
2.3 Retinoids and lipid metabolism

Accumulating evidence suggests that retinoids are involved in the development of NAFLD. A cross-section study reported that a high prevalence of NAFLD in 91 school children in Brazil and also found a trend between low serum retinol tends and hepatic steatosis (108). Another observational study of 145 obese subjects showed that individuals with NAFLD had significantly lower serum levels of β-carotene compared with those without NAFLD, although serum retinol was not different between the two groups (109). A study of 80 obese patients reported that serum retinol was inversely correlated with serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in obese patients with nonalcoholic fatty liver disease. Since no dietary retinoid intake was available in those human studies, it is hard to know if a low level of serum retinoid was due to decreased dietary retinoid intake or retinoid metabolism was impaired in subjects with NAFLD. Nevertheless, all these human studies indicate that retinoids may play a role in pathogenesis of NAFLD.

In animal studies, the findings are controversial. Aguilar et al. (74) showed that lipid droplets were accumulated in the hepatocytes in adult Wistar rats fed a vitamin A deficient (VAD) diet for 3 months. In addition, this study also found that vitamin A deprivation leads to liver injury as indicated by increased expression of hepatic fibronectin and collagen type IV in VAD rats. Consistent with above findings, hepatic lipid accumulation was significantly increased in mice fed VAD diet compared with those fed vitamin A sufficient (VAS) diet from day 10 of gestation to 9 weeks of age. Consistent with increased lipid and TG accumulation in VAD rat liver, this study also found different expression patterns of metabolic genes between mice on VAD and VAS. Specifically, genes encoding fatty acid oxidation, such as fatty acyl-CoA ligase 2, CPT1, medium-chain acyl CoA dehydrogenase, and peroxisomal acyl CoA oxidase, were significantly
decreased in VAD mice (110). Although these animal studies support the idea that retinol is inversely correlated with hepatic lipid accumulation shown in human studies, other investigators showed different findings. Oliveros et al. (75) found that vitamin A deficiency induced a hypolipidaemic effect by decreasing serum triacylglycerol, cholesterol and HDL-cholesterol levels in rats. Also, liver fatty acid synthesis was decreased, as was indicated by activity and mRNA expression of ACC, whereas fatty acid oxidation was increased, which was indicated by increased CPT1 expression. More interestingly, vitamin A supplementation ameliorated all the changes that occurred in VAD rats. The discrepancy of these findings may be explained by different species (rat vs. mouse), age of animals, and different diets.

Retinoic acid (RA), a bioactive metabolite of vitamin A, has been shown to regulate lipid metabolism by several groups. Palou et al. (111) showed that mice receiving subcutaneous injection of all-trans-RA (ATRA) had a 3-fold reduction of liver TG content compared with mice receiving vehicle. Consistent with TG change, ATRA treatment led to change of pattern of gene expression. Specifically, hepatic expression of genes related to fatty acid oxidation, such as CPT1, UCP2 and PPARα, were increased in mice receiving RA. By contrast, lipogenic genes, such as SREBP1c and FAS, were decreased in mice receiving RA. In a follow up study (112), the group further showed that ATRA induced expression of CPT1 and increased palmitate oxidation in HepG2 cells and concluded that ATRA favors fatty acid catabolism. Of note, this study also showed that ATRA significantly induced both SREBP1c and FAS in HepG2 cells, which had no any changes in total lipid accumulation assessed by Oil Red O staining. Induction of expression of SREBP1C and FAS genes by ATRA in HepG2 cells was supported by other groups (113, 114). This is inconsistent with Palou’s animal study showing a hepatic lipid reduction by ATRA (111). To explain the discrepancy, the authors proposed that the balance
between LXR:RXR and PPARα:RXR activities determines the roles of retinoids on lipid metabolism in liver. Of note, activation of PPARα:RXR increases transcriptional activities of fatty acid oxidation related genes, whereas LXR:RXR signaling increases expression of lipogenic genes. More specifically, in vivo, PPARα:RXR-mediated RA signaling is dominant so that RA treatment enhances fatty acid oxidation. By contrast, in cultured cells, the activities of PPARα:RXR and LXR:RXR are more balanced, therefore genes related to both fatty acids catabolism and anabolism are stimulated and the lipid content remains unchanged in HepG2 cells. However, the mechanism regulating the balance between those two signaling pathways remains unclear. Consistent with Palou’s animal study, Noy et al. (115) showed that high fat diet induced hepatic steatosis in mice was completely reversed upon 5-weeks of retinoic acid treatment. In addition, RA reduced abdominal and epididymal white adipose tissue mass, accompanied by a decrease in adipocyte cell size. The group further showed that activation of PPARβ/δ and RARs by retinoic acid contributes to the regulation of energy homeostasis. Therefore, these studies indicate that retinoids may be a therapeutic approach to treat metabolic diseases, such as obesity and NAFLD.

The involvement of retinoid metabolism in NAFLD has been confirmed by series of studies in Japan. In a case control study (116), expression of fifty-one genes regulating vitamin A metabolism was assessed by real time PCR in patients with NAFLD and in healthy individuals. The data showed that expression pattern of those genes are significantly different between NAFLD patients and health subjects. For example, genes involved in conversion between retinyl ester and retinol, such as CRBP1, LRAT, DGT1/2 and CES1, were highly expressed in NAFLD patients. Expression of retinal dehydrogenases, such as ADH1/2/3, RDH5/10/11, DHRS3 and RALDH1/3 were higher in NAFLD patients compared with healthy controls. This study
suggested that retinoid signaling is related to the pathogenesis of NAFLD. In an animal study (117), transgenic mice expressing dominant negative form RAR developed hepatic steatosis and hepatocellular carcinoma at 4 and 12 month age respectively. This study demonstrated that loss of RA signaling results in the development of hepatic steatosis and liver tumor. To study potential role of ATRA in the prevention of NAFLD, the group developed NAFLD model by feeding mice a high fat and high fructose diet and showed that all-trans-RA improved mouse hepatic steatosis. They further investigated possible mechanism preventing hepatic lipid accumulation and found that retinoic acid increased expression of lipolytic genes, such as PPARα and PPARβ and suppressed lipogenic genes, such as PPARγ, SREBP1 and FAS (118). Overall, data from animal study and cell culture work suggest that all-trans-RA is a potential therapy for the NAFLD.

Retinoids and vitamin A derivatives have been successfully used to treat some dermatologic diseases, such as severe acne, psoriasis and genodermatoses, and acute promyelocytic leukemia (119). However, vitamin A derivatives therapy leads to change of lipid profile. For instance, in a prospective study, patients with acne had increased plasma triglyceride, elevated LDL and decreased HDL after they received 13-cis-RA treatment (120). In another prospective study, the investigators observed that patients on 13-cis-RA treatment acquired hypertriglyceridemia and increased LDL/HDL ratio. In addition, elevated hepatic vitamin A content was observed in patients with diabetes more than 70 years ago. All these early observation indicated that retinoids have an impact on glucose and lipid metabolism.
Retinal is generated from the oxidation process by alcohol dehydrogenases on retinol. The only biological function of 11-cis retinal is involved in vision cycle. Recently, a series of studies by Plutzky et al. revealed that retinal and retinal dehydrogenase-1 can regulate carbohydrate and lipid homeostasis (121-124). In the first study, Plutzky et al. (121) showed that mice-lacking the retinaldehyde-catabolizing enzyme dehydrogenase 1 (Raldh1) resisted high-fat diet induced obesity and insulin resistance and had decreased lipid accumulation in liver and decreased total liver weight compared with wild-type mice. They also demonstrated that in ob/ob mice, administrating retinal or a Raldh inhibitor reduced fat and increased insulin sensitivity. In the second study, Ziouzenkova et al. (123) demonstrated that Raldh1 deficiency mice are protected from diet induced obesity and fatty liver through suppression of gluconeogenesis and lipogenesis in liver and enhancement of fatty acid oxidation in muscle. A more recent study by Kiefer et al. (124) showed that retinal promotes UCP-1 expression in white adipocytes by activation of RAR and recruitment of PGC-1α. Overall, data from Pluzky’s group support the idea that retinal dehydrogenase-1 and its substrate retinal regulate energy homeostasis, although those findings need to be replicated by other groups.

There is increasing evidence suggesting that retinol binding protein 4 (RBP4) plays a role in metabolic syndrome. A pioneering study by Kahn et al. (125) showed that serum RBP4 is elevated in both patients with obesity and type 2 diabetes and insulin-resistant mice induced by GLUT4 knockdown in adipose tissue. More interestingly, Kahn et al. (125) further demonstrated that decreasing serum RBP4 by some chemicals improved insulin sensitivity and glucose tolerance in obese mice fed on high-fat diet. The association between serum RBP4 and insulin resistance has been confirmed by many other investigators (125-127). In a cross section study,
the data showed that serum RBP4 levels were significantly correlated to liver fat but not with total body, visceral, or subcutaneous abdominal fat (128). Several other human studies also confirmed that serum RBP4 is correlated to NAFLD (129-131). However, a number of other human studies failed to find an association between RBP4 and insulin resistance. Thus, the relationship between RBP4 and insulin resistance is still debatable. Although the role of RBP4 on metabolic syndrome is controversial, several groups actively investigate the molecular mechanism by which RBP4 is connected to metabolic diseases. Knockdown of RBP4 expression in C57BL/6 mice by injection of RBP4 specific RNA oligonucleotide ameliorated mouse hepatic lipid accumulation induced by high fat diet, which supports the potential role of RBP4 in the pathogenesis of NAFLD (132). A most recent study showed that treatment with RBP4 activated SREBP1c gene expression and protein nuclear translocation in HepG2 cells, thereby enhancing expression of lipogenic genes, such as FAS, ACC1 and DGAT-2. As a result, TG synthesis in HepG2 cells were significantly induced (133).

3. PNPLA3/ADIPONUTRIN

3.1 Discovery of PNPLA3

Adiponutrin (ADPN) was initially discovered more than a decade ago in fully differentiated mouse 3T3-L1 and 3T3-F442 cell lines, which serve as a faithful model to study adipogenesis in vitro (134). In this study, investigators sought to identify novel genes involved in adipogenesis using a differential display technique. As expected, the investigators found that a great number of genes are differentially expressed during preadipocyte maturation. They specially explored the characteristics of a 3.2-kilobase mRNA, called ADPN, since its sequence was not listed on GeneBank at that time. This study revealed that ADPN is exclusively expressed in mouse
inguinal and epididymal white adipose tissues and brown adipose tissues, although subsequent studies showed that it is not true because ADPN can be found in variety of tissues, including brain, heart, intestine, stomach, skin, lung, spleen, testis and more. The discrepancy may be explained by different techniques; Northern blotting used by Baulande et al. (134) may not detect low levels of mRNA, but reverse transcription quantitative PCR used by other groups is very sensitive to low levels of mRNA. This pioneering study showed that ADPN gene expression is under the control of nutritional signals. For instance, ADPN is almost undetectable in fasted mice but is dramatically induced by high carbohydrate diet re-feeding. The expression of ADPN is also increased in obese Zucker rats compared to control lean rats. In addition, this study also showed that overexpressed exogenous ADPN localized to membrane. Since the newly identified transcript was exclusively expressed in adipose tissue and regulated by nutritional signals, it was designated as “ADPN”.

3.2 Structure of PNPLA3/ADPN

Baulande et al. (134) obtained a full length of cDNA of 3007bp encoding 413 amino acids with molecular mass of 48 KD. Sequence analysis of ADPN protein suggested that it contains four transmembrane domains. Database searches also identified three homologous human proteins, including glutamine synthetase 2 (GS2), transport secretion protein-2 (TTS2) and GS2-like protein, which did not have functional information at that time. Three years later, Jenkins et al. (135) searched for candidate TAG lipase/acylglycerol transacylase and identified a subfamily of putative iPLA2 enzymes, including ADPN, GS2, TTS2 and GS2-like proteins. All these members of this family contain specific motifs: the calcium-independent phospholipase A2 (iPLA2) dual signature nucleotide ((G/A)XGXXG), and lipase (GXSXG) consensus sequence motifs.
3.3 Functional analysis of ADPN in early studies

Jenkins et al. (134) was the first group to perform functional analysis for human ADPN in vitro, in which human ADPN was expressed in Sf9 cells and purified from cytosolic and membrane fractions. They found that human ADPN was capable of breaking down TG in triolein lipase assay and also catalyzing the synthesis of diolein or triolein from mono-olein (acyl donor) in the presence of mono-olein and diolein respectively. Consistent with Jenkins’s observation, Lake et al. (136) demonstrated that human ADPN immunoprecipitated from HEK293 cells possesses lipase activity as indicated by cleavage of 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester (DGGR), whereas mutant form of human ADPN had significant lower lipase activity compared to the wild-type protein. However, when human ADPN was overexpressed in HEK293 cells, it did not decrease the incorporation of radiolabeled oleic acids into triglycerides; rather, HEK293 cells transfected with human ADPN showed a trend toward increased TG content. In addition, Kershaw et al. (137) showed that knockdown of ADPN in 3T3-L1 by small interference RNA had no effect on glycerol and FFA release. If ADPN had lipase activity we would expect decreased TG content when it is overexpressed in cells and decreased glycerol and FFA release when ADPN is knock down. The discrepancy between results from cell free system in vitro and cell lines indicates that additional factors present in cells have an impact on lipogenic or lipolytic activities of ADPN or some compensatory mechanism is activated to balance loss or expression of human ADPN.

3.4 Nutritional regulation of ADPN in animals

Baulande et al. (134) not only discovered ADPN but also investigated its gene expression in several conditions. In vitro, ADPN mRNA is dramatically induced during the process of 3T3-L1 preadipocyte cell differentiation into mature adipocytes and the expression pattern of ADPN is
reminiscent of those of lipogenic genes, such as FAS, SREBP1c. In vivo, ADPN mRNA in epididymal fat pads was almost undetectable when Swiss mice were fasted for 19 hours. Refeeding mice for 8 hours rescued ADPN mRNA expression to the normal control levels. Furthermore, they showed that ADPN mRNA was increased by 30-50-fold in obese Zucker rats compared with their lean counterparts. Kershaw et al. (137) confirmed previous findings by showing that ADPN is induced during adipogenesis in 3T3-L1 and that ADPN expression was decreased by fasting and rescued by re-feeding. More interestingly, the group revealed that the pattern of adipose triglyceride lipase (ATGL) gene expression is opposite to that of ADPN. Taken together, studies from these two groups suggest that ADPN plays a role in adipogenesis and ADPN gene in adipose tissue is regulated by energy shift (e.g. starvation and refeeding).

Polson and Thompson (138, 139) examined the effect of four different diets with different macronutrient on ADPN gene expression in rats. In their first study, ADPN gene expression was analyzed in white adipose tissue in rats fed with a high sucrose diet for 3 hours after 21 hours fasting and was shown to be induced about 5-fold compared with pre-meal levels (138). Furthermore, they showed that induction of ADPN was blocked when rats were injected with actinomycin-D before the meal, suggesting that induction of ADPN gene is due to increased transcription but not mRNA stability. In the second study, the investigators determined the effects of both high fat diet (70% fat by weight) and high protein diets (70% protein by weight) on adipose ADPN gene expression. Similar to that seen in the first study, high protein diet induced ADPN gene expression by about 8-fold 3 hours after the meal. Conversely, the diet either high in saturated fat or polyunsaturated fat failed to induce ADPN gene expression. The combination of above studies shows that the diets with different macronutrients differentially regulate ADPN gene expression. Different response of ADPN to different diet is not likely
attribute to the energy content in those diets, since the studies showed that rats received same amount of total energy from different diets. What are the mechanism(s) underlying the effects of different macronutrient on ADPN gene expression? The investigators hypothesized that hormonal and/or neural signals triggered by different diets mediate ADPN gene expression. In addition, direct effect from macronutrient itself cannot be excluded.

3.5 Nutritional regulation of human ADPN
Possibly because of ethical considerations about collection of human adipose tissue, there were no data regarding human ADPN before 2004 until Liu et al. (140) conducted a study on ADPN expression in obese women and healthy control subjects. In this study, subcutaneous adipose tissue samples were collected by needle biopsy from 35 obese and 13 lean healthy women after an overnight fasting. Adipose ADPN gene expression levels between obese and lean subjects were not significant different. However, Johansson et al. (141) reported that levels of ADPN mRNA were significantly higher in obese subjects compared with lean controls in both subcutaneous and visceral adipose tissues. Liu et al. (140) also performed two energy restriction studies in those obese women to examine how energy shift regulates human ADPN gene expression. In the first energy restriction study, obese women were given a very low-calorie diet, which only provides 30% of required daily energy intake, for 2 days followed by refeeding with normal diet for another 2 days. The data show that ADPN gene expression was reduced by 36% in subcutaneous adipose tissue by the very low-calorie diet (VLCD), whereas refeeding rescued the levels of ADPN gene to the baseline. In the second energy restriction study, another group of obese women were give a low-calories diet, which corresponds to 70% of required energy intake, for 3 weeks. Consistent with 2-day energy restriction study, the 21-day energy restriction resulted in 58% decrease of ADPN mRNA in obese subjects. Data from this study clearly
showed that human ADPN gene expression is regulated by energy balance, which is consistent with observations in rodents showing ADPN gene is upregulated upon feeding and downregulated by starvation (134).

3.6 Regulation of ADPN by insulin

To further understand how hormonal signals regulate ADPN gene expression, Kershaw et al. (137) studied the effects of insulin on ADPN gene expression both in vitro and in vivo. The initial study showed that ADPN expression was suppressed when fully differentiated 3T3-L1 cells were incubated in serum-free medium, which indicates that some factor(s) in serum are critical for ADPN expression. Then they showed that supplementation with 1ng/ml insulin in serum free medium induced ADPN gene expression. Furthermore, they observed the similar effects of insulin on ADPN gene expression in mice, which were injected with the drug streptozotocin (STZ), which results in insulin deficiency as it kills pancreatic beta cells. Thus, both in vitro and in vivo data support the concept that ADPN gene expression in rodents varies in response to insulin signaling. Consistent with this animal study, in a human study, Moldes et al. (142) investigated the regulation of ADPN gene expression by insulin through the glucose clamp technique. In this study, 23 lean healthy males participated in three clamp experiments: euglycaemic hyperinsulinemic (EGHI), hyperglycemic euinsulinemic (HGEI) or hyperglycemic hyperinsulinemic (HGHI). In all three experiments, ADPN gene expression significantly increased compared to before clamp. Compared with the basic values, subcutaneous adipose ADPN gene expression was increased by 2 fold during HEGI clamp, 8 fold during EGHI, and 10 fold during HGHI. Taken together, data from animal and human studies showed that ADPN is regulated by insulin. Insulin is a very important hormone involved in regulation of carbohydrate and lipid metabolism. For instance, insulin stimulates lipogenesis through the activation of
lipogenic enzymes, such as FAS and SREBP1c. On the other hand, insulin inhibits lipolysis through the suppression of lipolytic enzymes, such as ATGL and HSL. The upregulation of ADPN by insulin may indicate that ADPN plays a role in lipogenesis.

In summary, ADPN was initially discovered in adipose tissue and regulated by energy shift. In vitro data suggest that ADPN has both lipase and transacylase activities.

3.7 NAFLD associate gene PNPLA3

A single nucleotide polymorphism (SNP) is defined as a nucleotide variation in a DNA sequence found in different individuals of the same species. For example, a SNP may substitute the nucleotide guanine (G) for the nucleotide cytosine (C) in a certain site of a gene. Single-nucleotide polymorphisms may locate within the coding region of genes or non-coding regions of genes. Because of degeneracy of the genetic code, SNPs within a coding region may not alter the sequence of the protein encoded by the gene (143).

Most studies about ADPN focused on its regulation and function in adipose tissue before Cohen and Hobbs’s group (144) reported a strong association between a SNP on human ADPN gene and hepatic lipid content in three populations compromising Hispanics, European American and African American. The variant is a replacement of cytosine (C) by guanine (G) that changes amino acid 148 in PNPLA3 from isoleucine to methionine. In particular, the highest frequency of the G allele was in Hispanics (49%) and the lower frequency G allele was observed in European Americans (23%) and African Americans (17%). The allele frequencies in these three ethnic populations match with the prevalence of NAFLD in those populations as another study showed that the prevalence of hepatic steatosis was highest among Hispanics (45%), lower in whites (33%) and blacks (24%) (7). Cohen and Hobbs’s study also examined the association of G allele with a number of other metabolic parameters, such as BMI, HOMA-IR, plasma concentrations of
triglyceride, total cholesterol, VLDL and HDL, but no significant association was observed between G allele with those parameters. Cohen and Hobbs’s study really impacts the research direction focus shifting from adipose tissue to liver in this field.

Subsequent studies confirmed the association of the PNPLA3 G allele with risk of NAFLD in different populations. A genetic association study in Finland reproduced the association of rs738409 G allele with liver fat content in 291 Finish individuals. This study also showed that there was no significant association between rs738409 G allele with BMI, fasting plasma glucose, insulin sensitivity, LDL, HDL. The result is consistent with Romeo’s study where liver fat but not other metabolic parameters were associated with rs738409 G allele. Similar findings were reported in Argentina, Germany, Austria, Italia, Japanese and Chinese populations (145-151).

The prevalence of NAFLD in children is about 10%, but the risk is significantly increased in obese children. In order to understand how the SNP on PNPLA3 affects the risk of NAFLD in children, Romeo first conducted a genetic association study in 475 overweight and obese children in Italy (152). In this study, a significant increased frequency of hepatic steatosis in children with rs738409 G allele (41%), compared to those with rs738409 C allele (13%) was observed. Similar to data generated from adults, this study failed to find an association between rs738409 G allele with the component of metabolic syndrome, such as serum insulin, plasma triglycerides, total cholesterol and HDL-cholesterol. Subsequent studies confirmed that rs738409 G allele predisposes children to liver steatosis in several ethnic populations (153-156).

Interestingly, a growing body of evidence also suggests that rs738409 G allele also plays a role in other liver injuries. Tian et al. (157) performed a genetic association study in 1221 alcoholic
users who were divided to 3 groups: normal liver function (control), alcoholic fatty liver disease (ALD) and alcoholic cirrhosis. This study showed that rs738409 G allele in PNPLA3 is strongly associated with ALD and cirrhosis. Consistent with Tian’s findings, another group showed that frequency of rs738409 G allele in PNPLA3 in alcoholic cirrhosis patients was significantly higher than those without steatosis and normal ALT/AST (158). In line with the concept that rs738409 G allele is associated with liver damage in alcoholic users, Nischalke et al. (159) found that rs738409 G allele is also a strong genetic risk factor for hepatocellular carcinoma in alcoholic cirrhosis.

In summary, the single variant rs738409 in PNPLA3 is attributable to a broad range of liver injuries, including simple hepatic steatosis, alcoholic fatty liver disease, cirrhosis, and hepatocellular carcinoma. The key questions are what the biological functions of PNPLA3 in the liver are and how the SNP in PNPLA3 contributes to those liver injuries.

Since PNPLA3/ADPN was discovered in mouse adipose tissue a decade ago (134), many investigators have paid a lot effort to understand its nutritional regulation and biological function. Especially, after the rs738409 SNP on PNPLA3 was repeatedly reported to associate with NALFD, it triggers great interest in this field to explore the role of PNPLA3 in glucose and lipid metabolism. Jenkins et al. (135) was the first to report that recombinant human ADPN has both triglyceride lipase and transacylase activities in vitro. Subsequent studies confirmed that ADPN possesses lipase (136) and hydrolytic activity (160) in vitro. However, its physiological function in vivo still remains controversial. In functional studies, He et al. (161) showed that overexpression of human wild type of PNPLA3 in mouse had no effect on TG content, whereas mutant PNPLA3 leads to increased lipid accumulation in mouse liver. The investigators argued that the mutant form PNPLA3 disrupts normal hydrolytic activity of wild type of PNPLA3,
thereby leading to hepatic lipid accumulation. Consistent with potential hydrolase activity of PNPLA3, Huang et al. (160) showed that purified human PNPL3 had hydrolytic activity for triacylglycerols, diacylglycerols and monoacylglycerols in vitro. In addition, a more recent study showed that PNPLA3 is involved in VLDL secretion in both human and in vitro studies (162). The authors proposed that the decreased hydrolase activity of 148M mutant form PNPLA3 resulted in decreased FFA supply for VLDL synthesis and subsequent TG accumulation. By contrast, Qiao et al. (163) showed that overexpression of mouse PNPLA3 in primary hepatocytes increased TG content and knockdown of PNPLA3 inhibited lipid accumulation induced by overexpression of SREBP-1c. The group concluded that PNPLA3 promoted lipogenesis in mouse primary hepatocytes by functioning as a lipogenic gene. Consistent with the lipogenic function of PNPLA3, Kumari et al. (164) revealed that both murine and human PNPLA3 can catalyze conversion of LPA to PA by functioning as a member of LPAAT enzymes. In addition, Kumashiro et al. (165) recently demonstrated that knockdown of PNPLA3 with specific antisense oligonucleotides prevented hepatic steatosis in high-fat-fed rats and reduction of PA and PA/LPA ratio were also observed in this study. More interestingly, two studies failed to identify any metabolic changes including TG metabolism in global PNPLA3 knockout mice, suggesting that PNPLA3 is not a major player in glucose/lipid metabolism or other compensatory factors are activated in response to the loss of PNPLA3 (166, 167).

In summary, how PNPLA3 contributes to the development of fatty liver disease is still a puzzle. For future studies, researchers have to apply different thinking and approaches to explore the function of PNPLA3. For instance, Sookian et al. (168) proposed a hypothesis that the 148M mutant form of PNPLA3 may a play a role in modifying miRNA target sites, thereby increasing fatty liver susceptibility. In addition, microarray analysis can be performed in vitro and in vivo.
models with overexpressing PNPLA3 or knockdown of PNPLA3 in order to identify some genes that can be compensatory for PNPLA3 abnormalities.
CHAPTER 2

STATEMENT OF HYPOTHESIS
A significant change in American dietary pattern has occurred over the last few decades due to increased consumption of carbohydrate and reduced fat intake. More specifically, fructose, the simple carbohydrate has increased from 3.9% of total energy intake in 1977 to 9.2% of total energy intake in 2000, accompanied by the rise of obesity, insulin resistance and NAFLD (169). In addition, studies indicate that some micronutrients, such as vitamins E, C and retinoids, are also regulators in the development of NAFLD (118, 170). Lastly, several lines of evidence suggest that weight reduction and increased physical activity led to an improvement in serum liver enzymes, and reduced hepatic fatty infiltration (171, 172). Therefore, we hypothesize that dietary factors, physical activity and hepatic genes are all involved in the development of NAFLD.

Accordingly, four aims were designed to test the hypothesis:

1. To investigate the roles of high simple carbohydrate diet, unsaturated fat and retinal supplementation in the development of NAFLD. This could be tested by examining hepatic lipid accumulation and inflammation markers in mice fed a high simple carbohydrate diet with or without supplementation of unsaturated fat and/or retinal in vivo. Specifically, we hypothesized that supplementation with lipid emulsion (LE), rich in C-18 unsaturated fatty acids, and/or the vitamin A metabolite (RAL) will prevent hepatic steatosis induced by a high carbohydrate liquid diet (parenteral nutrition formula, PN) in mice.

2. To clarify the roles of retinoids, including retinal, retinoic acid and β-carotene in the development of hepatocellular steatosis in the liver cells. This could be tested by investigating how aforementioned retinoids have an impact on hepatocellular steatosis induced by high glucose medium or fatty acids in HepG2 cells.
3. To explore the biological function of NAFLD associated gene, PNPLA3. This could be tested by investigating how nutrients, hormones and physical activity modulate PNPLA3 gene expression in mice and HepG2 cells. In addition, PNPLA3 gene knockdown in HepG2 cells by using PNPLA3 specific siRNA can provide direct evidence for understanding the biological function of PNPLA3.

4. To explore the potential role of fibroblast growth factor 21 in hepatic lipid metabolism. This could be tested by evaluating how nutritional, hormonal signals and physical activity regulate FGF21 expression in mice and in HepG2 cells.
CHAPTER 3

STUDY ONE

Adapted from

Ito K, Hao L, Wray AE, Ross AC. Lipid emulsion administered intravenously or orally attenuates triglyceride accumulation and expression of inflammatory markers in the liver of nonobese mice fed parenteral nutrition formula. J Nutr 2013;143:253-259
Abstract

NAFLD is characterized by pathological accumulation of lipid in liver in the absence of significant alcohol intake. Dietary factors play an important role in the development and progression of NAFLD. Specially, higher carbohydrate and lower fat diet has been shown to predispose individuals to develop NAFLD. We hypothesized that supplementation of lipid emulsion (LE), rich in C-18 unsaturated fatty acids, and/or the vitamin A metabolite retinal (RAL) will prevent hepatic steatosis induced by a high carbohydrate diet in mice. In Expt.1, mice were fed normal chow diet (NC), or high carbohydrate diet (HCD) only or HCD with supplementation of LE (200µL i.v.), RAL (1µg/ WB g), or both LE and RAL for 4 weeks. In Expt.2, mice were assigned to four groups receiving normal chow diet (NC), or HCD only, or HCD plus 4.0% LE (as percentage of total energy), or HCD plus 13.5% LE (as percentage of total energy) for 5 weeks. Both histological and biochemical lipid data showed that all HCD only mice developed hepatic steatosis compared with mice fed normal chow diet and supplementation of LE reduced lipid accumulation in a dose dependent manner. The hepatic total retinol was higher in the RAL-fed mice, but RAL did not reduce lipid accumulation induced by HCD diet. The transcripts for SREBP-1cFAS were strongly induced by HCD only diet, suggesting that HCD induced hepatic steatosis through enhanced de novo lipogenesis. However, supplementation of LE suppressed de novo lipogenesis, as both SREBP-1c and FAS were significantly decreased in LE supplementation groups. We did not observe hepatic macrophage infiltration in the hepatic steatosis model, but we found that the inflammation markers, CCL2 and APCS, were induced by HCD only diet and inhibited by LE supplementation. In summary, supplementation of LE effectively prevented mouse fatty liver induced by a high dextrose formula and suppressed hepatic inflammation, but the vitamin A metabolite, retinal did not.
Introduction

Most current studies exploring the potential therapy for NAFLD use high fat diet induced mouse model; however, the concept that dietary fat account for the development of obesity received challenge (173) and the high fat diet (60% fat) in animal models may not reflect the scenario in human. For the past few decades, the most significant change in American diet is an increase in refined-carbohydrates (173). Most recent study showed that carbohydrate intake as the percentage of total energy increased from 44.0% to 48.7%, fat intake as the percentage of total energy decreased from 36.6% to 33.7%, and protein intake as the percentage of total energy decreased from 16.5% to 15.7%, by comparison of NHANES I (1971-1975) and NHANES 2005-2006 data (174). This dietary change occurred in association with an increased prevalence of obesity in past three decades. The role of RA, a biologically active metabolite of vitamin A, in lipid metabolism is still under debate (175, 176). The only known function of retinal is to modulate vision cycle; however a recent study showed that retinal suppressed adiposity and prevented the development of hepatic steatosis in ob/ob mice, suggesting that retinal is also a regulator in lipid metabolism (121). Based on above observations, we aimed to examine if administration of unsaturated fatty and/or retinal will impact the development of fatty liver induced by a high simple carbohydrate diet in mice. For these studies, as a high carbohydrate diet we used a glucose-rich formula diet, which is used clinically for total parenteral nutrition. Here, we reported that supplementation of unsaturated fat prevented hepatic steatosis while inhibiting two main regulators, (SREBP1c and FAS and reducing inflammatory markers in mouse liver. However, we did not observe any beneficial effect of all-trans-retinal on hepatic lipid accumulation.
Materials and Methods

Animal protocols. Animal protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. In Expt. 1, 5- to 6-wk-old male C57/BL6 mice (Taconic, n=7–9/group) were divided into 5 groups: a group fed a stock rodent chow diet [normal chow (NC), Rodent Diet 5001, Lab Diet] as a reference group for comparison with the HCD only group. This diet contained 239 g/kg protein, 50 g/kg fat, 51 g/kg fiber, and 3020 kcal/kg. The other groups were: HCD only, HCD+RAL (RAL described below), HCD+LE (LE described below), and HCD+RAL+LE. The HCD formula was the only source of nutrition and hydration. HCD consisted of CLINIMIX E 5/20 sulfite-free injection (Baxter Healthcare) (see table 3.1) containing 20% dextrose, 5% amino acids, and electrolytes supplemented with multiple vitamins for infusion (INFUVITE Pediatric, Baxter) (see Table 3.2) and minerals and trace elements (8 mg/L zinc chloride, 6.4 mg/L cupric sulfate, 1.2 mg/L manganous sulfate, 80 mg/L chromium chloride, 176 mg/L sodium selenite, and 1.13 g/L ferrous sulfate). The HCD solution was added daily to calibrated bottles fed to 2–3 mice/cage. Body weight (BW) and the volume of PN formula used were recorded daily. For the RAL treatment, all-trans-retinal (Sigma-Aldrich and Santa Cruz Biotechnology) was dissolved in soybean oil (Sigma-Aldrich) and delivered directly into the mouth at a dose of 1 mg/g (BW orally each day); all other groups received an equivalent amount of soybean oil, calculated to provide sufficient essential FAs to prevent essential FA deficiency. For the LE treatment, mice in Expt. 1 received Intralipid 20% (Baxter) (see table 3.3) injected into the retro-orbital sinus, 100 µL/eye to both eyes daily. Mice were briefly anesthetized before injection using isoflurane-oxygen inhalation; a tetracaine analgesic drop was applied to each eye after injection. In Expt. 2, we further tested LE as the more promising of these agents for ameliorating the development of fatty liver in HCD-fed mice.
and we eliminated injections, which could be a source of inflammation. Twenty 5-wk-old male C57/BL6 mice, n=5/group, were divided into 4 groups: NC, as in Expt. 1; HCD solution as described above, which contained 0.5% of total energy as Intralipid for the prevention of essential FA deficiency; HCD+4% LE; or HCD+13.5%LE, equal to the percent of energy from fat in the NC diet. The HCD+LE solutions were mixed daily and fed in calibrated feeding bottles. Intake was measured daily and mice were weighed every 3–4 d. After 4 wk (Expt. 1) or 5 wk (Expt. 2), mice were individually killed with CO₂. BW, liver, and epididymal fat pad weights were recorded. Blood for hematocrit and plasma preparation was collected in heparinized syringes. Portions of liver and epididymal adipose tissue were snap frozen in liquid nitrogen and other portions were fixed in formalin or snap-frozen with Optimal Cutting Temperature compound (Sakura Finetek Tissue-Tek, Fisher) and stored at -80°C.

**Histology.** Hematoxylin and eosin (H-E) staining was performed on 5-mm sections of formalin-fixed, paraffin-embedded liver and epididymal adipose tissue. Oil Red O staining was used to detect hepatic lipid droplets. Briefly, 8-µm-thick, frozen sections of liver were air-dried for 15 min and fixed with 3.7% formaldehyde for 1 hour. An Oil Red O working solution was applied to each section and incubated for 30 min. To prepare the Oil Red O working solution, 12 mL of a stock solution consisting of 250 mg Oil Red O dye, 30 mL triethyl phosphate was mixed with 8 mL distilled water and filtered. Slides were counterstained with hematoxylin.

**Lipid assays.** Liver total lipid was extracted from 100 mg of liver using the Folch method (177). After solvent evaporation, the total weight was measured gravimetrically. The plasma TG concentration was quantified by a TG quantification kit (BioVision) and liver TG was assayed by a modification of the method of Sardesi and Manning (178). The lipid extract from 50 mg liver tissue was applied to a column of 3% water-deactivated aluminum oxide and the TG fraction was
eluted with 25% diethyl ether in hexane (178). Then 100-µL aliquots of the elute were used in duplicate for the TG assay, measured at 415 nm with triolein as a standard.

**Liver retinol analysis.** The concentrations of retinol in plasma and liver were quantified by ultra-performance liquid chromatography using an Acquity (Waters) system and a reverse-phase C-18 column. Plasma (20 µL) or lipids extracted from 100 mg liver (12) were saponified in 5% (wt:v) potassium hydroxide in ethanol containing 1% pyrogallol (179) and then analyzed by ultra performance liquid chromatography.

**qRT-PCR.** Total RNA was extracted from 100 mg liver tissue using Trizol reagent (Life Technologies), homogenized until the mixture became smooth, and the RNA was isolated. cDNA was synthesized from 1 µg total RNA in 20 µL of the reaction mixture using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). Either iQ SYBR Green Supermix (Bio-Rad) or Fast SYBR Green Master mix (Applied Biosystems) was used for qRT-PCR.

**Statistical analysis.** Data are reported as the mean ± SEM. Student’s t test was used to compare the HCD group with the NC group only. One way ANOVA with Tukey’s post hoc test was used to compare the groups that were fed HCD-based diets by using Prism5 software (GraphPad Software). P < 0.05 was considered significant.
Table 3.1 Composition of CLINIMIX

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**Table 3.2 Composition of INFUVITE Pediatric**

<table>
<thead>
<tr>
<th>Ingredients of multiple vitamins</th>
<th>Each 5ml contains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (Vitamin C)</td>
<td>80mg</td>
</tr>
<tr>
<td>Vitamin A (as palmitate)</td>
<td>2300 IU&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin D (cholecalciferol)</td>
<td>400IU&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thiamine (Vitamin B&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>1.2mg</td>
</tr>
<tr>
<td>Riboflavin (Vitamin B&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>1.4mg</td>
</tr>
<tr>
<td>Pyridoxine HCL (Vitamin B&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>1mg</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>17mg</td>
</tr>
<tr>
<td>Dexpanthenol</td>
<td>5mg</td>
</tr>
<tr>
<td>Vitamin E (dl-α-tocopheryl acetate)</td>
<td>7IU&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.2mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>140mcg</td>
</tr>
<tr>
<td>Biotin</td>
<td>20mcg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>1mcg</td>
</tr>
</tbody>
</table>

<sup>1</sup> Vitamin A 2,300 IU equals 0.7mg

<sup>2</sup> Vitamin D 400IU equals 10mcg

<sup>3</sup> Vitamin E 7IU equals 7mg
Table 3.3 The major component fatty acids in Intralipid

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Percentage of total fatty acids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic</td>
<td>44-62</td>
</tr>
<tr>
<td>Oleic</td>
<td>19-30</td>
</tr>
<tr>
<td>Palmitic</td>
<td>7-14</td>
</tr>
<tr>
<td>Linolenic</td>
<td>4-11</td>
</tr>
<tr>
<td>Stearic</td>
<td>1.4-5.5</td>
</tr>
</tbody>
</table>
Results

Liver weight, but not growth, was changed by HCD diet and LE treatment. In both Expt. 1 and Expt.2, we developed a mouse model of the high carbohydrate diet feeding for hepatic steatosis. We first evaluated the effects of HCD only diet and treatment with LE or retinal on growth and liver weight. In Expt. 1, all mice had a similar weight gain and final body weight (not shown). In both Expt. 1 and Expt.2, overall food intake among HCD groups was not significantly different. We could not determine food intake in NC mice, since we did not record how much they eat. In Expt. 2, the final body weight was same among all groups. Liver weight as percentage of body weight was significantly decreased in all HCD groups compared with NC mice in Expt.1. However, we observed that liver weight was increased in HCD only group compared with NC mice, and LE treatment produced liver weight that was significantly lower than that of HCD only mice ($P<0.05$) and comparable to that of NC mice. Hematocrit is in normal range in all mice in either experiment.

HCD induced hepatic steatosis is reduced by LE but not RAL. Histological examination showed that macrovesicular and microvesicular steatosis was observed in mice fed HCD only compared with NC mice in Expt.1 and Expt.2. Oil Red O staining showed numerous large and small lipid droplets in cytosol of hepatocytes in the periportal area. By comparison, this change in lipid accumulation was significantly attenuated in HCD mice with LE treatment but not with RAL in Expt. 1 (Fig 3-1). In Expt.2, LE dose dependently reduced lipid accumulation and 13.5% LE nearly completely prevented hepatic steatosis induced by HCD only diet (Fig 3-2).
FIGURE 3-1 Histology of mouse liver illustrating increased lipid accumulation in HCD-fed mice and reduced lipid accumulation in mice treated with RAL, LE, and both. A. Hematoxylin and eosin staining of formalin-fixed, paraffin-embedded sections. B. Oil Red O staining of frozen liver sections with hematoxylin counter staining. Red: lipid; purple: nuclei. Magnification 100x
FIGURE 3-2 Histology of liver from mice fed HCD with indicated amount of LE. A. Hematoxylin and eosin staining of formalin-fixed, paraffin-embedded sections. B. Oil Red O staining of frozen liver sections with hematoxylin counter staining. Red: lipid; purple: nuclei. Magnification 100x.
To further confirm the observations from histological examination, we quantified TG content in liver using lipid assay. Consistent with histological data, lipid assay revealed that TG content was about 10 fold higher in the HCD only group than in the NC mice. In Expt.1, only LE but not RAL reduced TG mass in HCD fed mice (Fig 3-3A). In Expt. 2, hepatic TG was significantly reduced in HCD with 4 and 13.5% LE (Fig 3-3B). In both experiments, liver total lipid content followed the same pattern as TG.
FIGURE 3-3 Liver TG concentration in mice in Expt. 1 (A) and Expt. 2 (B). Data shown are for individual mice with mean (wide bar) and SEM (narrow bars) for each group. Mean ± SEM, n = 7–9 (Expt. 1) or 5 (Expt. 2). *Different from HCD, P < 0.001. HCD-fed groups without a common letter differ, P < 0.05. LE, lipid emulsion; NC, normal chow; HCD, high carbohydrate diet; RAL, retinal.
Hepatic retinoid status in mice in Expt.1. In Expt.1, we measured liver total retinol and plasma retinol by UPLC. Liver total retinol was lower in HCD only compared with NC mice (P < 0.01), which may reflect the lower vitamin A content in HCD diet compared with normal chow diet. Total retinol was higher in mice that received RAL (1 µg/g BW) regardless of LE (P < 0.0001) (Fig 3-4A). By comparison, plasma retinol (not shown) did not differ among any of the groups.

To determine how different dietary composition of macronutrients and retinal alter the expression of genes involved in vitamin A metabolism, we quantified the relative expression levels of several genes related to retinoid homeostasis, including Cyp26A1, which catalyzes retinoic acid (RA) oxidation, LRAT, which esterifies retinol, retinol dehydrogenase (Rdh)1, and 3 retinal dehydrogenase genes, Aldh1a1, Aldh1a2, and Aldh1a3, which are implicated in RA biosynthesis. The real time PCR showed that CYP26A1 was upregulated by RAL treatment, which indicates the enhanced conversion of retinal to retinoic acid (Fig 3-4B). Interestingly, RBP4 was increased in HCD groups.
Liver total retinol

A

![Liver total retinol graph]

B

![CYP26 A1 graph]

FIGURE 3-4 Hepatic retinol status and CYP26A1 gene expression in mice in Expt. 1. A; Total retinol was measured from hepatic lipid extraction by UPLC. B; Cyp26A1 expression was measured in liver tissue by qRT-PCR. *P < 0.05 by t-test, a > b > c by one-way ANOVA.
**Hepatic genes related to FA synthesis, oxidation, and lipid mobilization.** It has been known that transcriptional control of lipogenic genes regulates fatty acid synthesis in liver. Those lipogenic genes include: ACC, FAS, SREBP1c, and LXR. The relative mRNA transcript levels for FAS, a major lipogenic gene, and for SREBP1c, known to be involved in the development of fatty liver, were determined in both experiments (Fig 3-5). FAS transcripts were higher in HCD only compared with NC mice (Fig 3-5A and B), as was FAS protein (Fig 3-5C). Whereas FAS mRNA was not significantly reduced by LE or RAL, FAS protein was lower in mice treated with LE (Fig 5-4C). SREBP1c followed a similar pattern to FAS protein (Fig 3-5E and F). We also measured the transcripts for Chrebp, Cpt1, Scd1, apolipoprotein (Apo) AI, ApoB, adiponectin receptor 2 (AdipoR2), uncoupling protein- 2 (Ucp2), and the reference gene tubulin (Tub); these mRNAs did not differ among groups (not shown).
FIGURE 3-5 Relative mRNA levels for FAS (Fasn) transcripts (A,B) and protein (C) and SREBP1c (Srebpf1) transcripts (E,F) in the liver of mice fed HCD with or without RAL and LE treatments in Expt. 1 (A,E) and 2 (B,C,F). The mean of the NC group was set to 1.0. Values are mean ± SEM, n = 7–9 (Expt. 1) or 5 (Expt. 2). *Different from PN, P < 0.05. HCD-fed groups without a common letter differ, P < 0.05. LE, lipid emulsion; NC, normal chow; HCD, high carbohydrate diet; RAL, retinal.
**Reduction in hepatic inflammatory markers by LE.** We did not observe significant macrophage infiltration in liver in HCD mice, but we found that some hepatic inflammatory markers were changed in HCD mice. We determined the mRNA transcript levels for CCL2 (Fig 3-6A) and APCS, an acute-phase protein produced in liver (Fig 3-6B). CCL2 and APCS transcripts were higher in the HCD only group than in the NC mice, whereas LE-treated groups had lower levels of these transcripts compared with HCD only. Other genes that were measured, TNF and IL1B, did not significantly differ (data not shown), whereas IL6 mRNA was below the limit of detection by qRT-PCR.
FIGURE 3-6 Relative mRNA levels for inflammatory markers Ccl2 and Apcs in the liver of HC-fed mice administered HCD formula with or without RAL and LE treatments in Expt. 1 (A) and 2 (B). Values are mean ± SEM, n = 7–9 (Expt. 1) or 5 (Expt. 2). *Different from PN, P < 0.005. PN-fed groups without a common letter differ, P < 0.05. LE, lipid emulsion; NC, normal chow; HCD, high carbohydrate diet; RAL, retinal.
Adipose tissue weight and adipocyte size. Epididymal adipose tissue weight was greater in the HCD only than in the NC groups. Whereas RAL had no effect, weight was lower in the LE group in Expt. 1 compared with HCD only, but not in Expt. 2 in which LE was given orally. Epididymal adipocytes were larger in HCD compared with NC mice in Expt. 1 and as illustrated for Expt. 2, but treatments with RAL or LE did not alter levels compared with the HCD group (Fig 3-7).
FIGURE 3-7 Mouse epididymal adipose tissue weight and cellularity in Expt.2. (A) Representative H-E–stained adipose sections (B) Adipose tissue weight per BW. Values are mean ± SEM, n = 5
*Different from HCD, P < 0.05. HCD-fed groups without a common letter differ, P < 0.05. BW, body weight; H-E, hematoxylin and eosin; LE, lipid emulsion; NC, normal chow; HCD, high carbohydrate diet; RAL, retinal.
Discussion

In the present study, from a nutritional perspective, we sought to examine the benefits of some macronutrients, C-18 unsaturated fatty acids and the micronutrient retinal on the prevention of hepatic steatosis induced by a liquid high carbohydrate diet.

Several types of animal model of NAFLD/NASH have been developed and reviewed extensively (180). These animal models provide useful tools for understanding the pathogenesis of NAFLD and developing new therapeutic strategies. However, each of these animal models does not recapitulate the pathogenesis of NAFLD in human. For instance, the high fat diet is commonly used to induce hepatic steatosis and NASH in experimental animals. However, the high fat diet, in which fat account for 60% of total energy, is an extremely fatty diet for rodents since the normal chow diet only contains 4-13.5% energy provided by fat, which is not relevant to human diet. In this study, we developed the mouse model of hepatic steatosis by feeding mice with high carbohydrate diet based on several reasons: Firstly, the dramatic changing of American food habits is an increase in absolute total energy and carbohydrate intake for the past two decades. In the 1977-1978 NFCS, the total energy intake and carbohydrate intake across the age groups was 1817 kcal/d and 193 g/d respectively; however, the total energy intake and carbohydrate intake increase to 2148 kcal/day and 272 g/day respectively in the 1999–2004 NHANES, or an 18% increase in daily energy intake and 41% increase in carbohydrate intake between the two surveys (181). Secondly, several studies showed that excessive carbohydrate intake and glycemic index of consumed carbohydrate influence the development of NAFLD (182). A human study showed that carbohydrate overfeeding for 3 weeks in over weight individuals significantly increased liver fat by 27%, whereas body weight was only increased by 2% (183). Another study compared the metabolic effects of fructose with that of glucose in healthy men and revealed that both fructose
and glucose, providing 30% excess energy, increased VLDL-TG and intrahepatocellular lipids (184). A subsequent study by the same group failed to observe increased plasma TG when 30% excess energy is provided by saturated fat, which indicates simple sugars may have specific effects on plasma TG concentration (185). Thirdly, de novo lipogenesis from glucose and/fructose is implicated in the pathogenesis of NAFLD. Hepatic fatty acids deriving from de novo lipogenesis in healthy individuals can be negligible when compared with those from peripheral lipolysis and diets. However, in NAFLD patients, TG from de novo lipogenesis is significantly increased (23). Donnelly et al. (23) showed that hepatic de novo lipogenesis contributes to as high as 26% of total TG in liver, which indicates that enhanced de novo lipogenesis plays an important role in the pathogenesis of NAFLD. Fourthly, parenteral nutrition comprising high dextrose often leads to hepatic steatosis in patients, who receive long-term PN therapy. Therefore, findings from this study are relevant to human parental nutrition therapy as we used PN formulas as mouse diet.

In the present study, we successfully developed a mouse model of simple hepatic steatosis by feeding mice with a high carbohydrate diet (see model in Fig 3-8). Our model was based on a previous study, which also used the same diet to induce mouse model of hepatic steatosis after 19 days of high carbohydrate feeding (186). In our experiments, we supplemented mice with soybean oil to prevent essential fatty acid deficiency, which also lead to hepatic steatosis due to impaired TG import and enhanced lipogenesis. By comparison, essential fatty acid deficiency could be a confounding factor in Alwayn’s mouse model of hepatic steatosis as no EFAs were added in the diet (186). Rodent models of high simple carbohydrate induced NAFLD were also developed by giving high fructose diet, in which fructose accounts for about 50-70% of total energy. Although lipid accumulation and inflammation were observed in this type of model, it
does not reflect human diet, in which only about 10% total energy come from fructose. In our model, the percentage of simple carbohydrates as total energy is increase by 19% in the high carbohydrates diet compared to the normal chow diet, which is comparable to American human diet change in past three decades. In our model, robust hepatic inflammation was not observed as filtration of macrophage was not detected. However, we found that the inflammatory marker, CCL2 and APCS gene, were significantly induced by high carbohydrate diet, which suggest that our model is in the inductive phase of hepatic inflammation. Since a 4 and 5-wk the duration of high carbohydrate feeding is relative to short compared with other studies, in which high fat or high fructose were given for 12-16 weeks, we expect that our model may progress to steatohepatitis if those mice were fed on HCD for longer time.
FIGURE 3-8 Mouse hepatic steatosis model induced by high carbohydrate diet
We found that HCD mice grew normally, as they had similar final body weight and hematocrit. This is most likely due to food intake of HCD group is comparable to that of control mice fed on normal chow. Although the HCD mice were not obese, their epididymal adipose tissue weight was slightly greater than control mice, which suggest that HCD diet also induced lipogenesis or fatty acids uptake in adipose tissue. In fact, basal rate of de novo lipogenesis in adipose tissue is very low and it is estimated that only about 2% of total daily deposited TG in adipose tissue comes from lipogenesis (187). Gene expression data showed that several lipogenic gene expressions in the epididymal adipose tissue was not altered by the HCD diet, suggesting that enhanced free fatty acid uptake from VLDL secreted from liver may contribute to the increased epididymal adipose tissue weight. We did not measure lipogenic genes expression in muscle in this experiment, but our data from other experiments showed that some lipogenic genes are induced by HCD feeding. Overall, these results suggest that the lipogenic effect of HCD was focused on the liver.

Retinal supplementation had no effect on mouse body weight, liver size, lipid or TG mass and inflammation markers in Expt. 1. Retinal is required for vision cycle and serves as the required intermediate metabolite for producing retinoic acid. A seminal study by Plutzky’s lab at Harvard University revealed that retinal is present in rodent adipose tissue and suppresses adipogenic gene expression and lipid deposition in adipocyte and hepatocyte. In this study, retinal treatment effectively improved insulin sensitivity, reduced fat pads size and reversed hepatic steatosis in ob/ob mice (121). Why did we not see the potential beneficial effects of retinal on prevention of hepatic steatosis observed by Plutzky et al. (120)? Of note, several aspects of our study and Plutzky’s study were different, including the mouse model of hepatic steatosis, the route of retinal administration, and the dosage of retinal. In Plutzky’s study (120), the transgenic ob/ob
mouse model of hepatic steatosis was used and 500nM retinal was administered by daily intraperitoneal injections. The routes of retinal treatment may influence its role in reducing lipid accumulation, since injecting retinal can bypass the intestine, whereas orally supplemented retinal was reduced to retinol and esterified to form retinyl esters, which is supported by our data showing that total liver retinyl ester increased in HCD groups supplemented with retinal. Most recent data from Plutzky’s lab showed that retinal regulates glucose and lipid metabolism by the suppression of gluconeogenesis and lipogenesis and the enhancement of fatty acid oxidation (123). Certainly, further studies are needed to understand the antiadipogenic and antilipogenic activities of retinal.

A growing body of evidence suggests that retinoids are involved in the development of NAFLD. Human studies showed that serum retinol was inversely correlated with prevalence of NAFLD and serum concentrations of ALT and AST in NAFLD patients (108). In a case control study (116), expression of fifty-one genes regulating vitamin A metabolism was assessed by real time PCR in patients with NAFLD and healthy individuals. The data showed that expression pattern of those genes are significant different between NAFLD patients and health subjects. In this study, genes involved in conversion between retinyl ester and retinol, such as CRBP1, LRAT, DGT1/2 and CES1, were highly expressed in NAFLD patients. Expression of retinal dehydrogenase, such as ADH1/2/3, RDH5/10/11, DHRS3 and RALDH1/3 was higher in NAFLD patients compared with healthy controls. Data from ours and others showed that the two lipogenic genes, SREBP-1c and FAS, were induced in hepatic cell incubated with all-trans-RA in vitro (113, 114, 188). In fact, hypertriglyceridemia was observed in patients with some dermatological diseases when they received vitamin A derivatives therapy (119, 120). However, other studies showed that hepatic lipid accumulation was suppressed by RA treatment in vivo.
Overall, the research of retinoids in glucose/lipid metabolism is active and controversial.

There was no noticeable evidence of macrophage infiltration in HCD mice. For most animal models of NAFLD/NASH induced by high fat diet or high fructose, inflammation could be observed. Kanuri et al. (189) reported that a high-fructose diet feeding for 8 weeks resulted in a 5-fold greater hepatic TG deposition, a doubling in CCL19 production, and increased number of neutrophil infiltration. Although, we did not observe macrophage infiltration in HCD mice, we found that the inflammatory markers, CCL2 and APCS were upregulated, which indicates that early inflammatory response is already induced by HCD diet. Of note, recent studies showed that CD8+ T cell infiltration precedes macrophage accumulation in adipose tissue in obese mice fed a high-fat diet for 16 weeks (190), thus we cannot exclude that CD8+ T cell infiltration is present in liver of HCD mice. The impact of high-carbohydrate diet on immune function is not fully understood. A study showed that dietary carbohydrates play critical roles in inflammatory response in the mouse liver (191). Inflammation is regarded as a factor leading to insulin resistance. We know that high carbohydrate is associated with insulin resistance and our findings support that inflammation is induced; indicating insulin resistance may exists in our mouse model.

Lipid emulsion, rich in oleic acid, linoleic acid and α-linolenic acid, effectively prevented TG accumulation driven by high carbohydrate diet in both Expt. 1 and Expt. 2. Excess carbohydrates are converted to TG in liver by enzymes involved in de novo lipogenesis, such as ACC-1FAS and SCD-1. It is well known that glucose and insulin regulate lipogenesis by activation of two main transcription factors, namely ChREBP and SREBP-1c. Although SREBP-1c is regulated at both transcriptional and post-translational levels, evidence suggests that SREBP-1c is regulated by
insulin mainly at transcriptional level (32). SREBP-1c activates the transcription of several lipogenic genes, such as ACC, FAS and SCD-1, which directly catalyze the formation of fatty acids. In both Expt.1 and Expt.2, we observed that mRNA of SREBP-1c and FAS were dramatically induced by HCD diet. One limitation of our study is that we did not measure insulin and glucose, but some other studies showed that excessive carbohydrates induced high level of insulin. ChREBP is a glucose responsive transcription factor, which activates genes containing carbohydrate response elements (ChREs). We did not observe any change of ChREBP gene expression (data not shown). This is most like due to that fact that ChREBP is regulated at post-translational level through a phosphorylation/de phosphorylation mechanism. Nonetheless, high carbohydrate diet led to TG accumulation through enhanced de novo lipogenesis indicated by increased SREBP1-c, FAS and ACC. The provision of sufficient amount of n-18 unsaturated fatty acids, oleic acid, linoleic acid and linolenic acid, nearly completely prevented hepatic steatosis induced by the HCD diet in Expt. 2, which is also indicated by the suppression of SREBP1c transcript and FAS protein. Indeed, the mechanisms by which dietary fat, especially PUFA, regulate expression of hepatic genes involved in glucose/lipid metabolisms have been delineated. A number of studies showed that PUFA inhibit fatty acid synthesis by suppressing SREBP1c, which is also supported by our data as lipid emulsion used in our study contains 53% linoleic acid and 7% linolenic acid. In fact, emerging data showed that MUFA and PUFA is potentially a therapy for NAFLD in human studies. Although studies showed that n-6 fatty acids have proinflammatory properties, a mixture of n-3 and n-6 fatty acids in an appropriate ratio may have favorable inflammation profile as indicated by reduction in CCL2 and APCS expression in mice supplemented with lipid emulsion.
In brief, our data clearly showed that replacement of a portion of carbohydrate with unsaturated fat prevented a high carbohydrate diet induced fatty liver in mouse. Since the increase in total energy intake in the American diet over the past several decades is mainly due to an increase in carbohydrate intake, we believe that carbohydrate reduction with unsaturated fat supplementation would provide beneficial preventive effect in NAFLD in developed countries.
CHAPTER 4

STUDY TWO

Do retinoids reduce lipid accumulation induced by high glucose or oleic acid in HepG2 cells?
Abstract

Evidence has shown that specific retinoids have an impact on lipid metabolism. This study examined the effects of RA, RAL and beta-carotene on lipid deposition in HepG2 cell line. HepG2 cells were incubated in medium with high glucose or oleic acid for 48 h and 24 h respectively and then treated with indicated retinoids for 24 hours. Both high glucose and oleic fatty acid induced hepatocellular steatosis in HepG2 cells; however, retinoids did not reduce lipid accumulation in HepG2 cells and gene expression data showed that retinoic acid stimulate lipogenic genes, FAS and SREBP1c. In conclusion, we did not observe that retinoids had beneficial effects on hepatic steatosis in the HepG2 cell line.

Introduction

The role of retinoids in lipid metabolism with their implication in metabolic disorders has attracted increasing attention in recent years (175, 176, 192-196). Several specific retinoids, including all-trans-RA and RAL, have been revealed to have beneficial effects on metabolic disorders. Studies by independent groups consistently showed that all-trans-retinoic acid treatment decreased body weight, corrected dyslipidemia; improve insulin sensitivity and hepatic steatosis (111, 115, 118, 197). The precursor of RA, retinal also was shown to have anti-adiposity effect in diet-induced obese mice (121). The effect of β-carotene on lipid metabolism was discovered in a study, in which β-carotene treatment inhibited adipogenesis of 3T3-L1 preadipocytes. In addition, some studies have shown that several enzymes catalyzing vitamin A metabolism also play a role in lipid metabolism. For instance, deletion of the retinal catabolizing enzyme-retinaldehyde dehydrogenase in mice suppressed high fat diet-induced obesity (121); and mice lacking β-carotene 15, 15’- monooxygenase 1 (BCMO1) developed fatty liver and had
elevated serum unesterified fatty acids (198). Finally, RBP4 has been implicated in the development of type 2 diabetes (125). Taken together, accumulating evidence suggests that retinoids, retinoid-related enzymes and transporting protein appear to be involved in lipid metabolism.

The effects of retinoids on hepatic lipid metabolism remain controversial. Kang et al. (110) firstly showed that VAD mice displayed altered lipid metabolism (110). Results showed that VAD mice had decreased expression of fatty acid oxidation genes and accordingly developed fatty liver. By contrast, McClintick et al. (199) found that there were an increase of expression of genes encoding fatty oxidation and a decrease of transcripts of genes encoding fatty acid synthesis in VAD rats. In in vitro studies, all-trans-RA treatment appears to facilitate fatty acid oxidation indicated by upregulation of CPT1-L in HepG2 cells (112). Conversely, other studies suggest that retinoic acid enhances fatty acid synthesis by showing that SREBP-1c was increased in HepG2 cells treated with RA (114, 188). In human studies, it was well recorded that retinoids and their derivatives induce hypertriglyceridemia (120, 200-202). The reasons for these contradictory finding remains unclear and may be explained by species differences (mice vs rat vs human).

Since one study showed that retinal had anti-adipogenic and anti-lipogenic functions in mice, indicating that the function of retinal is beyond the eyes and plays a role in lipid metabolism, we were interested in examining how retinal affects lipid accumulation in liver. We did not observe any beneficial effect of retinal on mouse fatty liver induced by high carbohydrate diet in our first study. Then we sought to investigate if retinal and other retinoids correct lipid accumulation induced by either high glucose or oleic acid in human hepatocellular carcinoma cell line. Here,
we report that we did not observe that retinoids had beneficial effects on hepatic steatosis in HepG2 cell line challenged with either high glucose or oleic acids.

**Methods**

**Cell culture.** HepG2 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 0.5% (v/v) penicillin-streptomycin in 75-cm² flasks at 37 °C in a 5% CO₂-air incubator. The cells were subcultured in 6-well plates and subject to experimental conditions when they reach approximately 70% confluence.

**Experimental conditions.** For experiments with high concentration of glucose, a certain amount of sterilized 2M glucose was added to give a final level of glucose in medium of 25mM. Cells were incubated in medium with 25mM glucose for 2 days. For experiment with 2-deoxy-D-glucose, 2M 2-deoxy-D-glucose was added to medium reach a level of 2-deoxy-D-glucose of 25mM. For experiments with fatty acids, cells were treated with 400nM of oleic acids and incubated for 24 hours. Control cells were treated with same amount of sterilized water or BSA. Then cells were exposed to retinoids for another 24 hours.

**Preparation of BSA-Bound Fatty Acids.** Oleic acids were bound to BSA by mixing 800 μL of ethanol with oleic acid (0.1mM) before adding 40 μL of 5M NaOH. The ethanol was evaporated and 4 mL of sterile PBS was added to suspend the fatty acids. Five milliliters of 24% (w:v) ice-cold fatty acid-free BSA was added. The ratio of the fatty acid to albumin is 5:1. PBS was added to final concentration of BSA-bound fatty acid of 10 mM. Aliquots of the solution were overlaid with nitrogen gas and stored at −20°C.
**Oil Red O staining.** Cells were washed with PBS and fixed with 10% buffered formalin for 1 hour. Then cells were washed with 60% isopropanol after fixation with formalin and incubated with Oil Red O working solution for 10 minutes. Remove all Oil Red O and wash with warm tap water for 4 times. After taking pictures, elute Oil Red O by adding 100% isopropanol and measure OD at 500nm.

**Total RNA Extraction.** Total RNA from HepG2 cells was isolated using QIAGEN RNeasy mini kit following the protocol provided by the manufacturer. RNA quantity was examined with standard 260/280 nm spectrophotometer readings.

**Reverse transcription.** The reverse transcription reaction was performed in a total volume of 20 µl and consisted of 4 µl 5x M-MLV RT buffer, 250 µM dNTP mix, 50 ng oligo dT15 primer, 20 U of ribonuclease inhibitor and 200 U M-MLV reverse transcriptase. The reaction was performed at 42°C for 30 min, 37°C for 30 min, and 94°C for 5 min. All reagents were purchased from Promega.

**Real Time PCR.** The iQ SYBR Green Supermix (Bio-Rad) was used for RT-PCR. The reaction took place in a total volume of 20 µl in 96 well plates. The PCR reaction conditions for each cycle were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec. Each value for the mRNA of genes was normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin expression analyzed in the same plate. The primers were made at the Nucleic Acid Facility at the Penn State University, University Park. The sequences of primers for target genes were listed on the following table.
Results

High glucose induced lipid accumulation in HepG2 cells. When HepG2 cells were cultured in medium with normal level of glucose (5.5mM), very few lipid droplets were observed (Fig 4-1A). We observed that lipid droplets were significantly induced when cells were incubated in medium with 25mM glucose for 48 hours (Fig 4-1B). By contrast, when HepG2 cells were cultured in medium with added 2-deoxy-D-glucose, lipid droplets were barely observed (Fig. 4-1C). The Oil Red O staining images were confirmed by the Optical Density (OD) of eluted Oil Red O measured at 500nm.
FIGURE 4-1 Lipid droplets in HepG2 cells cultured in medium with 5.5 mM glucose (A), 25 mM glucose (B), 2-deoxy-D-glucose (C), BSA (D) and 400nM oleic acids (E).
Oleic acids induced lipid accumulation in HepG2 cells. HepG2 cells were cultured in medium supplemented with 400nM oleic acid-BSA complex for 24 hours. Oil Red O staining showed that oleic acid dramatically induced lipid accumulation in HepG2 cells (Fig 4-1E). Other unsaturated fatty acids, such as linoleic, α-linolenic acids, were also capable of inducing lipid deposition. However, oleic acid could induce higher levels of cellular fat compared with linoleic and α-linolenic acids.

Retinoids could not reverse lipid accumulation induced either by high glucose or oleic acid.

HepG2 cells cultured in normal medium or medium with high glucose or oleic acid were treated with indicated several types of retinoids for 24 hours. Oil Red O staining showed that any of those retinoids could not reverse lipid accumulation in HepG2 cells (Fig 4-2 and Fig 4-3).
FIGURE 4-2 Effect of retinoids on hepatic steatosis in HepG2 cells. (A) Representative Oil Red O stained HepG2 cells under indicated conditions. (B) Optical density of Oil Red O eluted from stained HepG2 cells.
RA stimulated expression of lipogenic genes in HepG2 cells. We then examined expression of two key lipogenic genes, SREBP1c and FAS, in HepG2 cells treated with retinoic acids. Both SREBP1c and FAS tended to increase when cells were treated with 100nM RA, and significant difference was observed when cells were treated with 2µM RA (Fig 4-4).
FIGURE 4-3 Effect of retinal and retinoic acid on hepatic steatosis induced by oleic acid in HepG2 cells. (A) Representative of Oil Red O stained HepG2 cells under indicated conditions. (B) Optical density of Oil Red O eluted from stained HepG2 cells. One-way ANOVA with Tukey’s post hoc test was used to compare all pairs of treatment.
FIGURE 4-4 SREBP1c and FAS mRNA in HepG2 cells treated with indicated concentration of RA for 24 hours. One-way ANOVA with Tukey's post hoc test was used to compare each group.
Discussion

In our animal study (Expt.1), we did not observe any beneficial effects of retinal on mouse hepatic steatosis induced by high carbohydrate diet. It is possible that hepatic retinal is too low to impact on lipid accumulation as retinal was administered orally and could be converted into retinol in the intestine. Therefore, in the present study, we sought to examine the direct effects of retinal and other retinoids on the human hepatocellular carcinoma derived cell line, HepG2 cells. HepG2 cell possesses many functions that resemble the functions of human hepatocytes. For instance, it has been well known that this cell line is capable of making and secreting lipids, apolipoproteins, and lipoproteins (203, 204), and many researcher have used the cell line to study lipoprotein metabolism (205-207).

In order to be consistent with mouse model of hepatic steatosis induced by high carbohydrate diet in Expt.1, we established an in vitro hepatic steatosis model by incubating HepG2 cells with 25mM glucose. In our pilot study, when HepG2 cells were incubated with 25mM glucose for 24 hours, we did not observe lipid accumulation, although induction of expression of lipogenic genes occurred within 24 hours, suggesting that the change of lipogenic genes expression profile is most likely the cause but not the effect of lipid accumulation. As discussed in the third chapter in this dissertation, the western dietary pattern is characterized by increasing high carbohydrate intake. Our high glucose induced hepatocellular steatosis model using HepG2 cells could be a very useful tool to study NAFLD in vitro. In addition, we could establish the monounsaturated fatty acids induced hepatocellular steatosis model by treating HepG2 cells with oleic acids.

In this in vitro study, retinoids did not ameliorate hepatocellular steatosis induced either by high carbohydrate or fatty acids. In fact, we found that retinoic acid treatment resulted in a dramatic
increase in the gene expression of lipogenic SREBP-1c and FAS, suggesting that retinoic acid may favor fatty acid anabolism instead of catabolism. One previous study from our lab showed that total hepatic lipid was significantly higher in VA supplemented rats compared with VA marginal rats (208). Our gene expression data are also in line with other studies indicating that there was increased hepatic fatty acids catabolism in vitamin A deficient rats. For instance, McClintick et al. (199) reported that the lipogenic genes, FAS and SCD-1, were decreased in vitamin A deficient rats. Oliveros et al. (75) showed that vitamin A deficiency induced a hypolipidemic effect, as was indicated by decreased expression of acetyl-CoA carboxylase (ACC) and increased expression of carnitine palmitoyltransferase 1 (CPT1). However, other authors have reported that retinoids favor fatty acid catabolism and or inhibits lipogenesis. For example, Amengual et al. (111) showed that RA treatment resulted in a reduction in hepatic triacylglycerol content and circulating VLDL. Consistent with the lipid profile data, the authors found that RA treatment led to an increase in expression of genes involved in fatty acid oxidation (PPARα, CPT1, UCP2) and a decrease in expression of two key lipogenic genes, SREBP-1c and FAS. In brief, the role of retinoids on lipid metabolism remains controversial. The reasons for these conflict findings are unclear and may be explained by difference in species, strain, and age of animals.

In conclusion, in the present study, we successfully established an in vitro high carbohydrate induced-hepatocellular steatosis model using HepG2 cells. We did not observe that retinoids had beneficial effects on hepatic steatosis induced either by high glucose or oleic acid in HepG2 cell line. We, in fact, found that RA may favor fatty acid anabolism, as was shown by increased expression of lipogenic genes.
CHAPTER 5

STUDY THREE

Nutritional regulation of non-alcoholic fatty liver disease-associated gene PNPLA3

expression in vitro and in vivo
Abstract

A polymorphism of the patatin-like phospholipase domain containing 3 (PNPLA3, ADPN) gene, I148M, is strongly associated with non-alcoholic fatty liver disease. In order to understand the physiological role of PNPLA3/ADPN protein, we have investigated nutritional regulation of the expression level of PNPLA3 gene in mice and human hepatocytes. In our first animal study, C57BL/6 male mice were fed a normal chow diet (control), a high-carbohydrate liquid diet, or the same diet with lipid emulsion for 5 weeks. PNPLA3 gene expression was increased ~22-fold in the high-carbohydrate diet fed group compared to the control group. However, lipid emulsion completely reversed PNPLA3 induction by the high carbohydrate diet. Interestingly, the expression pattern of several lipogenic genes, such as SREBP1c, FAS and ACC-1, follows that of PNPLA3. In our second animal study, mice were assigned to normal chow (NC ~10% fat), high fat diet (HF ~60% fat) and high fat with voluntary exercise (HFE) for 16 weeks. Hepatic PNPLA3 gene expression was not changed in mice fed high fat diet although they developed hepatic steatosis; however voluntary exercise dramatically decreased hepatic PNPLA3 gene expression compared to normal chow and high fat diet fed mice. In HepG2 cells that were cycled between 5.5 and 25mM glucose (starvation and refeeding conditions), PNPLA3 was significantly decreased ~5-fold by glucose starvation and increased ~2.5-fold by glucose refeeding, while insulin and glucagon had no effect. Carbohydrate response element binding protein (ChREBP) expression correlated with PNPLA3 expression. Unsaturated fatty acids, oleic acid, linoleic acid, EPA and DHA (400 μM of each, 24 h), each significantly decreased PNPLA3 mRNA by ~50%, but linolenic acid did not. We conclude that PNPLA3 is regulated by carbohydrates and certain types of unsaturated fatty acids both in vitro and in vivo, and our data suggest that PNPLA3 plays a role in lipogenesis.
Introduction

NAFLD is an emerging public health problem worldwide. It was reported that NAFLD affects about one third of the general population in Western countries (209, 210). It is believed that multifactorial complex interactions between nutritional factors, lifestyle, and genetic background determine the development of NAFLD (211). To date, several candidate genes have been identified to predispose susceptibility to NAFLD (212). The first study showing the association between a nonsynonymous single nucleotide polymorphism of PNPLA3 (rs738409 C/G) and fatty liver was reported by Romeo et al. (144). The polymorphism in PNPLA3 (rs738409 C/G) leads to substitute Ile to Met at amino acid 148 of PNPLA3. A number of other independent studies confirmed the association in different populations (150, 213, 214) and showed that PNPLA3 is also associated with alcoholic fatty liver diseases (158) and hepatic injury, including hepatitis and hepatocellular carcinoma (145, 215, 216). In addition, the rs738409 variant in PNPLA3 is also associated with other metabolic disorders, such as type 2 diabetes and obesity. A recent article by Li et al. (214) showed that the M allele frequency is significantly associated with degree of steatosis. Therefore, understanding the biological function of PNPLA3 in NAFLD may provide new insight in the design of novel treatment strategies. In addition, emerging evidence that PNPLA3 variants are involved in other liver diseases, such as alcoholic liver disease and chronic hepatitis C, indicate that the benefit from exploring biology of PNPLA3 is beyond the treatment of NAFLD.

PNPLA3, also called ADPN, was originally discovered in mouse adipose tissue. Others and our studies revealed that PNPLA3 is also expressed in liver and other tissues (217). In humans, PNPLA3 expression is higher in liver than adipose tissue (217). PNPLA3 belongs to patatin-like
phospholipase domain containing family, which also includes ATGL, a TG hydrolase (135). It is reported that PNPLA3 has both TG hydrolase and transacylase activity in vitro as discussed below.

To date, several functional studies have been performed to investigate whether PNPLA3 function as a lipogenic enzyme or TG hydrolase. He et al. (161) demonstrated that wild type of PNPLA3 enzyme has triglyceride hydrolase activity in vitro assays and over expression of mutated form of PNPLA3 increased TG content in Huh7 cell and mouse liver. In addition, Huang et al. (160) showed that wild type PNPLA3 has a high hydrolytic activity for glycerolipids and the I148M substitution dramatically decreased its enzymatic activity. Thus, these studies support the idea that PNPLA3 is involved in lipid metabolism by functioning as a TG hydrolase. However, Qiao et al. (163) showed that overexpression of PNPLA3 increased TG content in primary hepatocytes, which indicates that PNPLA3 works as a lipogenic enzyme. Consistent with this study, Kumari et al. (164) reported that PNPLA3 functions as LPA acyltransferase, which catalyzes the conversion of LPA into PA. Kumari et al. (164) concluded that the I148M variant of PNPLA3 contributes to hepatic lipid accumulation due to a gain of function. Nevertheless, how PNPLA3 is involved in the pathogenesis of NAFLD is still an open question.

In this study we aim to explore how PNPLA3 mRNA is regulated by carbohydrates and fatty acids in mouse liver and HepG2 cells. Our data indicate that PNPLA3 gene expression is tightly controlled by glucose and certain types of unsaturated fatty acids and that PNPLA3 is likely involved in the lipogenesis pathway.
Materials and methods

Animal protocols. Animal protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. 5-week-old male C57BL/6 mice were housed on a 12:12 hours light/dark cycle and received food and water ad libitum. For the evaluation of hepatic PNPLA3 expression in response to high carbohydrate diet, experimental mice were fed on one of four diets as follows for 5 weeks: a regular rodent (normal chow) diet (58% carbohydrate, 13% fat and 29% protein); a high carbohydrate (HC) diet (76.8% CHO, 22.7% protein and 0.5% fat); a high carbohydrate diet low fat (HCLF) diet (73.8% CHO, 21.7% protein and 4.0% fat); a high carbohydrate normal fat (HCNF) diet (66.8% CHO, 19.7% protein and 13.5% fat). For evaluation of hepatic PNPLA3 expression in response to a high fat diet, experimental mice were assigned the following diet and treatment for 16 weeks: a normal chow diet; a high fat diet (25% carbohydrate, 60% fat and 15% protein) and a high fat diet with voluntary wheels (Table 4-1). At the end of the study, tissues were dissected after mice were anesthetized. Partial liver samples were embedded in OCT and the remaining liver samples were immediately frozen in liquid nitrogen and stored at -80°C. All other tissues are stored at -80°C.

Cell culture. HepG2 human hepatocarcinoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 0.5% (v/v) penicillin-streptomycin at 37°C in a 5% CO₂-air incubator. The cells were plated at approximately 80% confluence.

Experimental condition. For the starvation and refeeding experiment, HepG2 cells were cultured in glucose-free DMEM for 8h, and then glucose was added to medium, followed by several cycles of the change of glucose concentration in medium. In another sets of experiments, HepG2
cells were treated with 400nM of indicated fatty acids and allowed to grow for 24h. For the control group, cells were treated with BSA.

**Oil Red O staining.** Cells were washed with PBS and fixed with 10% buffered formalin for 1 hour. Then cells were washed with 60% isopropanol after fixation with formalin and incubated with Oil Red O working solution for 10 minutes. After removal of all Oil Red O the cells were washed with warm tap water for 4 times. After taking pictures, Oil Red O was eluted by adding 100% isopropanol and the absorbance was measured (OD at 500 nm).

**Total RNA Extraction.** Total RNA was extracted from mouse liver and adipose tissue using Trizol reagent (Life Technologies). Total RNA from HepG2 cells was isolated using QIAGEN RNeasy mini kit following the protocol provided by the manufacturer. Concentration of RNA was determined by UV absorbance spectrophotometry (NanoDrop’s ND-1000).

**Reverse transcription.** The reverse transcription reaction was performed in a total volume of 20 µl and consisted of 4 µl 5x M-MLV RT Buffer, 250 µM dNTP mix, 50 ng Oligo dT15 primer, 20 U of ribonuclease inhibitor and 200 U M-MLV reverse transcriptase. The reaction was performed at 42°C for 30 min, 37°C for 30 min, and 94°C for 5 min. All reagents were purchased from Promega.

**Real Time PCR.** The iQ SYBR Green Supermix (Bio-Rad) was used for RT-PCR. The reaction took place in a total volume of 20 µl in 96 well plates. The PCR reaction conditions for each cycle were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec. Each value for the mRNA of genes was normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or b-actin expression analyzed in the
same plate. The primers were made at the Nucleic Acid Facility at the Penn State University, University Park. The sequences of primers for target genes were listed on the following table.

**Western Blotting.** Whole cell lysates (30-50 µg) were separated by electrophoresis through 10% SDS-PAGE gels and transferred to PVDF membranes. The blots were then incubated with anti-PNPLA3 polyclonal antibody (1: 5000; Everest Biotech), anti-ß-actin monoclonal antibody (1: 10000; Santa Cruz). For all blots, horseradish peroxidase-conjugated secondary antibody were used. Then the protein bands were visualized by ECL Western blotting analysis system and exposed to X-ray film.

**RNA interference.** PNPLA3 siRNA was delivered into HepG2 cells using Thermo Scientific DharmaFECT transfection reagent following the protocol provided by the manufacturer. In brief, cells were plated into each well of a 24-well plate. After 24 hours, the medium was aspirated and cells were washed with PBS. Add prepared non-specific and PNPLA3 siRNA solution, which is the mixture of transfection reagent, siRNA and antibiotic-free medium, to cells. Incubate cells at 37°C in 5% CO₂ for 24 hours, followed by adding oleic acid-BSA complex or glucose and incubated for another 24 or 48 hours for mRNA analysis and lipid quantification analysis.

**Statistical analysis.** Data were analyzed in Prism 5 software. We applied one-way ANOVA or Student t test for statistical analysis. Significant difference was considered when P values were < 0.05. Data are expressed as means ± standard deviation.
Table 4.1 Composition of diets used in the high fat diet-induced NAFLD model

<table>
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<tr>
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<th>LF</th>
<th>HF</th>
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<tr>
<td><strong>Macronutrient composition</strong></td>
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</tr>
<tr>
<td>Protein, % of energy</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Carbohydrate, % of energy</td>
<td>70.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Fat, % of energy</td>
<td>10.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Energy (MJ/kg)</td>
<td>15.9</td>
<td>21.8</td>
</tr>
<tr>
<td><strong>Ingredient (g/kg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>189.6</td>
<td>258.4</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Corn starch</td>
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<td>0.0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>33.2</td>
<td>161.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>331.7</td>
<td>88.9</td>
</tr>
<tr>
<td>Cellulose</td>
<td>47.4</td>
<td>64.6</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>23.7</td>
<td>32.3</td>
</tr>
<tr>
<td>Lard</td>
<td>19.0</td>
<td>316.6</td>
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<tr>
<td>Mineral mix</td>
<td>9.5</td>
<td>12.9</td>
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<tr>
<td>Vitamin mix</td>
<td>9.5</td>
<td>12.9</td>
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<tr>
<td>Choline bitartrate</td>
<td>1.9</td>
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RESULTS

Tissue distribution pattern of PNPLA3. PNPLA3 was initially discovered in adipose tissue using an mRNA differential display technique (134). Subsequent studies showed that PNPLA3 was also detected in other tissues, such as liver, heart and muscle (218). We are interested in exploring the tissue distribution pattern of PNPLA3 gene expression in mice fed regular chow diet. Real time PCR of total RNA showed that PNPLA3 mRNA was detected in all tissues that we collected from 10-week-old male C57BL/6 mice (Fig 5-1). Specifically, PNPLA3 mRNA was highest in epididymal adipose tissue and lowest in stomach. Notably, hepatic PNPLA3 mRNA is ~ 250-fold lower compared to adipose tissue, suggesting that PNPLA3 is not a major player in liver metabolism under normal nutrition status. By contrast, in human hepatic PNPLA3 expression is highest and skin PNPLA3 is secondary to hepatic PNPLA3 (217). The discrepancy of tissue distribution of PNPLA3 between human and rodent may suggest that PNPLA3 has different physiological function in different species. Interestingly, a recent study reported that polymorphisms in chicken PNPLA3 were not associated with any fat related traits. Thus, the biological function of PNPLA3 has species specificity (219).
FIGURE 5-1 Expression pattern of PNPLA3 in mouse tissues. Relative levels of PNPLA3 mRNA in various types of 10-week old mouse was measured by quantitative real-time PCR. Each bar represents the mean of duplicate measurements.
Hepatic PNPLA3 expression was increased in mouse fed with liquid high carbohydrate diet. A number of studies have shown that nutritional and hormonal signals regulate PNPLA3 expression. However, there was no data showing PNPLA3 gene expression levels in fatty liver although the human studies associated the polymorphism in PNPLA3 with fatty liver. In order to determine how PNPLA3 gene expression is regulated in fatty liver and how energy shifts (high carbohydrate diet, high fat diet and physical activity) affect hepatic PNPLA3 gene expression profile, we developed two type of animal model of fatty liver disease, namely high carbohydrate (model HC) and high fat diet (model HF) induced fatty liver disease model. In model HC, mice were fed with either normal chow (NC) or high carbohydrate diet (HCD) or high carbohydrate diet with low fat (HCD +4% LE) or high carbohydrate with normal fat (HCD +13.5% LE) for 5 weeks. Hepatic PNPLA3 mRNA levels in mice fed on HCD were significantly increased by ~6 folds compared to mice on normal chow (Fig 5-2A). By contrast, HCD+LE diet ameliorated induction of PNPLA3 mRNA by high carbohydrate diets and PNPLA3 mRNA was brought back to basic level by HCD + 13.5% LE (Fig 5-2A). Western blotting data showed that PNPLA3 protein matched with its transcript. Interestingly, liver total lipid content was significantly correlated with PNPLA3 mRNA levels (Fig 5-2B). We also examined some gene expression profile involved in lipid droplets formation and lipogenic pathway. SREBP1c is a key transcriptional factor that regulates many genes involved in lipid metabolism. ACC-1 gene is responsible for formation of malonyl-CoA in the process of de novo fatty acid synthesis. Interestingly, both SREBP1c and ACC-1 showed the similar patterns to that of PNPLA3 (Fig 5-2C and D). By contrast, patatin-like phospholipase domain containing 2, PNPLA2 was not different among those diets (Fig 5-2E). PNPLA2, adipose triglyceride lipase (ATGL) can break down TG. Next, we measured PNPLA3 gene expression in mouse epididymal white adipose
tissue. The result showed that PNPLA3 was not different among those diets (Fig 5-2F). Thus, the different regulation of PNPLA3 in adipose and liver tissues indicates that it may have different roles in those tissues.
FIGURE 5-2. Regulation of PNPLA3 expression by high carbohydrate diet. Liver total RNA from mice fed chow, HC, HCLF and HCNF diet were subject to real time PCR for (A) PNPLA3 (C)Srebp1-c (D)ACC-1 (E) PNPLA2/ATGL. Bars with different letters indicate significant difference with each other. Epididymal adipose PNPLA3 expression (F) was also determined using real time PCR. Correlation of total lipid content and hepatic PNPLA3 was analyzed (B).
Hepatic PNPLA3 was not changed in mice fed a high fat diet but suppressed by exercise. Since an HF diet is widely used to develop obesity and metabolic syndrome, including fatty liver in rodents, we investigated how the PNPLA3 gene is regulated in fatty liver induced by high fat diet. Mice were assigned to normal chow (NC ~10% fat), high fat diet (HF ~60% fat) and high fat with voluntary exercise (HFE) for 16 weeks. Surprisingly, PNPLA3 expression was not increased in mouse fatty liver induced by high fat diet (Fig 5-3A). Inconsistent with our data, Basantani et al. (166) showed that hepatic PNPLA3 expression was increased by 12 folds in FVB mice fed high-fat (~42% fat) diet for 2 weeks. Strikingly, voluntary exercise suppressed PNPLA3 gene expression by 10 fold compared to normal chow and high fat diet groups (Fig. 3A). We also examined the key lipogenic gene, SREBP1c and FAS and the data showed that these two genes were not changed by high fat diet (Fig 5-3B and C), suggesting that lipogenesis does not play a major role in fatty liver induced by high fat diet.
FIGURE 5-3 Regulation of hepatic PNPLA3 in mice fed a high fat diet. Liver total RNA from mice on chow, HF and HF+ExE were subject to real time PCR for (A) PNPLA3 (B) SREBP-1c (3) FAS expression quantitation. statistical difference is dotted as * (p<0.05) using t-test.
PNPLA3 gene expression was very sensitive to glucose level in HepG2 cells. Next, we investigated PNPLA3 gene expression in HepG2 cell exposed to so-called starvation-re-feeding conditions. Briefly, HepG2 cells were initially incubated in DMEM without glucose for 8 hours, then the cells was treated with either low glucose (5.5mM) or high glucose (25mM) for a certain period time. With changing glucose level in DMEM, PNPLA3 gene expression was also modulated. In brief, PNPLA3 gene expression was increased by high glucose and suppressed by low glucose (Fig 5-4A). More interestingly, we found that ChREBP also had similar pattern to that of PNPLA3 (Fig 5-4B). The correlation analysis showed that PNPLA3 is significantly correlated with ChREBP in mRNA levels (Fig 5-4C).
FIGURE 5-4  Regulation of PNPLA3 in HepG2 cells. HepG 2 cells were exposed to so called starvation-refeeding condition. Briefly, HepG2 cells was initially incubated in DMEM without glucose for 8 hours, then the cells was treated with either low glucose (5.5 mM) or high glucose (25 mM) for a indicated period time. Real time PCR was performed to determine mRNA levels of PNPLA3 (A) and ChREBP (B). Correlation of PNPLA3 and ChREBP was analyzed (C).
**PNPLA3 gene response to insulin in HepG2 cells.** Studies showed that PNPLA3 gene expression was regulated by insulin in 3T3-L1 adipocytes (137) and in human adipose tissue (142). In addition, Sharleen et al. (220) showed that insulin increased promoter activity of human ADPN gene in Chinese hamster ovary cells. Therefore, we examined whether hepatic PNPLA3 is regulated by insulin in HepG2 cell line, which expresses the insulin receptor protein. In an initial experiment, cells were incubated in low glucose or high glucose with various concentration of insulin (1nM -100nM). After a 8 hour or 24 hour period incubation, insulin did not have significant effect on PNPLA3 gene expression (Fig 5-5A). To examine whether FBS in medium would have an effect on PNPLA3 regulation by insulin, cells were incubate in DMEM medium either without or with 5% FBS. After a 24 h period incubation, again we did not observe that insulin had any effect on PNPLA3 expression (not shown). Fibroblast growth factor 21, which was used as a positive control for the delivery efficiency of insulin, was significantly induced 2 fold by 10nM insulin (Fig 5-5B). Taken together, these data suggest that hepatic PNPLA3 does not response to insulin stimulation in human hepatocytes.
FIGURE 5-5 Homonal regulation of PNPLA3 in HepG2 cells. HepG2 cells were incubated in low glucose or high glucose with 10nM insulin for either 8 hours or 24 hours period. Real time PCR was performed to measure PNPLA3 expression (A) and fibroblast growth factor 21, a positive control for insulin delivery was also determined (B). Cells were incubated in low glucose with 0.1µM glucagon for 24 hours and PNPLA3 expression was quantified by real time PCR (C).
PNPLA3 gene response to glucagon in HepG2 cells. As our in vivo data showed that exercise suppressed PNPLA3 expression, we hypothesized that exercise would stimulate glucagon secretion, thereby decreasing PNPLA3 gene expression. In vitro, we tested whether glucagon regulates PNPLA3 expression in the HepG2 cell line. Cells were incubated in low glucose with 0.1μM glucagon for 24 hours. The real time PCR data showed that glucagon had no effect on PNPLA3 expression (Fig 5-5C). Other concentrations of glucagon were also applied, but we still failed to observe any effects (data not shown).

PNPLA3 gene expression and unsaturated fatty acids. The major component fatty acids in Intralipid are linoleic acid, oleic acid and linolenic acid. Since we found that Intralipid completely reversed PNPLA3 induction by high carbohydrate diet in mice, we further investigated whether certain types of fatty acid had effects on PNPLA3 gene expression in HepG2 cells. HepG2 cells were treated with 400nM of fatty acids either with low glucose or high glucose for 24 hours. Interestingly, both oleic acids and linoleic acid suppressed PNPLA3 gene expression in both low glucose and high glucose levels (Fig 5-6A and B). However, α-linolenic fatty acid had no effect on PNPLA3 gene expression (Fig 5-6C). All these fatty acids resulted in lipid accumulation shown by Oil Red O staining. We further explored effects of both EPA and DHA on PNPLA3 gene expression. Interestingly, we found that both EPA and DHA suppressed PNPLA3 gene expression (Fig 5-6D).
FIGURE 5-6 Regulation of PNPLA3 by unsaturated fatty acids in HepG2 cells. HepG2 cells were treated with 400nM of indicated fatty acids or BSA either under low glucose or high glucose conditions for 24 hours. Bars with different letters are considered significant different with each other by One-way ANOVA.
**Effects of PNPLA3 knockdown on glucose or lipid metabolism.** Since others and our studies show that PNPLA3 gene expression is regulated by nutritional signals and the data implicate PNPLA3 may function as a lipogenic enzyme, we next studied whether the knockdown of PNPLA3 in HepG2 cells would affect glucose or lipid metabolism. As shown in Figure 5-7A, compared to negative control scrambled siRNA, PNPLA3 siRNA treatment decreased PNPLA3 mRNA expression by ~65% in HepG2 cells. Oil Red O staining showed that the abundance of lipid droplet was the same between negative control siRNA and PNPLA3 siRNA treated cells incubated with high glucose medium, although high glucose increased lipogenesis in both groups compared to low glucose medium (Fig 5-8A). No significant difference in lipid content was observed between negative control siRNA and PNPLA3 siRNA treated cells incubated with oleic acids (Fig 5-9A). Interestingly, we found that FAS mRNA was increased by ~2 fold in PNPLA3 siRNA treated cells compared to control siRNA treated cells (Fig 5-7B).
FIGURE 5-7 Relative amount of mRNA of PNPLA3 (A) and FAS (B) in HepG2 cells treated with either non-targeting control siRNA or PNPLA3 specific siRNA. Student's t test was used to compare difference between experimental and control groups.
FIGURE 5-8 Effect of PNPLA3 knock down on glucose metabolism in HepG2 cells. (A) Oil Red O stained HepG2 cells treated with indicated siRNA under either low glucose or high glucose condition. (B) Optical density of Oil Red O eluted from stained HepG2 cells. One-way ANOVA with Tukey’s post hoc test was used to compare all pairs.
FIGURE 5-9 Effect of PNPLA3 knock down on lipid metabolism in HepG2 cells. (A) Oil Red O stained HepG2 cells treated with indicated siRNA. (B) Optical density of Oil Red O eluted from stained HepG2 cells. One-way ANOVA with Tukey’s post hoc test was used to compare all pairs.
Discussion

All genome-wide association studies in NAFLD determined the genotype of PNPLA3 in the subjects; however no data showing the hepatic expression in NAFLD patients or animal model were available when we started the study. In our HC induced mouse fatty liver model, we showed that PNPLA3 gene expression was dramatically increased in high carbohydrate model. Interestingly, the pattern of PNPLA3 gene expression is very similar to that of the lipogenic genes, such as SREBP1C, ACC and FAS, suggesting that PNPLA3 is involved in lipogenesis pathway. In HepG2 cells, PNPLA3 is quickly responsive to glucose starvation-refeeding condition. In agreement of our data, studies have shown that PNPLA3 is under the direct control of SREBP1c in response to glucose treatment (217, 221). By contrast, adipose triglyceride lipase, ATGL (PNPLA2), which shares 70% similarity with PNPLA3, was not changed in HC fed mice in the present study. In addition, many studies showed that nutritional regulation of PNPLA2 is always opposite of that of PNPLA3. Thus, it is not likely that PNPLA3 has lipid hydrolase activity as ATGL. To our knowledge, we are the first to report PNPLA3 gene induction in mouse fatty liver developed by feeding a high carbohydrate diet, although a number studies showed that PNPLA3 gene is responsive to glucose in vitro (220, 222) or high carbohydrate diet in vivo in rodent or human adipose tissues (134, 142).

In our high fat diet induced mouse fatty liver model, we did not observe any significant change of PNPLA3 gene expression in mouse fatty liver. This data support that the notion that PNPLA3 induction concurred with de novo lipogenesis since hepatic steatosis in mouse fed high fat is not through increased lipogenesis. In contrast, a study showed that hepatic PNPLA3 gene expression was dramatically induced in mouse fed Western-type diet for two weeks. In this model, exercise
effectively reversed high fat diet induced fatty liver, and in meantime, PNPLA3 gene expression was dramatically decreased. What’s the mechanism by which exercise modulates PNPLA3 gene expression? We hypothesized that glucagon stimulated by exercise is responsible for the reduction of PNPLA3. However, we failed to find any change of PNPLA3 gene expression in HepG2 cells treated with glucagon. Although it is not clear how exercise regulated PNPLA3 gene, the data indicate that PNPLA3 is very sensitive to energy shift.

In the first animal study, Intralipid rich in linoleic, oleic and linolenic acid suppressed PNPLA3 expression in mouse fed high carbohydrate. In vitro study, linoleic and oleic individually or combined suppressed PNPLA3 gene expression, but linolenic acid had no effect. Inconsistent with our data, Huang et al. (217) showed that oleate, linoleic acid increased stability of recombinant PNPLA3 protein, but had no effect on PNPLA3 mRNA in HuH7 cells. The inconsistency could be due to different cell line and source of PNPLA3 (endogenous vs exogenous). What is the mechanism by which the unsaturated fatty acids suppressed PNPLA3 gene expression? Based on the observations that PNPLA3 is under the direct transcriptional control of SREBP-1c and ChREBP and that PUFA suppress transcriptional activity of both SREBP-1c (223) and ChREBP (224), we think that decrease of PNPLA3 is due to inhibition of the two key lipogenic transcription factors by those unsaturated fatty acids.

To investigate the role of PNPLA3 in glucose and lipid metabolism in vitro we knocked down PNPLA3 gene using a PNPLA3 specific siRNA in HepG2 cells. Our data showed that PNPLA3 knockdown did not affect total lipid content when cells were incubated in normal medium or treated either with high glucose or oleic acids. Consistent with our data, Kershaw et al. (136) reported that siRNA knockdown of ADPN had no effect on hydrolysis of TG in 3T3-L1
adipocytes (137). In addition, overexpression of human ADPN in HEK293 cells did not change cellular TG content. If PNPLA3 is a lipogenic enzyme, decreased lipid content would be expected in PNPLA3 knockdown cells treated with high glucose. If PNPLA3 has triglyceride lipase activity, PNPLA3 knockdown would decrease lipolytic activity, thereby increasing TG accumulation in cells treated with OA. We failed to observe any change of lipid content in any case. The data indicate that PNPLA3 is not a major player in glucose and lipid metabolism or some compensatory mechanisms are acting to maintain homeostasis of glucose and lipid in face of PNPLA3 knockdown. Supporting the latter explanation, we observed up-regulation of FAS mRNA in PNPLA3 deficient cells. FAS is a critical enzyme in lipogenesis, so the compensatory regulation of FAS may contribute to unchanged lipid content in PNPLA3 deficient cells.

Since PNPLA3/ADPN was discovered in mouse adipose tissue a decade ago (134), many investigators have paid a lot of effort to understand its nutritional regulation and biological function. Especially, after the rs738409 SNP on PNPLA3 was repeatedly reported to associate with NALFD, it triggers great interest to explore the role of PNPLA3 in glucose and lipid metabolism. Jenkins et al. (135) was the first to report that recombinant human ADPN has both triglyceride lipase and transacylase activities in vitro. Subsequent studies confirmed that ADPN possesses lipase (136) and hydrolytic activity (160) in vitro. However, its physiological function in vivo still remains controversial. In functional studies, He et al. (161) showed that overexpression of human wild type of PNPLA3 in mouse had no effect on TG content, whereas mutant PNPLA3 leads to increased lipid accumulation in mouse liver. The investigators argued that the mutant form PNPLA3 disrupts normal hydrolytic activity of wild type of PNPLA3, thereby leading to hepatic lipid accumulation. In consistent with potential hydrolase activity of PNPLA3, Huang et al. (160) showed that purified human PNPL3 had hydrolytic activity for
TAG, DAG and MAG in vitro. In addition, a more recent study showed that PNPLA3 is involved in VLDL secretion in both human and in vitro studies (162). The authors proposed that the decreased hydrolase activity of the 148M mutant form of PNPLA3 resulted in decreased FFA supply for VLDL synthesis and subsequent TG accumulation. By contrast, Qiao et al. (163) showed that overexpression of mouse PNPLA3 in primary hepatocytes increased TG content and knockdown of PNPLA3 inhibited lipid accumulation induced by overexpression of SREBP-1c. The group concluded that PNPLA3 promote lipogenesis in mouse primary hepatocytes by functioning as a lipogenic gene. Consistent with the lipogenic function of PNPLA3, Kumari et al. (164) revealed that both murine and human PNPL3 can catalyze conversion of LPA to PA by function as a member of LPAAT enzymes. In addition, Kumashiro et al. (165) recently demonstrated that knockdown of PNPLA3 with specific antisense oligonucleotides prevented hepatic steatosis in high-fat-fed rats and reduction of PA and PA/LPA ratio were also observed in this study. More interestingly, two studies failed to identify any metabolic changes including TAG metabolism in global PNPL3 knockout mice, suggesting that PNPLA3 is not a major player in glucose/lipid metabolism or other compensatory factors are activated in response to the loss of PNPLA3 (166, 167).

For future study, microarray analysis can be performed in vitro and in vivo models with overexpressing PNPLA3 or knockdown of PNPLA3 in order to identify some genes that can be compensatory for PNPLA3 abnormality.
CHAPTER 6

STUDY FOUR

Regulation of FGF21 gene expression by nutritional signals and physical activity in vivo and in vitro
Abstract

Fibroblast growth factor 21 (FGF21), a newly identified hormone-like FGF member, regulates glucose and lipid metabolism. However, the regulation of FGF21 expression by nutrients, hormones and physical activity is not fully understood. We sought to explore how nutritional and hormonal signals and physical activity regulate FGF21 gene expression in liver and adipose tissue. In Expt. 1 and 2, C57BL/6 male mice were fed a stock nonpurified diet (NP), a purified high-carbohydrate (HCD) liquid diet, or the same diet with lipid emulsion (LE) for 5 weeks. Hepatic FGF21 gene expression was increased ~20-25 folds in the HCD group versus the control group. However, LE reversed FGF21 induction by the HCD diet in a dose-dependent manner. By contrast, adipose FGF21 was not changed. In experiment 3, C57BL/6 male mice were randomly assigned to 3 groups: a low-fat purified diet, a high-fat purified diet, or high fat-diet with voluntary running. After 16 weeks, FGF21 did not differ between low- and high-fat diets; however, voluntary exercise dramatically reduced hepatic FGF21 expression, as compared to the other two groups. In Expt.4, the data revealed that short term of physical activity had no effect on hepatic FGF21 expression, but suppressed muscle FGF21 expression. In human HepG2 liver cells that were cycled between 5.5 and 25 mM glucose (starvation and refeeding conditions), FGF21 was significantly decreased ~20-fold by glucose starvation and increased ~2.5-fold by glucose refeeding. In addition, insulin significantly induced FGF21, while glucagon had no effect. We conclude that FGF21 in liver is regulated by dietary carbohydrate, insulin, and physical activity.
**Introduction**

Fibroblast growth factor 21 (FGF21) belongs to an evolutionarily related FGF family, which includes 22 members. All FGF members contain ~150-300 amino acids and share 13-71 identity (225). The reason why FGF21 is numbered as the twenty first member of FGF2 family is that FGF21 was discovered from mouse embryos in 2000 (226). Unfortunately, its name is misleading as there is no any evidence showing FGF21 has activity in fibroblasts and promotes growth (227). The initial study showed that FGF21 mRNA was mainly expressed in liver by using Northern blotting method. Homology alignment of human and mouse FGF21 showed that they share 75% amino acid identity (226).

The initial cloning study revealed that FGF21 is predominantly expressed in liver and lower level of FGF21 mRNA was also detected in mouse thymus (226). Subsequent studies demonstrated that mouse FGF21 was expressed in adipose tissue (228), muscle (229), pancreas and testis (230). To date, there is no expression atlas of FGF21 in human tissues.

The first functional study on FGF21 reported that FGF21 facilitates glucose uptake in differentiated mouse 3T3-L1 and primary human adipocytes through upregulation of GLUT1 gene expression (231). The group showed that administration of recombinant FGF21 effectively reduced blood glucose and improved insulin sensitivity in three types of diabetic rodents, namely ob/bo mice, db/de mice and Zucker diabetic fatty rats. Furthermore, the group showed that transgenic FGF21 mice were resistant to high-fat/high carbohydrate diet induced obesity (231).

In a follow up study, the group showed that FGF21 administration in diabetic monkeys improved metabolic parameters, such as a decline in fasting plasma glucose and insulin, a decrease in LDL and an increase in HDL (232). Several other groups also confirmed the role of FGF21 in
prevention of diet induced obesity (233-237). Most interestingly, a recent study revealed that FGF21 extends lifespan in mice (238). Overall, FGF21 has been shown to have beneficial effects on metabolic disorders.

During fasting and starvation, free fatty acids are released from adipose tissue and transported into liver, where fatty acids are oxidized to acetyl-CoA through β-oxidation in mitochondria. Acetyl-CoA is the substrates for the synthesis of ketone bodies, including acetone, acetoacetic acid, and β-hydroxybutyrate. Current in vitro and in vivo data indicate that FGF21 plays a role in ketogenesis (239-241). In 2007, three groups independently demonstrated that FGF21 was dramatically induced by fasting (239-241). Peroxisome proliferator-activated receptor α (PPARα) plays a critical role in the adaptive response to starvation by regulating the transcription of several genes involved in FFA transport and oxidation. Inagaki et al. (239) showed that both PPARα agonist and fasting strongly induced FGF21 mRNA in mouse liver and human hepatocytes. Furthermore, Inagaki et al. (239) found that serum β-hydroxybutyrate and liver total ketone body production were significantly increased in FGF21 transgenic mice, which could be explained by increased ketogenic enzymes, such as CPT1a and 3-hydroxy-3-methylglutaryl-CoA synthase 2. In addition, Inagaki et al. (238) showed that FGF21 stimulates lipolysis by upregulation of the transcription of genes encoding adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in adipose tissue.

In a microarray analysis to investigate the change in hepatic gene expression in mice fed on a high-fat, low-carbohydrate ketogenic diet (KD) for 30 days, Badman et al. (241) identified 43 upregulated genes, of which FGF21 mRNA was shown to be increased >25 fold confirmed by real time PCR. Then Badman et al. (240) focused on the functional study on FGF21 by performing a series of in vitro and in vivo experiments. Firstly, Badman et al. (240) showed that
hepatic FGF21 was induced by 24h fasting and returned to normal levels at 8 h of refeeding. Secondly, they showed that FGF21 knockdown mice had significant higher TG accumulation in liver, paralleled with lower serum ketone body, than wild type mice when they were put on the same KD diet. Finally, they revealed that the levels of mRNA encoding acetyl-CoA dehydrogenases (ACAD), CPT1 and acyl-CoA oxidase (ACOX1), were reduced as the result of FGF21 knockdown in mice. Consistent with Inagaki and Badman’s findings, Lundåsen et al. (240) showed that FGF21 mRNA was induced by fasting or treatment with PPARα agonist. Overall, these data support that FGF21 plays an important role in regulating ketogenesis during starvation.

The pharmacological and physiological actions of FGF21 depend on the propagation of FGF21 signaling. As other typical FGF members, FGF21 mediates biological functions by activating cell surface tyrosine kinase FGF receptors. However, FGF21 does not directly interact with FGFR as evidence shows that FGF21 activity depends on βKlotho (242-249). The βKlotho gene encodes a single-pass transmembrane protein and was predominantly expressed in liver and white adipose tissue (250). Kharitonenkov et al. (251) proposed a mechanism of FGF21 signal transduction, which needs two-step motion. In the first step, the C-terminus of FGF21 binds to βKlotho, which lead to a conformational change in FGFR, allowing FGF21 via the N-terminus to interact with FGFR. The formation of FGF21/FGFR/KLB complex triggers the intrinsic tyrosine kinase activity of FGFR, followed by FGFR cross-phosphorylation, downstream signal transduction and a cellular functional response.

Although pharmacological function of FGF21 has been extensively studied, the physiological role of FGF21 in energy homeostasis is still not fully understood (252, 253). Nutritional regulation of FGF21 could provide important clues for understanding its physiological function.
FGF21 has been implicated in NAFLD as recent studies showed that serum FGF21 was increased in NAFLD patients and a positive correlation between FGF21 and intrahepatic TG content was established (254-256). Fibroblast growth factor 21 is induced by endoplasmic reticulum stress, a condition observed in NAFLD and other diseases (257). Enhanced metabolic action of FGF21 may partially explain the benefits of exercise and caloric restriction on NAFLD outcomes (258). Most study on nutritional regulation of FGF2 was focused on how fasting and refeeding condition regulate FGF21 expression. Several studies showed that FGF21 gene expression is remarkably induced in the mouse liver by fasting (239, 240) and suppressed by refeeding (241). Higher hepatic expression of FGF21 was also reported in mice feed high-fat, very low-carbohydrate ketogenic diet (KD) (241).

To our knowledge, there are no studies showing hepatic FGF21 gene expression in mouse models induced by simple high carbohydrate diet and high fat diet. In the present study, we aimed to investigate FGF21 response to hepatic steatosis induced by two different diets in vivo. We also looked at how physical activity modulates FGF21 expression and finally we investigated how glucose and hormones regulate FGF21 in HepG2 cells in vitro.

**Materials and Methods**

**Animal protocols.** Animal protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. In Expt. 1, 5- to 6-wk-old male C57/BL6 mice (Taconic, n = 7–9/group) were divided into 3 groups: a group fed a stock rodent chow diet [normal chow (NC), Rodent Diet 5001, Lab Diet] as a reference group for comparison with the HCD only group. Of note, diets used in Expt. 1, 2,3, and 4 are expressed in energy percent. This diet contained 239 g/kg protein, 50 g/kg fat, 51 g/kg fiber, and 3020 kcal/kg. The other two
groups were: HCD only and HCD+LE (LE described below). HCD consisted of CLINIMIX E 5/20 sulfite-free injection (Baxter Healthcare) containing 20% dextrose, 5% amino acids, and electrolytes supplemented with multiple vitamins for infusion (INFUVITE Pediatric, Baxter) and minerals and trace elements (8 mg/L zinc chloride, 6.4 mg/L cupric sulfate, 1.2 mg/L manganous sulfate, 80 mg/L chromium chloride, 176 mg/L sodium selenite, and 1.13 g/L ferrous sulfate). The HCD solution was added daily to calibrated bottles fed to 2–3 mice/cage. Body weight (BW) and the volume of PN formula used were recorded daily. All mice, except control group, received 20µl soybean oil, calculated to provide sufficient essential FAs to prevent essential FA deficiency. LE was injected into the retro-orbital sinus, 100 µL/eye to both eyes daily in Expt.1. Mice were briefly anesthetized before injection using isoflurane-oxygen inhalation; a tetracaine analgesic drop was applied to each eye after injection.

In Expt. 2, twenty 5-wk-old male C57/BL6 mice, n = 5/group, were divided into 4 groups: NC, as in Expt. 1; HCD liquid diet as described above, which contained 0.5% of total energy as Intralipid for the prevention of essential FA deficiency; HCD+4% LE; or HCD+13.5%LE, equal to the percent of energy from fat in the NC diet (). The HCD+LE solutions were mixed daily and fed in calibrated feeding bottles. Intake was measured daily and mice were weighed every 3–4 d. After 4 wk (Expt. 1) or 5 wk (Expt. 2), mice were individually killed with CO₂. Body weight, liver, and epididymal fat pad weights were recorded. Blood for hematocrit and plasma preparation was collected in heparinized syringes. Portions of liver and epididymal adipose tissue were snapfrozen in liquid nitrogen and other portions were fixed in formalin or snap-frozen with Optimal Cutting Temperature compound (Sakura Finetek Tissue-Tek, Fisher) and stored at -80ºC.
In Expt. 3, male C57BL/6J mice (4 weeks old) were assigned to low fat diet (LF, containing 10% energy from fat), high fat diet (HF, containing 60% energy from fat) (see table 4.1), high fat diet plus access to voluntary running wheel (HF+Ex). Since voluntary running is a treatment, we wanted to minimize variability by eliminating mice that had limited propensity to run to better examine the effect of running on markers of metabolic syndrome. For this reason, after a 1 wk acclimation period, mice were individually screened for voluntary running behavior for 24 h in a cage fitted with a mouse running wheel apparatus (Wheel for Rodents, Techniplast, Exton, PA, USA) as previously described (259). Mice with running activity at or above the 25th percentile (approximately 2.25 km/d) were selected for this study and randomized to low fat diet (LF, containing 10% energy from fat), high fat diet (HF, containing 60% energy from fat), high fat diet plus access to voluntary running wheel (HF+Ex). Mice were maintained on experimental treatments for 16 wk. Body weight and food consumption were recorded weekly. Rate of body weight gain was calculated by regression analysis of the body weight record.

At the end of the study, mice were fasted for 7 h (7:00-14:00), anesthetized with ether and killed by exsanguination. Plasma was isolated by centrifugation at 3200 x g for 15 min and stored at -80°C for later analysis. Livers were harvested, rinsed with saline, and weighed.

In Expt. 4, male C57BL/6J mice (5 weeks old) were assigned to normal chow diet (NC), high carbohydrate diet (HCD), HCD+ 13.5% LE, HCD plus access to voluntary running wheel (HCD+Exe), and HCD+ 13.5% LE+Exe. Mice were maintained on experimental treatments for 1 week.

Cell culture. HepG2 human hepatocarcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 0.5% (v/v) penicillin-
streptomycin at 37 °C in a 5% CO2-air incubator. The cells were plated at approximately 80% confluence.

**Total RNA Extraction.** Total RNA was extracted from mouse liver and adipose tissue using Trizol reagent (Life Technologies). Total RNA from HepG2 cells was isolated using QIAGEN RNeasy mini kit following the protocol provided by the manufacturer. Concentration of RNA was determined by UV absorbance spectrophotometry (NanoDrop’s ND-1000).

**Reverse transcription.** The reverse transcription reaction was performed in a total volume of 20 µl and consisted of 4 µl 5x M-MLV RT Buffer, 250 µM dNTP mix, 50 ng Oligo dT15 primer, 20 U of ribonuclease inhibitor and 200U M-MLV reverse transcriptase. The reaction was performed at 42°C for 30 min, 37°C for 30 min, and 94°C for 5 min. All reagents were purchased from Promega.

**Real Time PCR.** The iQ SYBR Green Supermix (Bio-Rad) was used for RT-PCR. The reaction took place in a total volume of 20 µl in 96 well plates. The PCR reaction conditions for each cycle were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec. Each value for the mRNA of genes was normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin expression analyzed in the same plate. The primers were made at the Nucleic Acid Facility at the Penn State University, University Park.

**Statistical analysis.** Data are reported as the mean ± SEM. Student’s t-test was used to compare the HCD group with the NC group only. One way ANOVA with Tukey’s post hoc test was used to compare the groups that were fed HCD-based diets by using Prism5 software (GraphPad Software). P < 0.05 was considered significant.
Results

**FGF21 gene expression pattern in 10-week-old male mice fed normal chow.** The initial cloning study showed that FGF21 is predominantly expressed in mouse liver and subsequent studies also demonstrated that FGF21 was detected in adipose, muscle and pancreas. We are interested in looking at FGF21 gene expression pattern in whole body. Consistent with previous studies, FGF21 is present in muscle, liver and adipose tissue. Surprisingly, FGF21 transcripts were also detected in other tissues that we collected from male mice in fed status. Of note, FGF21 gene expression is highest in testis and higher in brain than in muscle and liver (Fig 6-1).
FIGURE 6-1 Expression pattern of FGF21 in mouse tissues. Relative levels of FGF21 mRNA in various types of tissues in 10-week old mice was measured by quantitative real-time PCR. Each bar represents the mean of duplicate measurements.
FGF21 gene expression in high carbohydrate diet induced mouse model of NAFLD. It was shown that hepatic FGF21 expression was dramatically induced by a ketogenic diet consisting of 78.9% fat, 9.5% protein, and 0.76% carbohydrate (241). We were curious to know how a lipogenic diet modulates FGF21 gene expression. We examined FGF21 expression in three independent animal studies, which are different in the percentage of fat and the duration of feeding. In Expt.1, quantitative PCR (qPCR) showed that FGF21 mRNA was increased by more than 25-fold in livers of mice that had been fed HCD diet consisting of 77% carbohydrate, 22% protein and 0.5% fat for 4 weeks. However, supplementation of unsaturated fatty acids (4% lipid) significantly attenuated FGF21 mRNA induction by the lipogenic diet (Fig 6-2A). Then we postulated that increasing fat component in the high carbohydrate diet would lead to further reduction of FGF21 induced by the lipogenic diet. In Expt. 2, we again observed hepatic FGF21 mRNA induction in mouse fed HCD for 5 weeks. Consistent with our hypothesis, we found that supplementation of unsaturated fatty acids suppressed FGF21 gene expression in a dose dependent manner (Fig 6-2B). In order to confirm the upregulation of FGF21 gene expression in response to the HCD diet, we performed a short-term experiment, in which mice were on HCD diet for only 1 week. In Expt.4, we found that hepatic FGF21 mRNA was increased by 5-fold in mice fed HCD diet and returned to the control levels in mice fed HCD with 13.5% lipid (Fig 6-2C). We also examined FGF21 expression in adipose tissue in Expt. 2 and Expt. 4 and did not observe any difference among those diets (Fig 6-2D and E). Muscle is one of the most important tissues involving energy homeostasis. We measured FGF21 expression in muscle tissue in Expt. 4 and found that FGF21 was upregulated by HCD diet and suppressed by HCD with 13.5% lipid, which is reminiscent of regulation of hepatic FGF21.
Figure 6-2: Relative amount of FGF21 mRNA in liver and adipose tissue in Expt 1 (A), Expt 2 (B, C) and Expt 4 (D and E). One way ANOVA with Tukey’s post hoc test was used to compare all groups.
Modulation of FGF21 gene expression by physical activity in high fat diet induced mouse model of NAFLD. Several human studies showed that serum FGF21 was significantly higher in NAFLD compared with healthy control individuals. We are interested in examining how FGF21 gene is regulated by a high fat diet and physical activity in a mouse model of NAFLD. Of note, liver tissues used for gene expression analysis came from another independent study on prevention of NAFLD by green tea (unpublished data), which is coded as Expt.3 in this manuscript. In Expt.3 mice were randomly assigned to low fat diet, high fat diet and high fat diet with access to voluntary running wheel and maintained on the experimental treatments for 16 weeks. Liver histology data revealed that mice on high fat diet developed steatosis and voluntary exercise reversed lipid accumulation (data not shown). Our qPCR data showed that the high fat diet had no effect on FGF21 expression; however voluntary exercise dramatically suppressed FGF21 gene expression (Fig 6-3).
FIGURE 6-3 FGF21 expression in liver in obese mice. Mice were randomly assigned to low fat diet, high fat diet, and high fat diet with access to voluntary running wheel and maintained on the experimental treatments for 16 weeks. One way ANOVA with Tukey’s post hoc test was used to compare all groups.
Modulation of FGF21 gene expression by physical activity in mice fed high carbohydrate diet. Since we found that voluntary exercise suppressed hepatic FGF21 expression in high fat diet fed mice in Expt.3, we were wondering if voluntary exercise could modulate FGF21 expression when mice are on high carbohydrate diet. Therefore, two additional groups with access to voluntary running wheel were included in Expt. 4. The real time PCR data showed that hepatic FGF21 expression was induced by the short-term high carbohydrate diet feeding and suppressed by lipid emulsion supplementation (Fig 6-4A and B). Unexpectedly, 1-week-long voluntary exercise had no effect on FGF21 expression in liver (Fig 6-4B). By contrast, muscular FGF21 gene expression was suppressed by voluntary exercise in HCD only group (Fig 6-4D). Histology of liver and biochemical analysis of hepatic lipid showed that lipid accumulation is more pronounced in HCD with 1-week-long voluntary exercise compared with HCD only group (Fig 6-5).
FIGURE 6-4 FGF21 expression in liver (A and B) and muscle (C and D). Mice were assigned to normal chow diet (CN), high carbohydrate diet only (HCD), high carbohydrate diet supplemented with 13.5% lipid emulsion (HCD+13.5% LE), HCD diet with access to running wheel (HCD+ Exe) and HCD+13.5% LE with access to running wheel and maintained on above conditions for 8 days.
FIGURE 6-5 Oil Red O stained liver sections (A) and biochemical analysis of TG in liver (B). Mice were assigned to normal chow diet (CN), high carbohydrate diet only (HCD), high carbohydrate diet supplemented with 13% lipid emulsion (HCD +13.5% LE), HCD diet with access to running wheel (HCD+ Exe) and HCD+13.5% LE with access to running wheel and maintained on above conditions for 8 days.
**Regulation of FGF21 gene expression by glucose in HepG2 cells.** Since our data showed that high carbohydrate diet regulate hepatic FGF21 expression in vivo, we hypothesized that glucose would regulate FGF21 gene expression in hepatocytes. To test the hypothesis, we first examined FGF21 gene expression in HepG2 cells cultured in medium with different levels of glucose for 24 hours. As expected, FGF21 expression was dramatically induced by 10-fold by medium with 25mM glucose compared to 5.5mM glucose. Surprisingly, we found that 2-deoxy-D-glucose dramatically was shown to increase FGF21 mRNA by 28-fold in HepG2 cells (Fig 6-7A). Next, we determined gene expression of FGF21 in HepG2 cell exposed to a so called starvation-refeeding condition. Briefly, HepG2 cells was initially incubated in DMEM without glucose for 8 hours, then the cells were treated with either normal glucose (5.5mM) or high glucose (25mM) for a certain period time. With changing glucose level in DMEM, FGF21 gene expression was also altered (Fig 6-7D).
FIGURE 6-6 Regulation of FGF21 expression by glucose in HepG2 cells. HepG2 cells were incubated in DMEM with either 25mM glucose or 2-Deoxy-D-glucose for 24 hours and total RNA was subject to real time PCR for detection of transcripts for FGF21(A), ChREBP(B) and SREBP1c. HepG2 cells were cultured under glucose starvation-refeeding condition and FGF21mRNA was measured (D). One way ANOVA with Tukey’s post hoc test was used to compare all groups.
Regulation of FGF21 gene expression by insulin and glucagon in HepG2 cells. To investigate whether FGF21 expression can be influenced by hormonal signals, HepG2 cells were incubated in DMEM with either 10nM insulin or 100nM glucagon for 24 hours, and then expression of FGF21 was examined by real time PCR. The results revealed that insulin could significantly up-regulate FGF21 expression (Fig 6-7B); by contrast, glucagon had no effect on FGF21 expression in HepG2 cells (Fig 6-7A).
FIGURE 6-7 Relative amount of FGF21 mRNA in HepG2 cells treated with glucagon (A) and insulin (B). HepG2 cells were incubated in DMEM with either 10nM insulin or 100nM glucagon for 24 hours, and then expression of FGF21 was examined by real time PCR. Student's t test was used to compare the difference between control and experimental groups.
Discussion

The FGF21 mRNA expression pattern may provide clues for understanding the biology of FGF21. We detected abundant levels of FGF21 mRNA in reproductive organ (testis), the central nervous system (brain) and metabolic tissues (liver, kidney, muscle and adipose tissue). Most studies on FGF21 have focused on its peripheral effects on liver and adipose tissue, but the presence of FGF21 mRNA in brain indicates that FGF21 may act on central nervous system, thereby centrally regulating energy homeostasis. Systemic administration of FGF21 in diet-induced obese mice significantly increased agouti-related peptide (AGRP) and neuropeptide Y (NPY) mRNAs in the hypothalamus (233). In addition, a study revealed that FGFR1c, FGFR2c, FGFR3c are very highly expressed, with moderate level of βKlotho, in brain (230). Taken together, we believe that endogenous source of central FGF21 may play an important role in regulating whole body energy homeostasis. Most interestingly, we detected the highest level of FGF21 mRNA in testis. Abnormal FGF signaling has been implicated in cancer development and progression in reproductive system, such as overexpression of FGF2 and FGF6 in prostate cancer, and overexpression FGF8 in breast and prostate cancers (260). However, to our knowledge, there is no data showing how FGF21 is involved in reproductive function. Finally, FGF21 was detected in kidney, which was known to responsible for ~50% total glucose production during prolong starvation (261). A study showed that PEPCK, the rate-limiting enzyme of hepatic gluconeogenesis, was down-regulated in FGF21 transfected hepatocytes and up-regulated in FGF21 knockdown hepatocytes, suggesting that FGF21 suppresses gluconeogenesis in hepatocytes (262). By contrast, another group reported that FGF21 increased hepatic glucose production by inducing gluconeogenic gene expression in vivo (263).
Nevertheless, it is reasonable to postulate that FGF21 modulate gluconeogenesis in kidney during fasting/starvation.

Given the fact that FGF21 is present in blood (264-266) and capable of targeting many organs, FGF21 is believed to exert its pharmacological and physiological effect on human metabolism in a classical endocrine manner. However, there is no available technique to confirm that FGF21 exists in its active form in blood, so the concept that FGF21 functions as an endocrine is challenged (251). Since our data revealed that FGF21 is broadly expressed in multi-systems, including reproductive organ (testis), the central nervous system (brain) and metabolic tissues, we believe that FGF21 mediate pleiotropic biological response in a paracrine/autocrine/endocrine manner. FGF21 expression analysis in human tissues is needed as the biology of FGF21 may be different between mice and human.

In the present study, we examined how glucose regulates human FGF21 gene expression in human liver hepatocellular cells, HepG2 cell line. Surprisingly, we found that glucose or 2-deoxy-D-glucose strongly induced FGF21 expression, suggesting that FGF21 is regulated by both feeding and starvation signals. It has been reported that FGF21 gene was significantly induced by fasting or PPARα agonist in mice by several groups (239, 241, 263). Increased FGF21 regulates gluconeogenesis, fatty acid oxidation and ketogenesis, thereby making glucose during fasting or starvation. Here, we revealed that the feeding signal, glucose also increased expression of human FGF21 in HepG2 cells. Consistent with our in vitro data, our animal studies (Expt.1, 2 and 3) also showed that hepatic FGF21 gene was induced in response to high carbohydrate diet feeding. Recently, three groups also reported that glucose can induce FGF21 expression in rodent hepatocytes (267-269). Furthermore, Uehanso et al. (268) showed that ChREBP is required to stimulate human FGF21 gene expression by glucose. Consistent with this
finding, our data showed that ChREBP is correlated to FGF21 mRNA. Thus, data from animal
study and human liver cell line studies clearly showed that FGF21 gene is regulated by both
fasting and glucose feeding signals. In line with animal studies, in a human study, serum FGF 21
levels were increased by 74% after a 7 day fast in 5 female subjects (270). On the other hand,
some human studies have reported that serum FGF21 levels are higher in obese individuals and
patients with type 2 diabetes (265, 271, 272). Additional human studies, such as observational
and interventional studies, are needed to investigate how high carbohydrate diet regulates
systemic and local FGF21. However, an important question why FGF21 is paradoxically induced
by both fasting and feeding signals remains unanswered. If FGF21 functions as an inducer to
increase gluconeogenesis, fatty acid oxidation and ketogenesis, it is not expected to increase
under the condition of high carbohydrate feeding as there is no need to make more glucose.
There are several explanations for this phenomenon. The first explanation is that FGF21 may
regulate lipogenesis induced by high carbohydrates. An evidence supporting this explanation
comes a study showing that overexpression of FGF21 in HepG2 cells down-regulates several key
genes involved in de novo fatty acid synthesis, including SREBP-1c, ACC1, FAS and
SCD1(267). Second possibility is that FGF21 may play a role in regulating the formation and/or
secretion of VLDL in response to aberrant hepatic TG synthesis. Indeed, several human studies
showed that serum FGF21 was elevated in patients with NAFLD (254-256). Last but not least,
hepatic FGF21 is secreted to blood and regulate glucose uptake by extra-hepatic tissues, such as
adipose and muscle, in an endocrine manner. This explanation is based on one observation that
FGF21 stimulates glucose absorption by mouse 3T3-L1adipoctyes through up-regulation of
GLUT1 (231). Under any of those conditions, increased FGF21 leads to attenuation of TG
accumulation in liver (see model Fig 6-8). Further studies are warranted to elucidate the role of FGF21 in hepatic lipid metabolism.
FIGURE 6-8 Model of regulation of FGF21 by nutrients, hormones and physical activity
A growing number of studies have investigated the mechanism(s) by which FGF21 regulates insulin sensitivity (235, 273-277), however little is known about how insulin acts on FGF21. In the present study, we examined for the first time how insulin regulates FGF21 gene expression in human liver cell line. We showed that physiological levels of insulin significantly induced FGF21 expression. It was known that insulin suppresses gluconeogenesis; thereby inhibiting hepatic glucose output (278). So it is likely that FGF21 plays a role in insulin mediated gluconeogenesis pathway. Future studies are needed to examine how FGF21 deficiency in vitro and in vivo affect insulin pathway in liver. A recent study revealed that FGF21 expression was significantly induced in response to insulin stimulation in C2C12 myocytes (229). Furthermore, the researchers showed that FGF21 induction by insulin is in a PI3-kinase-dependent manner (229). Besides, it was shown that FGF21 increased the levels of insulin mRNA and protein in islets from healthy rats, augmented insulin secretion and synthesis in diabetic islet and improved pancreatic β cell survival (279). Altogether, our data and others suggest that there is a crosstalk between insulin and FGF21 signaling.

We found that consumption of HCD diet with lipid emulsion suppressed FGF21 expression. Gene expression data also showed that lipid emulsion, rich in C-18 unsaturated fatty acids, inhibited several lipogenic genes, such as SREBP1C, FAS, ACC1, and PNPLA3. So it is reasonable to postulate that FGF21 is also a regulator in lipogeneis pathway. What is the mechanism that C-18 unsaturated fatty acids regulate the transcription of FGF21? The peroxisome proliferator-activated receptor-α (PPARα) is a nuclear receptor that regulates the expression of genes involved in fatty acid oxidation in response to starvation and high fat consumption (280). It is well known that PPARα mediates transcriptional activity of FGF21gene as PPARα knock out suppresses the ability of ketogenic diet and starvation to induce
hepatic FGF21 gene expression (239, 241). A recent study revealed that several unsaturated fatty acids, including oleic acid, linoleic acid, and EPA, enhanced FGF21 gene expression in rat hepatocytes via functioning as ligands for PPARα (281). Instead of stimulating FGF21, lipid emulsion, rich in oleic acids and linoleic acids, were shown to suppress FGF21 gene expression in vivo in our study. Consistent with our finding, the suppressive effects of FFA on FGF21 was found in a study showing that the mixture of palmitic acid and oleic acid down regulated FGF21 mRNA in HepG2 cells (267). Alternatively, suppressed FGF21 is the consequence of decreased lipogenesis instead of the direct effect of C-18 unsaturated fatty acids on PPARα activity.

We did not observe any effect of the high fat diet on hepatic FGF21 gene expression in Expt. 3. Compared with the ketogenic diet, which only contain 0.78% carbohydrate, the HFD used in our study is rich in carbohydrate (20% CHO, 20% protein and 60% fat). This observation suggests that hepatic FGF21 may remain stable when gluconeogenesis is not needed. One limitation of our Expt. 3 study is that we did not measure the level of plasma FGF21. Several human and animal studies showed that plasma FGF21 was elevated in obese individuals or mice. However, elevated plasma FGF21 could be derived from other tissues, such as adipose, muscle and pancreas. In Expt. 3, we found that voluntary wheel running exercise strongly inhibited hepatic FGF21 expression in HFD mice, providing additional evidence to support that FGF21 is a regulator in energy homeostasis. However, we did not observe the suppressive effects of exercise on FGF21 in Expt. 4, in which HCD fed mice had access to running wheels for 1 week. The discrepancy could be explained by the different duration of exercise (1 week in Expt. 4 vs 16 weeks in Expt. 3) and the diet nature (HCD vs HFD). Consistent with the finding from our Expt. 3 study, a recent study show that voluntary wheel running exercise suppressed hepatic FGF21 mRNA by ~65% in Otsuka Long-Evans Tokushima Fatty (OLETF) rat (258).
Conversely, serum FGF21 was shown to be significantly increased after two weeks of physical activity in young healthy women (282).

In conclusion, our data demonstrated that hepatic FGF21 is regulated by nutritional and hormonal signals and physical activity in mice, providing new insights into the physiological roles of FGF21 in energy homeostasis.
CHAPTER 7

SUMMARY AND DISCUSSION
Non-alcoholic fatty liver disease (NAFLD) is characterized by pathological accumulation of lipid in liver in the absence of significant alcohol consumption. NAFLD affects 20-30% of the adult population and 3-10% of the childhood population in developed nations. NAFLD is associated with insulin resistance and increased risk of cardiovascular diseases (CVD). As with other complex diseases, it is believed that multi-factors including genetic background, dietary factors and physical activity, contribute to the development of NAFLD. While hepatic lipid accumulation can be simplified as an imbalance between the pathways of lipid input and output, the nutritional and hormonal signals that regulate hepatic lipid metabolism are very complicated and have been the subject of research for several decades. Therefore, a series of studies were performed to investigate the effects of dietary factors, including carbohydrate, unsaturated fat, retinal (a vitamin A metabolite) on the development of NAFLD from animal, cellular, and molecular levels.

STUDY 1

**The effects of unsaturated fat and retinal on the prevention of NAFLD induced by a high simple carbohydrate diet in mouse**

Study one investigated the effects of supplementation of lipid emulsion (LE), rich in C-18 unsaturated fatty acids, and/or the vitamin A metabolite retinal (RAL) on the development hepatic steatosis induced by a high carbohydrate diet in mice. Mice were assigned to several types of diets: normal chow diet (NC), high carbohydrate only diet (HCD), high carbohydrate only diet plus supplementation of lipid emulsion (HCD+4% LE/13.5% LE), high carbohydrate diet with supplementation of retinal (HCD+RAL) and high carbohydrate diet with both LE and RAL (HCD+LE+RAL). The main findings were that the HCD only diet induced hepatic steatosis
and the supplementation of lipid emulsion reduced lipid accumulation, but retinal had no effect on hepatic steatosis. Gene expression data revealed that the HCD only diet induced hepatic steatosis via stimulation of two key lipogenic genes, sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS). By contrast, supplementation of lipid emulsion suppressed SREBP-1c and FAS. In addition, our data showed that hepatic inflammation is initiated as inflammation markers, CCL2 and APCS were significantly induced by the HCD only diet (Fig 7-1). The results indicate that carbohydrate reduction with unsaturated fat supplementation would provide beneficial preventative effect in NAFLD.

We successfully developed an animal model of NAFLD by feeding mice with a high carbohydrate diet. Our data revealed that enhanced de novo lipogenesis is mainly responsible for liver fat accumulation. Our model is quite unique at least in terms of diet and feeding time compared with other common animal models of NAFLD. By feeding a high carbohydrate diet (77% CHO), we were able to observe pronounced hepatic lipid accumulation within only 4-5 weeks. By contrast, it takes about at least 16 weeks to establish an animal model of NAFLD by feeding mice with a high fat diet. Compared to the commonly used high fat diet induced NAFLD model, which often has steatohepatitis and fibrosis, our model is useful to study simple steatosis, the earliest stage of NAFLD. However, there are several limitations in this study. First, our model did not show metabolic abnormalities, such as obesity and dyslipidemia, which are usually associated with NAFLD. Second, we did not monitor glucose and measure insulin sensitivity in our mouse model of hepatic steatosis, so we don’t know what roles insulin signaling plays in lipid accumulation in our model. Third, we did not measure AST or ALT to test liver function. In addition, we did not determine how VLDL secretion is changed in our model, although we checked transcripts for microsomal TG transfer protein, apoproteins (apoA1
and apoB). Nevertheless, our model provides a useful and convenient tool to study pathophysiology and treatment of NAFLD.

Our findings support the premise that both carbohydrate quantity and quality contribute to the development of hepatic steatosis. Indeed, a growing body of evidence suggests that low fat, high carbohydrate diet is associated with increased risk of developing NAFLD. In fact, from 1971 to 2000, calories intake from carbohydrate diet significantly increased in the US, accompanied by the rise of obesity, insulin resistance and NAFLD. Consumption of simple sugars with high glycemic index, and more specifically fructose, has increased in the past decades in America. Both human and animal studies showed that excessive fructose intake is linked to the development of metabolic disorders, such as diabetes, NAFLD and cardiovascular disease. Therefore, our study suggests that reduction of simple carbohydrates may provide benefit for prevention of NAFLD and other metabolic disorders.

STUDY 2

**Retinoids did not reduce hepatocellular steatosis induced by high glucose or oleic acid in HepG2 cells**

Study two examined the effects of retinoic acid, retinal and beta-carotene on lipid deposition in HepG2 cell line. Hepatocellular steatosis was induced by either high glucose or oleic acid in HepG2 cells. However, retinoids did not reduce lipid accumulation in HepG2 cells and gene expression data showed that retinoic acid stimulate lipogenic genes, FAS and SREBP1c in a dose dependent manner (Fig 7-1). The results indicated that retinoids did not have beneficial effects on treatment of hepatocellular steatosis induced by high glucose or oleic acid in vitro in HepG2 cells, and in fact, retinoic acid may contribute to lipid anabolism.
In study two, we successfully established a hepatocellular steatosis model induced by high glucose using HepG2 cell line. We examined the expression of several lipogenic genes in HepG2 cells treated with high glucose and found that those lipogenic genes were up-regulated before lipid accumulation was detected by Oil Red O staining method, indicating that alteration of those genes is the cause but not the consequence of hepatocellular steatosis. In addition, our model may provide a useful in vitro tool to study oxidative stress in type 2 diabetes as a recent study showed that high levels of glucose led to reactive oxygen species (ROS) accumulation and DNA damage in HepG2 cells (283).

Our results showed that none of the administered retinoids could reduce lipid accumulation induced by high glucose or oleic acid, which suggests that retinoids does not facilitate lipid oxidation or lipid accumulation may have overridden any effect of retinoids on lipid oxidation. In addition, in this study, retinoids were administered after hepatocellular steatosis was established, therefore, the result could not exclude that retinoid may inhibit lipogenesis or TG synthesis, which already take place before lipid accumulation is detectable by Oil Red O staining method.

In brief, the role of retinoids, in particular RA, in lipid metabolism is still controversial. My study showed that RA enhanced lipogenesis as indicated by induction of two lipogenic genes, SREBP1c and FAS. In addition, RA used clinically is known to induce hypertriglyceridemia. On the other hand, some studies showed that RA treatment improved animal model of NAFLD. Certainly, further studies are needed to understand the impact of retinoids on the development of NAFLD.
STUDY 3

**Nutritional regulation of non-alcoholic fatty liver disease-associated gene PNPLA3 expression in vitro and in vivo**

Study three determined PNPLA3/ADPN gene expression in mice and HepG2 cells and investigated the biological function of PNPLA3 in vitro in HepG2 cell line. In the first animal study, mice were fed a normal chow diet (control), a high-carbohydrate liquid diet, or the same diet with lipid emulsion for 5 weeks. PNPLA3 gene expression was increased ~22-fold in the high-carbohydrate diet fed group compared to the control group. However, lipid emulsion completely reversed PNPLA3 induction by the high carbohydrate diet. Interestingly, the expression pattern of several lipogenic genes, such as SREBP1c, FAS and ACC, follows that of PNPLA3. In the second animal study, mice were assigned to normal chow (NC ~10% fat), high fat diet (HF ~60% fat) and high fat with voluntary exercise (HFE) for 16 weeks. Hepatic PNPLA3 gene expression was not changed in mice fed high fat diet although they developed hepatic steatosis; however voluntary exercise dramatically decreased hepatic PNPLA3 gene expression compared to normal chow and high fat diet fed mice. In HepG2 cells that were cycled between 5.5 and 25 mM glucose (starvation and refeeding conditions), PNPLA3 was significantly decreased ~5-fold by glucose starvation and increased ~2.5-fold by glucose refeeding, while insulin and glucagon had no effect. ChREBP expression was correlated with PNPLA3 expression. Unsaturated fatty acids, oleic acid, linoleic acid, EPA and DHA (400 nM of each, 24 h), each significantly decreased PNPLA3 mRNA by ~50%, but linolenic acid did not. Lastly, PNPLA3 knockdown did not affect total lipid content when HepG2 cells were incubated in normal medium or treated either with high glucose or oleic acids, although we observed that PNPLA3 knockdown increased FAS expression. The results suggest that hepatic PNPLA3 serves
as regulator in carbohydrate and lipid metabolism and plays a role in lipogenesis but not lipolysis pathway.

We have identified PNPLA3 in a screen for genes regulated by a high carbohydrate diet in our first study. The literature has documented that there is an association between the I148M variant in PNPLA3 and hepatic steatosis, but the precise physiological role of PNPLA3 remains unknown. Some studies suggest that PNPLA3 functions as a lipolytic enzyme, but other studies support that PNPLA3 bears a transacylase activity. We sought to explore how nutritional signals regulate PNPLA3 gene expression, which would provide important clues for understanding the biological function of PNPLA3. Based on expression pattern of PNPLA3 under a variety of nutritional challenges, we conclude that PNPLA3 is involved in lipogenesis in mouse liver (Fig 7-1). Although our data support the role of PNPLA3 in lipogenesis pathway, we don’t have evidence to exclude it may function as a lipolytic enzyme. How do we explain conflicting data on biological function of PNPLA3? The discrepancy may be explained by difference in species (mouse vs rat), cell lines (adipocytes vs heptocytes), and homology of PNPLA3 (mPNPLA3 vs hPNPLA3) among different research groups. Another possibility is that PNPLA3 may possess dual functions, namely lipolytic and lipogenic enzyme, which depends on nutritional status.

When we knocked down PNPLA3 in HepG2 cells, we did not observe any change in the phenotype in HepG2 cells. However, we observed up-regulation of FAS mRNA in PNPLA3 deficient cells, suggesting that some compensatory mechanisms were activated to maintain homeostasis of glucose and lipid. There are several directions for future study on PNPLA3. First, “omics” technologies, such as proteomics, metabolomics, and microarray analysis, can be performed in vitro and in vivo models with overexpressing PNPLA3 or knockdown of PNPLA3 in order to identify some molecules that can be compensatory for PNPLA3 abnormality. Second,
we observed that FAS was upregulated in PNPLA3 deficient cells, so we need to investigate how knockdown of both FAS and PNPLA3 affect lipogenesis in HepG2 cells. In addition, it would be very interesting to investigate how FAS interact with PNPLA3, thereby regulating glucose/lipid metabolism. Lastly, based on the quick response of PNPLA3 to nutritional challenge, we postulate that PNPLA3 may function as a hormone, which regulates nutrition status. Future study on PNPLA3 proteins structure analysis and presence of PNPLA3 in bloodstream can test above hypothesis.

Overall, study three demonstrated that PNPLA3 is a new player in glucose and lipid metabolism. Biological function of PNPLA3 must be fully understood before it can be considered a therapeutic target in the prevention/treatment of NAFLD.

STUDY 4

Regulation of fibroblast growth factor 21 (FGF21) gene expression by nutritional signals and physical activity in vivo and in vitro

Study four investigated the regulation of FGF21 expression by nutrients, hormones and physical activity in mice and HepG2 cells. In Expt.1 and 2, C57BL/6 male mice were fed normal chow diet (control), a high-carbohydrate liquid diet (HCD), or the same diet with lipid emulsion for 4-5 weeks. Hepatic FGF21 gene expression was increased ~20-25 fold in the HCD group versus the control group. However, LE reversed FGF21 induction by the HCD diet in a dose-dependent manner. By contrast, adipose FGF21 was not changed. In experiment 3, C57BL/6 male mice were randomly assigned to 3 groups: a low-fat diet, a high-fat diet, or high fat-diet with voluntary running. After 16 weeks, FGF21 did not differ between low- and high-fat diets; however, voluntary exercise dramatically reduced hepatic FGF21 expression, as compared to the
other two groups. In Expt.4, the data revealed that short-term of physical activity had no effect on hepatic FGF21 expression, but suppressed muscle FGF21 expression. In human HepG2 liver cells that were cycled between 5.5mM and 25mM glucose (starvation and refeeding conditions), FGF21 was significantly decreased ~20-fold by glucose starvation and increased ~2-fold by glucose refeeding. In addition, insulin significantly induced FGF21, while glucagon had no effect. We conclude that FGF21 in liver is regulated by dietary carbohydrate, insulin, and physical activity (Fig 7-1).

Since FGF21 has been shown to have potent insulin-sensitizing actions in rodents, it has received great interest from pharmaceutical industry. However, the biology of FGF21 and its mechanism of action in body remain incompletely understood. Study four investigated distribution and nutritional regulation of FGF21 in mouse. There are several new findings from our study four. Firstly, this study revealed that FGF21 is present not only in metabolic tissues (liver, adipose, muscle and kidney) but also in the central nervous system (brain) and reproductive organ (testis).

Up to date, all data on FGF21 are focused on metabolic organs, but it is reasonable to postulate that FGF21 also plays an important role in reproductive function based the facts that FGF21 is highest expressed in the reproductive organ and that several other FGF family members, such as FGF1, 2, 4, 8, and 9, have been shown to be involved in testicular maturation, Sertoli cell proliferation and differentiation (284-286). In addition, the presence of FGF21 in brain may indicate that FGF21 centrally regulates food intake. Secondly, our study for the first time revealed that FGF21 is strongly induced by a very low fat, high carbohydrate “lipogenic” diet. Combined with findings that a very low carbohydrate, high fat “ketogenic” diet strongly induces FGF21 expression (241), we can conclude that hepatic FGF21 plays important roles in coordinating energy homeostasis under various nutrition crisis. Thirdly, this study showed that
nutritional regulation of FGF21 is different between adipose tissue and liver, suggesting that biological function of FGF21 is depend on tissue specificity. Fourthly, to our knowledge, we are the first to discover that FGF21 was regulated by nutritional stimuli and physical activity in skeletal muscle, which indicates that FGF21 may function as a myokine. Lastly, our study for the first time revealed that insulin regulates FGF21 in hepatocytes, indicating that FGF21 is involved in insulin modulated gluconeogenesis in liver. For future study, it is worthy of investigating how FGF21 knock out/down affects insulin signaling in hepatocytes.

These four studies support several conclusions (shown in Fig 7-1). First, a high-carbohydrate diet promotes the development of fatty liver through increase de novo fatty acid synthesis as indicated by up-regulation of several lipogenic genes, such as FAS, SREBP1c and ACC1. The data also implicate that reduction of simple carbohydrate intake may be beneficial for patients with NAFLD. Second, compared with the high-carbohydrate diet, the diet supplemented with monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) may be preferable when it is not coupled with increased total energy intake. Third, the data support the idea that retinoic acid, the bioactive metabolite, plays a role in lipogenesis as indicated by enhanced expression of lipogenic genes, such as FAS and SREBP1c in liver cells treated with RA. Fourth, the data revealed that the two hepatic genes, PNPLA3 and FGF21, are new players in hepatic lipid metabolism. More specifically, PNPLA3 may function as a lipogenic enzyme in fatty acid synthesis pathway and FGF21 may suppress the production of fatty acids.

In conclusion, my studies showed that the interaction of dietary factors, including simple carbohydrate, unsaturated fat and retinoids, and hepatic genes (PNPLA3, FGF21) contributes to the lipid accumulation in liver and that the appropriate nutrient balance in diets should be considered for prevention of NAFLD.
FIGURE 7-1 Model of hepatic lipid metabolism regulated by dietary factors and hepatic genes. Please see text for identification of abbreviation and discussion of model.
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LEI HAO

EDUCATION

Ph.D. in Nutritional Sciences
The Pennsylvania State University, University Park, PA, August, 2013

Master of Medical Sciences (M. MS) in Pediatrics
Peking University, Beijing, China, 2003

Bachelor of Medicine (M.D.) in Clinical Medicine
Weifang Medical College, Shandong, China, 2000

RESEARCH EXPERIENCE

Graduate Research Assistant
Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA, August 2006 – 2013

Research Assistant
National Center for Women and Children’s health, Chinese Center for Disease Control and Prevention, Beijing, China, July 2004 – July 2006

Graduate student
Peking University, Health Science Center, Beijing, China, September 2000 – June 2003

TEACHING EXPERIENCE

Teaching Assistant, NUTR 445 (Nutrient Metabolism)
August 2010 – December 2010

PUBLICATIONS

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HONORS & AWARDS

Woot-Tsuen Scholarship in Nutrition (2012)