The CREBH in hepatic lipid and lipoprotein metabolism: Implication for the pathogenesis of hyperlipidemia and metainflammation

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The CREBH in hepatic lipid and lipoprotein metabolism: Implication for the pathogenesis of hyperlipidemia and metainflammation

by

Miaoyun Zhao

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
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For the Degree of Master of Science

Major: Interdepartmental Area of Nutrition

Under the Supervision of Professor Qiaozhu Su

Lincoln, Nebraska
December, 2014
The CREBH in hepatic lipid and lipoprotein metabolism: Implication for the pathogenesis of hyperlipidemia and metainflammation

Miaoyun Zhao, M.S.
University of Nebraska, 2014

Advisor: Qiaozhu Su

Hyperlipidemia is reemerging as an important cardiovascular disease risk factor and other metabolic disorders, such as obesity, insulin resistance and type-2 diabetes. Hepatic de novo lipogenesis is controlled by a family of the b-zip transcription factors, the sterol regulatory element-binding proteins (SREBPs), which targets downstream genes involved in free fatty acids, triglycerides and cholesterol synthesis. Regulation of SREBPs signaling is controlled by a cluster of the ER membrane-bound proteins, the insulin induced gene-1 (Insig-1) and gene-2 (Insig-2) and the SCAP protein. The cAMP responsive element binding protein H (CREBH) is a recently identified member of this family. CREBH is selectively and highly expressed in the liver and small intestine which is actively involved in lipid and glucose metabolism. However, its underlying molecular mechanisms are not fully understood. Here we demonstrated CREBH inhibits hepatic lipid de novo synthesis through modulating the expression of insulin induced gene-2 (Insig-2) isoform-a, a liver specific isoform of Insig-2, which is involved in the activation of sterol regulatory element-binding proteins (SREBPs). Metabolic cues, such as fasting, glucagon and cAMP agonist, activated CREBH which in turn inhibited SREBP-1c and SREBP-2 activation via upregulate the abundance of Insig-2a in hepatocytes. Depletion or suppression of CREBH expression by refeeding inhibited Insig-2a expression, which in turn hyperactivated SREBP-1c and -2, leading to the activation of hepatic de novo
lipid synthesis, accumulation of lipids in hepatocytes and systemic hyperlipidemia. We further demonstrated that depletion of CREBH reduced both mRNA and protein expression of apolipoprotein B (apoB). In vitro, transient expression of CREBH cDNAs in McA cells induced significant increase of apoB mRNA and protein expression, which indicated the positive regulatory impact of CREBH on apoB biosynthesis. This study establishes the CREBH-Insig-2a as a novel metabolic pathway that regulates hepatic de novo lipogenesis. This novel finding provides new mechanistic insight into the pathogenesis of hyperlipidemia in metabolic diseases.
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>apoB</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density-lipoprotein</td>
</tr>
<tr>
<td>CM</td>
<td>chylomicron</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density-lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density-lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL-receptor</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density-lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>apoA-I</td>
<td>apolipoprotein A-I</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>DGAT-1</td>
<td>diacylglycerol acyltransferase-1</td>
</tr>
<tr>
<td>DGAT-2</td>
<td>diacylglycerol acyltransferase-2</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>NC</td>
<td>negative control</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>TBST</td>
<td>1x Tris-buffered saline with tween</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>two-way analysis of variation</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor class B type 1</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>McA</td>
<td>McA-RH 7777 cells</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
</tbody>
</table>
Chapter one

General Introduction

1.1 Obesity and Hyperlipidemia

Obesity is often defined simply as a condition of abnormal or excessive fat accumulation in adipose tissue, which usually leads to health impairments\textsuperscript{1,2}. According to the current worldwide statistical data from the WHO, at least 2.8 million people die each year because of being overweight or obese, and overweight or obesity leads to an estimated 35.8 million (2.3\%) of global Disability-Adjusted Life Years. In the United States, two thirds of adults are obese or overweight, and thirty-six percent of US adults are obese. This places a heavy burden on public health care because many cannot lose sufficient weight to improve their health with lifestyle interventions alone\textsuperscript{3}. The emergence of overweight and obesity as the predominant challenges to global health is undisputed, and obesity rates are highly correlated with many incurable diseases\textsuperscript{4,5}.

Over several decades, research has elucidated that overweight and obesity have adverse metabolic effects on blood pressure, cholesterol, and triglycerides, and can lead to dyslipidemia, glucose intolerance, and insulin resistance, and even increased risk of some cancers, including breast, colon, prostate, kidney, and gall bladder\textsuperscript{3,6,7,8}. In summary, morbidity and mortality rates increase with the prevalence of obesity, which has been a great health burden for the world\textsuperscript{2,6}. Despite the wealth of information gathered over the last several decades about lipid metabolism and its relevant-diseases, the mechanisms connecting them are not yet fully understood \textsuperscript{2,9}. 
Obesity is a commonly acknowledged risk factor for various metabolic disorders or diseases, and people with abdominal obesity are more inclined to have hyperlipidemia, which is widely recognized as one of the main co-morbidities in obesity\textsuperscript{10,11}. Hyperlipidemia indicates abnormally elevated levels of one or more lipids and/or lipoproteins in the blood, and elevated levels of low-density-lipoprotein is the most common form of hyperlipidemia\textsuperscript{12}. In the United States, it is estimated that more than thirty percent of the adult population suffers from hyperlipidemia\textsuperscript{13}. Hyperlipidemia can lead to excess lipids in the arteries, which is a risk factor for the development of atherosclerosis and CVD\textsuperscript{7,14}; there is also strong evidence showing a link between lipid abnormalities and CVD\textsuperscript{14,15}. Hyperlipidemia progresses in association with obesity, type 2 diabetes, and other metabolic abnormalities, including hypertension and non-alcoholic fatty liver diseases (NAFLD), together referred to as the “metabolic syndrome”, which is a great threat to human health in the 21st century\textsuperscript{10,11,15}. Moreover, hyperlipidemia and its relevant complications exact tremendous economic burden on public health sources\textsuperscript{6}.

Hyperlipidemia and/or excess lipid accumulation can initiate a low-grade and chronic inflammatory response in adipose tissue, which is a causative factor of insulin resistance\textsuperscript{12}. Hyperlipidemia can also result in the activation of the unfolded protein response and mitochondrial dysfunction, which can further trigger metabolic inflammation\textsuperscript{13}. Metabolic inflammation may then lead to macrophage recruitment, which could increase adipose tissue lipolysis, leading to excess lipid delivery and ectopic lipid accumulation. This would further lead to impairments in insulin signaling and contribute to insulin resistance\textsuperscript{16}. Therefore, a vicious cycle occurs between lipid metabolism and inflammation. To our knowledge, though many genetic and
environmental factors have been shown to be involved in the process of hyperlipidemia, the mechanism of hyperlipidemia is still not clearly known \textsuperscript{15,17}.
1.2 Lipid and lipoprotein metabolism

1.2.1 Lipids

Lipids constitute a wide group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others. The main biological functions of lipids include storing energy, signaling, and acting as structural components of cell membranes. The property that sets lipids apart from other major nutrients is their solubility in organic solvents such as ether, chloroform, and acetone. All lipids are hydrophobic and mostly insoluble in blood, so they require transport within hydrophilic, spherical structures called lipoproteins.

Structurally, lipoproteins consist of two parts with a single layer of phospholipid and cholesterol molecules on their outside, surrounding a central core of lipids. Since the polar part of each phospholipid faces out, the outside of the lipoprotein particle is polar and therefore compatible with the surrounding water environment. On the other hand, the nonpolar portion of each phospholipid faces inward, and thus is compatible with the very nonpolar ingredients of the core of the lipoprotein. The nonpolar lipid core includes mainly triacylglycerol and cholesteryl ester. Lipoproteins also possess surface proteins (called apoproteins or apolipoproteins) that are cofactors and ligands for lipid-processing.

1.2.2 Lipoprotein metabolism

Lipoprotein fractions can be classified as chylomicron (CM), very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), or high-density lipoprotein (HDL) according to the ratio of lipid to protein within the particle as well as the different proportions of lipid types—triacylglycerol, cholesterol, cholesteryl esters, and phospholipids. Such differences influence the
density of the particle, which has become the physical characteristic used to differentiate and classify the various lipoproteins\textsuperscript{18,19}. Table 1 summarizes several physical and chemical characteristics of the human lipoproteins.

Apolipoproteins, the protein components of lipoproteins, tend to stabilize the lipoproteins as they circulate in the aqueous environment of the blood, but they also have other essential roles\textsuperscript{19,20}. In addition, apolipoproteins confer specificity on the lipoprotein complexes, allowing them to be recognized by their specific receptors\textsuperscript{19}. A partial listing of the apolipoproteins, the lipoproteins with which they are associated, and their postulated physical functions, is listed in Table 2.
Table 1. Major classes of human plasma lipoproteins: some properties

(Adapted from Lehninger Principles of Biochemistry 6th edition)

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Density (g/mL)</strong></td>
<td>&lt; 0.95</td>
<td>0.95-1.006</td>
<td>1.019-1.063</td>
<td>1.063-1.225</td>
</tr>
<tr>
<td><strong>Diameter (Å)</strong></td>
<td>1,000-10,000</td>
<td>300-800</td>
<td>200-250</td>
<td>50-120</td>
</tr>
<tr>
<td><strong>Apolipoprotein constituents</strong></td>
<td>apoB48 apoA-I apoA-II apoA-IV apoC-I apoC-II apoC-III apoE</td>
<td>apoB100 apoA-I apoA-II apoA-IV apoC-I apoC-II apoC-III apoE</td>
<td>apoB100 apoA-I apoA-II apoA-IV apoC-I apoC-II apoC-III apoE</td>
<td></td>
</tr>
<tr>
<td><strong>Protein (wt %)</strong></td>
<td>2</td>
<td>10</td>
<td>23</td>
<td>55</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>85</td>
<td>50</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 2. Apolipoproteins of the human plasma lipoproteins

(Adapted from Lehninger Principles of Biochemistry 6th edition)

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Lipoprotein association</th>
<th>Function (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>HDL</td>
<td>Activates LCAT; interacts with ABC transporter</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>HDL</td>
<td>Inhibits LCAT</td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>CMs, HDL</td>
<td>Activates LCAT; cholesterol transport/clearance</td>
</tr>
<tr>
<td>ApoB48</td>
<td>CMs</td>
<td>Cholesterol transport/clearance</td>
</tr>
<tr>
<td>ApoB100</td>
<td>VLDL, LDL</td>
<td>Binds to LDL receptor</td>
</tr>
<tr>
<td>ApoC-I</td>
<td>VLDL, HDL</td>
<td></td>
</tr>
<tr>
<td>ApoC-II</td>
<td>CMs, VLDL, HDL</td>
<td>Activates lipoprotein lipase</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>CMs, VLDL, HDL</td>
<td>Inhibits lipoprotein lipase</td>
</tr>
<tr>
<td>ApoD</td>
<td>HDL</td>
<td></td>
</tr>
<tr>
<td>ApoE</td>
<td>CMs, VLDL, HDL</td>
<td>Triggers clearance of VLDL and CM remnants</td>
</tr>
</tbody>
</table>
Chylomicrons (CMs) are the primary form of lipoprotein formed from exogenous or dietary lipids, and they are responsible for the transport of dietary lipids into the circulation. Enterocytes of the intestine resynthesize triacylglycerides, and together with phospholipids, cholesterol, and apolipoprotein 48 (apoB48) (human) or apoB100 (rodents), assemble them into CMs. CMs are secreted into the lymph and reach the plasma through the thoracic duct\textsuperscript{21,22}. Once the CMs reach peripheral tissues, lipoprotein lipase (LPL) will hydrolyze their triacylglycerides; the products – fatty acids (FA) – then enter cells. The remaining smaller particles are called chylomicron remnants, which acquire some cholesterol esters from HDL\textsuperscript{21} (see below). The change in particle size also uncovers apoE, which mediates the remnant binding to the apoB/E receptor and to the LRP in the liver\textsuperscript{21}.

Lipoproteins other than CMs transport endogenous lipids, which are circulating lipids that do not arise directly from intestinal absorption and are instead processed through other tissues, such as liver, muscle and adipose tissue. Triglyceride-rich VLDL is synthesized and secreted from the liver, a process that requires apoB100. In plasma, the triglyceride in VLDL is broken down into free FA and glycerol by LPL and its cofactor, apolipoprotein C-2 (apoC-2). Through this lipolysis, VLDL rapidly loses much of its triglyceride and becomes IDL, which will be further hydrolyzed and turn into LDL. Through the action of the LDL receptor, LDL is cleared from the plasma.

Alteration in hepatic lipoprotein secretion can induce disorders in lipid metabolism and an imbalance between lipogenesis and lipoprotein assembly can lead to many diseases, including, but not limited to hypertriglyceridemia, hepatic steatosis, cardiovascular diseases, and diabetes.
Fig. 1a. Lipoprotein metabolism schematics. Lipoproteins are like little vehicles that carry and transport fat from one part of the body to the next. CMs are produced in the cells of the intestines and carry fat that has been digested in the intestines into the body via the lymphatic system. VLDL is a carrier for fat that has been produced in the liver. LDL is derived from VLDL and IDL is the intermediate product of VLDL conversion to LDL. LDL is mainly sent back to the liver and its contents recycled.
Fig. 1b. Schematic diagram of LDL and its only structural protein apo100. The figure illustrates the proposed organization of apoB-100 on the LDL particle surface: blue, β structure; red, α-helical structure; darker blue and red, structures on the front of the sphere; lighter blue and red, structures on the back of the sphere. Adapted from JP Segrest, et al. 2001, J lipid Research.
1.2.3 Very-low-density lipoprotein (VLDL) and apolipoprotein B (apoB)

ApoB100 is the main structural protein of VLDL; both are synthesized in the lumen of the rough endoplasmic reticulum (ER) and secreted from the liver. Endogenous triglycerides, phospholipids, cholesterol, and cholesteryl esters can be transported in VLDL particles to peripheral tissues and organs, such as adipose tissue, muscles, and heart. The biogenesis of VLDL and its secretion into the circulatory system by the liver plays an essential role in lipid homeostasis since a major portion of triglycerides synthesized de novo is used for the formation of VLDL. Triglyceride in the ER appears to manage the intracellular fate of newly synthesized apoB100 protein because higher lipid levels can protect apoB from degradation. ApoB protein, expressed in the liver and intestine, has two forms: apoB100 and apoB48. In the human liver, only apoB100 is translated, whereas in the mouse liver, both forms are translated. ApoB is nonexchangeable (it never leaves the VLDL particle), which makes it an ideal indicator of VLDL production\(^\text{18}\). Emerging evidence has shown that high levels of lipids in the liver can lead to apoB accumulation and cause ER stress\(^\text{23,24,25}\).

ApoB serves as the basic scaffolding upon which TG-rich lipoproteins, VLDL and chylomicrons, and cholesterol-rich LDL are assembled (Fig. 1b.\(^\text{26}\)). Each lipoprotein particle contains just one apoB molecule. Full-length apoB, apoB100, one of the largest known proteins, is synthesized in the liver as a 4536-amino acid polypeptide (550 kDa)\(^\text{26}\). Through an RNA editing event that converts the Gln2153 codon to a stop codon, a truncated form (apoB48) containing 48% of the protein from the N-terminal is produced from the same RNA. In human, the RNA editing event only occurs in the intestine but not in the liver\(^\text{27}\). Thus, human CMs carry apoB48 whereas VLDL and LDL carry apoB100. In some rodents, including mice and rats, both forms of apoB are produced in the liver.
1.3 ER dysfunction and ER-resident proteins

1.3.1 ER dysfunction

The ER is the major site in the cell responsible for synthesis of lipids and sterols, as well as protein folding, maturation, quality control, and trafficking. The ER also plays important roles in stress response and cell metabolism. Specifically, in response to several stress stimuli, ER-transmembrane signaling molecules, including nuclear receptors, transcription factors, and cellular enzymes, can regulate lipid metabolism and/or glucose biosynthesis. Accumulation of misfolded or unfolded proteins in ER induces ER stress and results in refolding or degradation of the proteins, which is termed unfolded protein response (UPR). The UPR signaling network has been shown to involve three core elements: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein-1 (IRE1). Activation of these three pathways of the UPR leads to translation arrest and degradation of misfolded proteins, the expression of ER molecular chaperones, and the expansion of the ER membrane to decrease the load of proteins and increase the protein-folding capacity in the ER. UPR signaling was originally described as a system to evade cellular damage in acute ER perturbation. However, more recent advances have revealed that UPR signaling provides important signals for regulating cellular physiology. In summary, UPR signaling functions to restore and maintain homeostasis in the ER or to induce apoptosis if ER stress remains unmitigated. Recent studies implicate hepatic ER stress as a central abnormality associated with the development of metabolic syndrome, obesity, hepatic insulin resistance, and hepatic steatosis. However, the exact mechanisms remain obscure and treating obesity-related diseases by focusing on mitigating ER stress deserves further investigation.
1.3.2 SREBPs

Sterol regulatory element binding proteins (SREBPs), a family of ER membrane-binding transcription factors, mainly mediate lipid homeostasis by regulating the expression of a range of lipogenic genes, which are highly involved in the balance of endogenous cholesterol, FA, and triacylglycerol synthesis\(^ {35,36,37} \). The three main SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2, have different essential roles in lipid synthesis\(^ {38} \). Recent transgenic and knockout (KO) mice studies indicate that SREBP-1c is particularly involved in FA synthesis and insulin-induced glucose metabolism (in lipogenesis), whereas SREBP-2 is more specific to choleseterogenic gene synthesis; the SREBP-1a isoform seems to be implicated in both pathways\(^ {38,39} \).

Structurally, SREBPs consist of four domains with two membrane-spanning regions, and they function depending on the alteration of their structure. Initially, SREBPs are synthetized as approximately 120kDa inactive precursors that are bound to the ER membrane. When sterol is deprived, SREBPs translocate into the Golgi, escorted by SREBP cleavage-activating protein (SCAP), and this process requires cleavage by two distinct proteases in the Golgi apparatus. Finally, the SREBP precursors are cleaved by a sequential two-step cleavage process to release the NH (2)-terminal active domain (nuclear SREBP, roughly 70kDa) into the nucleus to activate their target genes, which are mainly involved in cholesterol and FA synthesis\(^ {36} \). It has been established that SCAP functions as a cholesterol sensor and is required for cleavage of SREBPs.
Fig. 2. SREBPs function as a control for lipid synthesis. SREBPs play a key role in transcriptional regulation of cholesterol metabolism in response to cholesterol levels in the cell. When cholesterol is abundant in the cell, the SREBPs are retained in the ER. When cholesterol levels decrease, SREBPs are cleaved and released to act as transcription factors, binding to the promoters of genes such as the LDL receptor and HMG CoA Synthase. The SREBPs are cleaved and activated by two proteases in the Golgi apparatus, S1P (Site 1 protease) and S2P (Site 2 protease). S1P cleaves the SREBP region and S2P cleaves in the transmembrane region of SREBPs. Regulation by sterols is provided by SCAP. SCAP activates S1P when sterols are low, inducing SREBP activation, and does not activate S1P when sterol levels increase.
1.3.3 Insulin induced genes (Insigs)

Insigs are also ER-resident proteins that contain six transmembrane segments that are highly involved in lipid synthesis\textsuperscript{40,41}. As mentioned before, SREBPs and SCAP together form a complex and then move into the Golgi to activate lipogenesis genes. However, if Insigs combine with this complex, this transportation will be obstructed and therefore reduce lipid synthesis. In other words, when cellular cholesterol levels are high, Insigs proteins bind and trap SCAP, retaining it in the ER and preventing it from escorting SREBPs from the ER to the site of proteolytic activation in the Golgi complex. Therefore, the INSIG-SCAP-SREBP pathway plays a crucial role in the feedback regulation of lipid metabolism and may be an intriguing factor in obesity development.

Research shows that Insigs include two closely related membrane proteins Insig-1 and Insig-2, known for their function as a promoter for ER retention of SCAP, and negative regulation of SCAP and HMG-CoA reductase\textsuperscript{42,43,44}. Though both Insig-1 and Insig-2 can principally regulate cholesterol feedback, they show some distinct differences. Firstly, though Insig-2 protein has 59% amino-acid sequence identity to Insig-1, Insig-2 performs a divergent role in regulating cholesterol homeostasis. Another important distinction is that high expression level of Insig-1 can retain the SCAP–SREBP complex in ER, even when sterols are completely depleted. However, Insig-2 requires that at least some sterols be present. Moreover, they are inversely regulated by insulin in the liver. Specifically, when insulin decreases upon fasting, Insig-1 expression also decreases, while Insig-2 expression increases. On the other hand, in refed animals, rising insulin levels promotes downregulation of Insig-2 and upregulation of Insig-1\textsuperscript{43,45}. 
It has been reported that Insig-2 has two isoforms: a liver-specific transcript of Insig-2, designated Insig-2a, and another transcript designated Insig-2b. They arise through the use of different promoters that produce different noncoding first exons that splice into a common second exon. Although the Insig-2a and -2b mRNAs encode identical proteins, they differ in patterns of regulation. Insig-2a is the predominant transcript in the liver of fed animals, and it is selectively down-regulated by insulin. Insig-2a mRNA increases when mice are fasted, and it declines when they are refed. The transcript also increases in the liver of rats whose insulin-secreting pancreatic beta cells have been destroyed by streptozotocin, and it is reduced when insulin is injected. The insulin-mediated fall in Insig-2a may allow SREBP-1c to be processed, thereby allowing insulin to stimulate FA synthesis, even under conditions in which hepatic cholesterol levels are elevated.

1.3.4 CREBH

Cyclic AMP-responsive element-binding protein H (CREBH, encoded by the gene CREB3L3) is a recently found ER-bound bZIP transcription factor that is highly and specifically expressed in the liver and the small intestine. CREBH is activated and then processed in the Golgi apparatus by proteolytic cleavage of the transmembrane domain by S1P and S2P proteases, which release the N-terminal portion of the protein that enters the nucleus to act as a transcription factor. In the nucleus, activated CREBH binds to cAMP response elements (CRE) and activates transcription driven by CRE-containing promoters, such as the rat phosphoenolpyruvate carboxykinase promoter. The expression of CREBH is dependent on hepatocyte nuclear factor 4α, which is a nuclear hormone receptor that is necessary for initiating and maintaining hepatocyte differentiation and liver function. Originally, CREBH was estimated to be activated by ER stress in a manner analogous to that of the canonical UPR
pathway initiator-ATF6. However, subsequent independent studies demonstrated that ER stress failed to induce the proteolytic activation of CREBH, which further questioned the hypothesis that CREBH is involved in the ER stress response. A novel study showed that CREBH processing is not increased by ER stress in CREBH KO mice compared to control mice.

CREBH is structurally and functionally related to the SREBPs. Recent studies have shown that CREBH is actively involved in lipid metabolism, and can be activated by free FA, fasting, inflammatory cytokines, and peroxisome proliferator activated receptor α (PPARα). Depletion of CREBH causes hyperlipidemia and severe NAFLD upon high-fat diet in rodent models. Recent studies also showed that fenofibrate decreases hepatic lipid synthesis through induction of CREBH, which behaves as a novel negative regulator of SREBP-1c production and hepatic lipogenesis. However, the underlying molecular mechanisms for the action of CREBH in lipid metabolism and the development of NAFLD still remain unknown. Thus, accumulating evidence suggests a potential link between hepatic ER stress and drug-induced fatality; however, the function of the ER stress protein CREBH, which is highly expressed in the liver, has not yet been extensively studied.

1.4 Metainflammation

1.4.1 Metainflammation

The association between lipid accumulation and metainflammation is widely acknowledged. Metainflammation was first introduced by Gokan Hotamisligil from the Harvard School of Public Health, and he used the term “metainflammation” to describe this state of low-grade inflammation, which leads to inflammatory response in many tissues and has been postulated to play an important role in many metabolic abnormalities like insulin resistance, type 2 diabetes,
Hyperlipidemia can initiate metainflammation in adipose tissue, which results in insulin resistance. Metainflammation may further trigger lipid abnormalities. Therefore, a vicious cycle occurs between lipid metabolism and inflammation. The strong and consistent relationships between makers of inflammation and risk of CVD have been confirmed by both epidemiological and clinical studies. Inflammatory mechanisms can lead to atherosclerosis through underlying pathophysiological mechanisms, and TNFα has been implicated in this process as an inflammatory cytokine. However, the exact mechanism is still unclear.

1.4.2 TNFα

Emerging evidence has accumulated over the past several decades that inflammation is implicated in lipid abnormalities. A major proinflammatory cytokine, TNFα, has been implicated in this process. Specifically, TNF is a critical cytokine, highly involved in both physiological and pathological processes. The TNF family is made up of many members with various roles, including TNF alpha (TNFα), TNF beta (TNFβ), CD40 ligand (CD40L), and Fas ligand (FasL). TNF-α plays a critical role in the pathogenesis of both acute and chronic inflammatory diseases by binding two ubiquitously expressed membrane receptors: p55 (TNF receptor 1) and p75 (TNF receptor 2). TNFα has been found to be involved in the regulation of several biological processes in liver, muscle and adipose tissue, including but not limited to immune-inflammatory and lipid homeostasis. Intraperitoneal injection of TNFα into C57B6 mice leads to an obvious accumulation of fat in the liver. In addition, administration of TNFα can rapidly increase serum triglyceride and triglyceride-rich lipoprotein levels in diabetic rats. TNFα can also stimulate significant expression and function of the LDL receptor. Furthermore, many studies have revealed that TNFα can directly block tyrosine phosphorylation of insulin
receptor and interfere with insulin signal transduction, leading to decreased insulin sensitivity\textsuperscript{78,79}. It has also been shown that TNFα can induce ER stress\textsuperscript{53,80,81} and ER stress can also induce the activation of CREBH\textsuperscript{53,82}. Specifically, TNFα can prompt CREBH mRNA expression in a time-dependent manner\textsuperscript{53}. Meanwhile, increased TNFα can directly stimulate hepatic assembly and secretion of VLDL. However, it is unknown whether or not TNFα regulates apoB levels by altering the expression of CREBH.
1.6 Study Aims

Specific Aim 1:
Determine the impact of CREBH signaling via insig 2a on hepatic lipogenesis and systemic hyperlipidemia. We will use Western blots to measure the relevant protein levels, including CREBH, Insig-1, Insig-2, and SREBPs. At the same time, we also plan to measure mRNA levels of CREBH, Insigs, SREBPs, and several SREBPs target genes.

Specific Aim 2:
Determine the impact of CREBH signaling on hepatic VLDL secretion via apoB expression. Firstly, we will determine whether overexpression of CREBH in McA-RH 7777 (McA) cells induces mRNA and protein expressions of ApoB. Next, we will test if overexpression of apoB induced by TNFα is capable of reducing hepatic lipid load to rescue CREBH-null mice from TNFα-induced CREBH expression.
Chapter Two

Materials and Methods

1. Cell culture and reagents

1.1 Cell lines and cell culture conditions

Rat hepatoma McA cells and human hepatoma HepG2 cells were cultured in 5% CO2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc. -BRL, Grand Island, NY) supplemented with 10% or 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 100 units/ml penicillin/streptomycin (Invitrogen, San Diego, CA). Equal numbers of cells were seeded onto tissue culture plates (60-mm culture dish). When cell confluence reached approximately 60%, the cells were subjected to serum starvation for 12 h in medium without FBS and treated as indicated below. Cells were subsequently processed for the isolation of RNA and protein.

1.2 Transfections

Transient transfections of cells with mammalian expression vectors were performed using Lipofectamine (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Briefly, 0.5×10^6 McA cells were plated in each well of a 6-well plate the day before transfection. The next day, 2 µg of DNA was first diluted in 100 µl serum-free medium followed by the addition of 8 µl Lipofectamine reagent. This mixture was then mixed well and incubated at room temperature (RT) for 20 minutes. During this time, 12 µl of Lipofectamine reagent was diluted in another 100 µl of serum-free medium per sample. At the end of 20-minute incubation, the diluted Lipofectamine reagent was added to the DNA mixture and incubated at RT for another 20 minutes. During this time, cells were deprived of serum supplements and incubated in 1.5 ml serum-free medium. At the end of the second 15-minute incubation, the DNA/Lipofectamine mixture was gently mixed into each well of a 6-cm plate of cells. The cells
were then incubated at 37°C for 5 hours, then 2 ml of medium supplemented with 10% FBS was added to each plate. The plates were then incubated at 37°C for 24 hours before further treatments.

1.3 Reagents

Forskolin (TOCRIS, Bristol, United Kingdom) was dissolved in Dimethyl sulfoxide (DMSO) to a final concentration of 10 mM. Glucagon (EMD4 Biosciences, Millipore, MA) was dissolved in 5% Acetic Acid to a final concentration of 1mg/ml, and the final working solution is 1µg/ml. Recombinant human TNF-a (Life technologies, Grand Island, NY) was dissolved in saline to a final concentration of 10µg/ml. Poloxamer (Sigma-Aldrich Canada Ltd., Oakville, ON) was dissolved in saline to have a 20% solution. The pH of each solution was adjusted to 6.8–7.4, and each solution was freshly prepared on the day of use.

2. Animals and animal experimental protocols

C57BL/6J mice were obtained from Jackson Laboratory, Maine, US. Mice were fed a standard chow diet containing 13.2% fat, 24.6% protein, and 62.1% carbohydrate (kcal/100 kcal) (#5053, Lab Diet). Heterozygous CREBH mice were kindly provided by Dr. Zhang Kezhong as a gift. Mice were housed in a 12 hours light/dark cycle with ad libitum access to food and water and euthanized by isoflurane. Both male and female mice around 2 months old were used in each experiment.

For some experiments, both wild type (WT) and CREBH KO mice were divided into fasted and refed groups. The fasted group was fasted 13 hours with free access to water and anesthetized under isoflurane. At the same time, the refed group was also fasted for 13 hours with free access to water and then fed an ad libitum diet for 8 hours before sacrifice. A baseline fasting blood sample was obtained from the submandibular vein. In Olive oil gavage treatment, mice were
fasted overnight for 5 hours with free access to water, followed a baseline fasting blood sample was collected and Poloxamer 407 was administrated 2.5ul/g by ip injection. Both WT and CREBH KO mice were fed 200µl virgin olive oil (Great Value, Walmart) by gavage and sacrificed 3 hours after the gavage. In TNFα administration experiment, both WT and CREBH KO mice were divided into two groups and then mice were fasted overnight for 13 hours with free access to water. One group was treated with recombinant human TNFα ( ), and the other group (control) was treated with saline. TNFα was injected ip at 0.001mg/kg, and the controls received the same volume of saline at the same time of day. After treatment for 5 hours, all the mice were killed. At the moment of sacrifice, mice were anesthetized under isoflurane and blood was directly collected from the heart. Blood samples were collected in heparin coated capillary tubes and centrifuged at 3000 rpm for 30 min and stored at −80°C for further analysis. Tissues, including liver, intestine, and adipose tissue, were collected, resected, and snap-frozen in liquid nitrogen and stored at −80°C for further analysis. Liver and intestinal tissues were then analyzed by quantitative reverse-transcription polymerase chain reaction (PCR), real-time PCR and immunoblots analyses. All protocols for animal care and experiments were approved by the Ethical Committee of University of Nebraska- Lincoln.

3. Immunoblots analysis

Following treatment, media was collected and cells were washed with ice-cold 1x PBS, rinsed totally three times, then lysed in solubilising buffer consisting of 150 mM sodium chloride (NaCl) (Sigma-Aldrich Canada Ltd., Oakville, ON), 10 mM tris (hydroxymethyl) aminomethylene (Tris) (pH 7.4) (Bio-Rad Laboratories Ltd., Mississauga, ON), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Canada Ltd., Oakville, ON), 1% Nonidet P-40 (Sigma-Aldrich Canada Ltd., Oakville, ON), and protease inhibitor cocktail tablet (Roche Applied Science, Laval, QC.).
Cells were then scraped and passed through a 23G needle 5 times. Cell lysates were centrifuged in a Heraeus Biofuge Pico centrifuge (Thermo Electron Corporation Canada, Gormley, ON) at 13,000 rpm for 15 minutes, then supernatants were transferred to new microfuge tubes and mixed with 2X Laemmli sample buffer consisting of 2% sodium dodecylsulfate (SDS) (Bio-Rad Laboratories Ltd., Mississauga, ON), 20% glycerol (Sigma-Aldrich Canada Ltd., Oakville, ON), 250 mM Tris-HCl, 5% 2-mercaptoethanol (Sigma-Aldrich Canada Ltd., Oakville, ON), and 0.01% bromophenol blue (BDH Chemicals Ltd., Poole, England). This was followed by boiling for 5 minutes at 100°C. Before further analysis, samples were stored at -80°C for detection of all other proteins. Media samples were also centrifuged and the supernatants were transferred to new microfuge tubes before addition of 4X Laemmli sample buffer, boiling for 5 minutes at 100°C and storage at -80°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% acrylamide gels for Histone H3 detection, 6% acrylamide gels for apoB detection, 10% acrylamide gels for SREBPs, and 12% for all other proteins, with running buffer (25 nM Tris, 192 mM glycine [Sigma-Aldrich, St. Louis, MO], 0.1% SDS) and a mini-PROTEAN electrophoresis system (Bio-Rad Laboratories Ltd., Mississauga, ON). Gels were transferred onto polyvinylidene fluoride (PVDF) membranes (PerkinElmer, Woodbridge, ON) with transfer buffer (190 mM glycine, 25 mM Tris, 10% methanol) and a wet transfer apparatus (Bio-Rad Laboratories Ltd., Mississauga, ON).

Membranes were blocked in either 5% milk (Bio-Rad Laboratories Ltd., Mississauga, ON) in 1x Tris-buffered saline with tween (TBST; 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 [Sigma-Aldrich Canada Ltd., Oakville, ON]) or 1% BSA in 1x TBST for at least 2 hours at room temperature or overnight 4°C, with shaking on an orbital shaker. Membranes were then shaken
with primary antibodies, diluted 1:1000 in either 5% milk or 2% BSA, overnight at 4°C. The following antibodies were used: anti-apoB and anti-albumin (Midland Bioproducts, Boone, IA); anti-β-actin (Sigma-Aldrich, St. Louis, MO); anti-CREBH, anti-SREBP-1, anti-SREBP-2, anti-Insig-1, and anti-Insig-2 (Santa Cruz, Dallas, TX); and anti-histone H3 (Thermo Scientific, Waltham, MA). Membranes were washed 3 times for 5 minutes in 1x TBST, then shaken for 1 hour at room temperature with the appropriate secondary antibody – either donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (GE Healthcare, Buckinghamshire, UK), sheep anti-mouse IgG-HRP (GE Healthcare, Buckinghamshire, UK) or rabbit anti-goat IgG-HRP (Santa Cruz, Dallas, TX). All secondary antibodies were diluted 1:5000. Membranes were washed 6 times for 5 minutes in 1x TBST then covered with enhanced chemiluminescence reagents (Amersham Biosciences, Pittsburgh, PA) to expose membranes onto autoradiography film (Denville Scientific Inc., Metuchen, NJ) using exposure cassettes (Eastman Kodak Company, Rochester, NY). Film was developed with an SRX-101A film processor (Konica Minolta Business Solutions (Canada) Ltd., Mississauga, ON) and bands were quantified by densitometry using Alpha Ease FC 4.0 software (Alpha Innotech, San Leandro, CA).

4. mRNA analysis by real-time PCR

Total RNA from rat liver was extracted using TRIzol (Invitrogen, Carlsbad, CA) and used for the synthesis of cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). mRNA for relevant genes and 18S rRNA were estimated by quantitative real-time PCR using a SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA). Quantitative RT-PCR was performed with a Prism 7300 Sequence Detecting System (Applied Biosystems, Carlsbad, CA). The primers used were listed in the Appendix.
5. Quantification of hepatic TG

Total lipids were extracted from the liver according to the method of Folch, J. Briefly, lipids were extracted from homogenized tissues with chloroform-methanol (2:1, v/v). After centrifugation, the organic phase was collected, dried under nitrogen, and then dissolved in 1% Triton X 100 in CHCl₃. Aliquots were assayed for triglyceride and cholesterol levels using a colorimetric TG and Cholesterol assay kit (Pointe Scientific Inc.).

6. Statistical analysis

In all cases, at least three independent experiments were performed and the data are presented as the mean ± standard error of the mean for triplicates within one representative experiment. If a representative experiment is shown, all three experiments showed similar results. For pairwise comparisons, the student t-test was used. Probability values of less than 0.05 are considered statistically significant and are indicated with a single asterisk (*). Likewise, probability values less than 0.01 are indicated with two asterisks (**).
Chapter Three

Results

Part 1

Determine the impact of CREBH signaling via insig 2a on hepatic lipogenesis and systemic hyperlipidemia

Previous studies have shown that CREBH, a liver-enriched ER-bound transcription factor, is highly involved in glucose and lipid metabolism. Specifically, both full-length CREBH (CREBH-F) and nuclear CREBH (CREBH-N) could enhance production of the cytosolic isoform of phosphoenolpyruvate carboxykinase promoter activity in hepatic cells and further delineate its physiological relevance in gluconeogenesis. To further confirm the potential involvement of CREBH in hepatic gluconeogenesis, we measured its expression levels in C57BL/6J mouse liver.

Interestingly, CREBH expression was significantly induced during fasting and was reduced upon refeeding, a characteristic regulatory pattern known for genes involved in gluconeogenesis (Fig. 1a). Furthermore, mRNA levels for hepatic CREBH (Fig. 1b) confirmed this phenomenon, showing a strong correlation between CREBH expression and gluconeogenic potential in liver. Indeed, we observed increased appearance of both full-length and nuclear CREBH under fasting, confirming that CREBH could be involved in the transcriptional process of hepatic gluconeogenesis.

Additionally, to further verify the regulation of the two Insigs transcripts by insulin, we also detected the expression of Insigs in this fasting or refeeding experiment. It is well known that food consumption can regulate hepatic lipid synthesis and fasting can decrease the synthesis of cholesterol as well as FA. As discussed before, when insulin is reduced upon fasting, Insig-1
expression decreases while Insig-2 expression increases. Consistent with previous studies, the protein expression of Insig-2 was significantly diminished by refeeding animals, whereas Insig-1 protein expression increased dramatically after refeeding (Fig. 3a). In summary, Insig-2 and Insig-1 protein expressions appear to be inversely regulated in the liver of mice subjected to fasting and refeeding. Furthermore, both hepatic Insig-1 mRNA and protein were simultaneously increased in the refed mice compared to the fasted mice (Fig. 3b). However, though Insig-2a mRNA was decreased significantly by refeeding, Insig-2b mRNA was unaffected (Fig. 3b). This phenomenon is consistent with the fact that Insig-2a is highly and specifically expressed in hepatocytes while Insig-1 is ubiquitously expressed. At the same time, we found that SCAP mRNA levels were significantly increased under refeeding conditions (Fig. 3b).

To identify a mechanism for CREBH regulation, we cultured HepG2 cells in media without FBS in order to mimic fasting signaling. Under refed conditions, protein expressions of both the full-length and nuclear forms of CREBH were decreased significantly (Fig. 4), suggesting an involvement of CREBH in the process of fasting signaling.
Fig. 3. CREBH and Insigs expression in C57BL/6J mouse liver under fasting and refeeding conditions. (A) WT mice were divided into fasted and refed groups. The fasted group was fasted 13 hours with free access to water and anesthetized under isoflurane. At the same time, the refed group was also fasted for 13 hours with free access to water and then fed an ad libitum diet for 8 hours before sacrifice. After sacrifice, liver extracts were assayed by immunoblotting for the membrane-bound precursor (CREBH-F) and mature nuclear (CREBH-N) forms of CREBH, as well as Insig-1 and Insig-2. (B) Blots are representative of three independent experiments. Both CREBH and Insigs bands were quantified by densitometry and intensities are shown relative to the loading control, β-actin. (C) Relative mRNA levels of hepatic CREBH, Insig-1, Insig-2a, **
Insig-2b, and SCAP were measured using real-time RT-PCR. Data were expressed as means ± SEM (n = 6), *P < 0.05, **P < 0.01.

**Fig.4.** CREBH expression is induced during fasting in HepG2 cells. HepG2 cells were fasted by culturing cells without FBS for 18 hours and refed by culturing in normal media with 5% FBS for 8 hours, after which cells were lysed and assayed by immunoblotting for the membrane-bound precursor (CREBH-F) and mature nuclear (CREBH-N) forms of CREBH, Insig-1 and Insig-2. Insig-1 and Insig-2 showed no significant changes (data not shown). Data were expressed as means ± SEM (n = 6), *P < 0.05, **P < 0.01.
The results of Fig. 3 and 4 suggested strongly that both CREBH and Insigs could be greatly influenced by fasting. To further test this hypothesis in vivo, both WT and CREBH KO mice were subjected to a fasting and refeeding protocol. Hepatic Insig-1 mRNA levels in both WT and CREBH KO mice were simultaneously increased under refeeding conditions, whereas Insig-2b was unaffected (Fig. 5). Specifically, Insig-2a mRNA was decreased by refeeding in WT and CREBH KO mice, and CREBH KO mice showed significant difference both in fasted and refed conditions compared to WT mice. This phenomenon was in accordance with the fact that Insig-2a is highly and specifically expressed in hepatocytes whereas Insig-2b is ubiquitously expressed. At the same time, we found that SCAP mRNA levels were increased under refed conditions in WT mice, although the difference did not reach statistical significance in CREBH KO mice. SCAP protein levels were consistent with the mRNA results (Data not shown). These data demonstrated that Insig-2a expression could be specifically regulated by both CREBH and insulin levels while not Insig-2b.

Activation of Insig-1 and Insig-2a signaling upon exposure to fasting and refeeding urged us to further investigate de novo lipid synthesis pathways in CREBH KO mice. Previous studies had shown that CREBH is a key metabolic regulator of hepatic lipogenesis, FA oxidation, and lipolysis under metabolic stress; after feeding an atherogenic high-fat diet, compared to control mice, a massive accumulation of hepatic lipid metabolites and a significant increase in plasma TG levels were observed in CREBH KO mice. We first determined whether SREBPs activation level was higher in CREBH KO mice since SREBP-1 directly regulates free FA synthesis in the liver. Indeed, expression of full-length SREBP-1c precursor (SREBP-1c-p) protein was markedly increased in CREBH KO mice (Figure 6A). Concurrently, the active nuclear form of SREBP-2
(SREBP-2-n), which regulates cholesterol metabolism, was significantly induced upon refeeding treatment in CREBH KO mice (Figure 6A).

**Fig.5.** Inverse changes in Insig-2a and Insig-1 in the liver of mice subjected to fasting and refeeding. WT and CREBH KO mice were divided into fasted and refed groups. The fasted group was fasted 13 hours with free access to water and sacrificed under isoflurane. At the same time, the refed group was also fasted for 13 hours with free access to water and then fed an ad libitum diet for 8 hours before sacrifice. Finally, total RNA from liver samples was pooled and subjected to real-time PCR quantification. Data were expressed as means ± SEM (n = 3), *P < 0.05, **P < 0.01.
**Figure A**

Protein expression levels of SREBP-1c-p, SREBP-1c-n, SREBP-2-n, and β-actin/Histone in WT and CREBH KO models under fasted and refed conditions. *p < 0.05, **p < 0.01.

**Figure B**

Relative intensity of SREBPs protein (ratio to actin/Histone) in various conditions. *p < 0.05, **p < 0.01.

**Figure C**

Relative mRNA expression of SREBP-1c and SREBP-2 in liver. **p < 0.01.
Fig.6. Refeeding induces hepatic full-length and nuclear SREBP protein expression and mRNA levels in both WT and CREBH KO mice. WT and CREBH KO mice were divided into fasted and refed groups. WT and CREBH KO mice were divided into fasted and refed groups. The fasted group was fasted 13 hours with free access to water and anesthetized under isoflurane. At the same time, the refed group was also fasted for 13 hours with free access to water and then fed an ad libitum diet for 8 hours before sacrifice. (A) Protein masses of precursor (p) and nuclear (n) SREBP-1c as well as nuclear SREBP-2 were detected by immunoblots analysis of cell lysates. (B) Immunoblots were quantified by densitometry and precursor proteins were normalized to β-actin, while nuclear proteins were normalized to Histone H3. (C) Total RNA was extracted from liver tissues to detect mRNA expression of SREBP-1c as well SREBP-2. Results are shown as means ± SD for two experiments that were performed in triplicate and n of 3 per group for animal study. *P<0.05.
To further explore the foundations of lipogenesis in WT and CREBH KO mice when they were exposed to fasting and refeeding situations, we measured the mRNAs of a number of SREBP-1c target genes that are involved in hepatic de novo FA synthesis, including FA synthase (FASN), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase-1 (SCD-1). Consistent with the activation of SREBP-1c upon refeeding, transcription of FASN, ACC, and SCD-1 mRNAs was significantly increased in CREBH KO mice compared to WT mice (Figure 7A). In parallel to the effects on mRNA expression of SREBP-1c target genes, we also observed the same increase in SREBP-2 target genes, including Hydroxymethylglutaryl Coenzyme A Synthase (HMGCS), HMG-CoA reductase (HMGCR), and LDL receptor (LDLR) (Figure 7B). Synthesis of triglyceride is a fundamental biochemical pathway, essential for nutrient utilization and energy storage. The enzyme catalyzing the final and committed step in the triglyceride biosynthetic pathway is diacylglycerol acyltransferase (DGAT). DGAT1 and DGAT2 are two of the several enzymes with DGAT activity that were cloned and characterized at the molecular level. Specifically, DGAT2 has been identified as the enzyme that catalyzes the de novo synthesis of triacylglycerols from newly synthesized FA and nascent diacylglycerols. DGAT levels were detected and remained unchanged between different groups (Figure 7C).

Glucose homeostasis is tightly regulated to meet fuel requirement in mammals. During fasting, secretion of pancreatic hormone, glucagon, adrenal hormone, and glucocorticoid is induced to enhance hepatic glucose production. Previous studies demonstrated that this process is partly accomplished through activation of gluconeogenesis, resulting from the transcriptional activation of gluconeogenic genes such as the cytosolic isoform of phosphoenolpyruvate carboxykinase or glucose-6-phosphatase. Additionally, cAMP response element has been found in gluconeogenic gene promoters, underscoring the importance of these transcriptional machineries in this pathway.
When HepG2 cells were exposed to glucagon treatment, both protein and mRNA levels of CREBH were increased. In the presence of glucagon, expression of full-length CREBH was slightly increased, but not to a level that was statistically significant (Figure 8). Subsequently, we confirmed that Insig-2 expression was also increased accordingly. However, Insig-1 protein and mRNA showed no changes, which were in accordance with the former results.

Previous studies demonstrated that CREBH is nutritionally regulated and activated by FA and PPAR alpha. To identify a novel mechanism for CREBH regulation, we treated HepG2 cells with stimuli known to mimic fasting signals, particularly forskolin. Forskolin is a cAMP agonist that can significantly enhance both mRNA and protein levels of CREBH. Of interest, when CREBH expression was increased, Insig-2a mRNA levels increased accordingly. The increase in Insig-2a mRNA translated into a significant increase in Insig-2 protein (Figure 9). Surprisingly, this was not the case with Insig-1, as we failed to show a change in Insig-1 protein mass or mRNA (Figure 9). This may be due to the rapid degradation of Insig-1 protein. These results suggested that CREBH might regulate Insig-2a when CREBH was induced by Glucagon. Indeed, a chromatin immunoprecipitation (ChIP) assay further confirmed the physical association between CREBH and the Insig-2a promoter (data from Dr. Wang Hai, not shown). These data identified Insig-2a as a target gene of CREBH.
Fig. 7. mRNA levels of SREBP target genes in both WT and CREBH KO mice under fasting and refeeding conditions. (A) Total RNA was extracted to detect mRNA expression of SREBP-1
target genes, FASN, ACC, and SCD-1 reductase. Transcript levels were normalized to internal control 18S ribosomal RAN. (B) The mRNA expressions of SREBP-2 target genes, HMGCS, HMGCR, and LDLR, were also investigated. Transcript levels were also normalized to internal control 18S ribosomal RAN. (C) The mRNA expressions of DGAT1 and DGAT2 were also examined. Results are shown as means ± SD for one representative experiment performed in triplicate. Three experiments were performed in triplicate and showed similar results. *P<0.05, **P<0.01.
Fig. 8. The regulatory role of glucagon-induced expression of CREBH. (A) HepG2 cells were cultured without FBS overnight, and then treated with 1ng/ml glucagon for 12 hours. Both mRNA and proteins were collected for further analysis. (B) Immunoblots were quantified by densitometry and CREBH precursor protein was normalized to β-actin. (C) mRNA levels of Insig-2a and SCAP were also detected. Results are shown as means ± SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.
Fig. 9. The regulatory role of forskolin-induced expression of CREBH. HepG2 cells were cultured without FBS overnight, and then treated with 10 µM forskolin 12 hours. Both mRNA and protein were collected for further analysis. (B) Immunoblots were quantified by densitometry and all proteins were normalized to β-actin. (C) mRNA levels of CREBH, Insig-2a, and SCAP were also detected. Results are shown as means ± SD for two experiments that were performed in triplicate. *P<0.05, **P<0.01.
Part 2
Determine the impact of CREBH on hepatic VLDL assembly and secretion via increased expression of apoB

ApoB is a key structural protein of VLDL particles produced in the liver, and therefore apoB levels are an ideal indicator of VLDL production. Perturbation of apoB protein synthesis can result in either accumulation of lipids in hepatocytes or VLDL overproduction. McA cells were transfected with the empty vector, CREBH 7.1 and CREBH DN to see their respective roles in apoB expression. Preliminary studies have demonstrated that depletion of CREBH decreases apoB mRNA expression (Figure 8B), whereas forced expression of CREBH in McA cells enhances expression of apoB at the transcriptional and translational levels (Figures 10B and C). These data indicated that the abundance of CREBH can influence apoB expression.
Fig. 10. Forced expression of CREBH in McA cells induces mRNA and protein expressions of ApoB. McA cells were seeded onto 6-well plates, and then transfected with 2 μg plasmids. M were transfected with 2 μg empty DNA vector p3×FLAG-CMV7.1 (Vector), vector expressing the active nuclear form of CREBH (CREBH 7.1) or vector expressing only the CREBH bZIP domain (CREBH dominant negative domain, CREBH DN), respectively. Five hours after transfection, cells and media were collected for protein and mRNA analysis. (A) A representative western blot analysis showing the protein levels of CREBH in transfected McA cells. (B) Transfection efficiency was further shown by polymerase chain reaction. (C) After transfection, the apoB expression was detected in media.
ApoB is required for VLDL formation and hence influences lipid transportation out of the liver. Firstly, in order to test if CREBH posed an influence on apoB and VLDL formation, we treated both WT and CREBH KO mice with virgin olive oil by gavage after 5 hours fasting. In order to eliminate the influence of LPL, we treated mice with LPL inhibitor Poloxamer before gavage. Plasma apoB levels were obviously lower in CREBH KO mice, relative to WT mice, suggesting a regulatory role for CREBH in apoB expression (Figure 1).

![Fig.1](image1)

**Fig.1.** ApoB expression in mice after gavage administration of fat in the form of virgin olive oil. In Olive oil gavage treatment, all mice were firstly fasted overnight for 5 hours with free access to water, followed a baseline fasting blood sample was collected and Poloxamer 407 was administrated 2.5ul/g by ip injection. Finally, both WT and CREBH KO mice were fed 200µl virgin olive oil (Great Value, Walmart) by gavage and sacrificed 3 hours after the gavage, and blood were collected again. (A) WT and CREBH KO mice (WT, n=6; KO MICE, n=4) were fasted for 5 hours. Basal line blood was collected, and apoB immunoblots were performed on plasma. (b) Mice were subjected to oral gavage with virgin olive oil. Blood was collected 3 hours after gavage, and apoB immunoblots were performed on plasma.
To further assess the potential involvement of CREBH in hepatic apoB expression in the liver, we treated both WT and CREBH KO mice with TNFα. As discussed before, TNFα can prompt CREBH mRNA expression, and TNFα treatment in animal experiments can directly stimulate hepatic assembly and secretion of VLDL. After fasting for 13 hours, both the WT and CREBH KO mice were treated with saline or TNFα. Then both Triglyceride and Cholesterol were measured from liver tissue. Triglyceride content increased in both WT and CREBH KO mice after TNFα treatment, though there is no significant difference between the two groups. However, there is an obviously increase in CREBH KO mice compared with WT mice after TNFα treatment (Figure 12). These data indicated that TNFα can induce lipids accumulation in the liver, and there is more lipids appeared in CREBH KO mice compared with WT mice.
Fig. 12. Hepatic triglyceride and cholesterol content in both WT and CREBH KO mice treated with TNFα. All mice were fasted for 13 hours, followed by ip injection of either TNFα or Saline. Mice were sacrificed 5 hours after treatment and the liver samples were homogenized to detect lipid contents. Results are shown as means ± SD for two experiments, n=3, *P<0.05, **P<0.01.
Chapter Four
Discussion
Part 1

Insig-2a Links CREBH Signaling to Hepatic Lipogenesis and Systemic Hyperlipidemia

In the current study, we examined the effects of CREBH on liver lipid metabolic signaling and regulation of genes involved in hepatic de novo FA synthesis. Initially, CREBH was identified as a hepatocyte-specific bZIP transcription factor belonging to the CREB/ATF family. CREBH was also known as a gene whose expression is dependent on hepatocyte nuclear factor 4α, which is highly involved in initiating and maintaining hepatocyte differentiation and liver function. Recent studies further demonstrate that CREBH mediates both the regulation of glucose and lipid metabolism. It has been well-established that secretion of insulin is controlled by nutritional signaling, such as fasting and refeeding conditions and mRNA expression of Insig-1 and Insig-2 are positively or negatively regulated by insulin, respectively. In addition, Insig-1 itself is a target of nSREBPs, and its mRNA rises and falls coordinately with nSREBPs levels. Hepatic Insig-1 protein level was low in the liver after 13 hours fasting, but became strongly induced after 8 hours of refeeding. On the other hand, Insig-2 is negatively regulated by insulin, though it is constitutively expressed at a low level in cultured cells. Consistent with our expectation, Insig-2 protein level was significantly decreased in the C57BL/6J mice fed a normal chow diet after 13h fasting. Furthermore, hepatic Insig-1 mRNA was simultaneously increased in the refed mice compared to the fasted mice, while Insig-2a mRNA was decreased significantly by refeeding. Our data clearly suggest that Insigs can be inversely regulated by insulin. Interestingly, when CREBH expression was induced by glucagon and cAMP agonist-forskolin in HepG2 cells, both mRNA and protein levels of Insig-2a expression were upregulated. On the other hand, the
expression of Insig-1 remained unchanged even though CREBH was induced. This is might because Insig-2 is a stable protein, whereas Insig-1 is a short-lived protein. Depletion or suppression of CREBH expression by refeeding inhibited Insig-2a expression, which in turn hyperactivated SREBP-1c and -2, leading to the activation of hepatic de novo lipid synthesis, accumulation of lipids in hepatocytes and systemic hyperlipidemia.

Previous results had shown that CREBH is a key metabolic regulator of lipid homeostasis, including hepatic lipogenesis, FA oxidation, and lipolysis, under metabolic stress. SREBPs are master transcription factors that regulate hepatic de novo lipogenesis and perturbation of SREBPs function is closely related to the onset of hyperlipidemia. Our study demonstrated that expression of Insig-2a was significantly decreased in the CREBH KO mice compared to its wild type littermate which led to the hyperactivation of SREBP-1c and SREBP-2. Consistence with the aberrant activation of SREBPs in the CREBH KO mice, the downstream target genes which involved in FA synthesis and lipid metabolism was activated, such as FASN, ACC, SCD-1, HMGCR, HMGCS, and LDLR. Activation of these genes led to the subsequently activation of hepatic lipid synthesis and the consequently systemic hyperlipidemia. Specifically, we noticed that both mRNA and proteins of SREBPs were higher in CREBH KO mice under refeeding conditions, which indicated that CREBH was essential in lipid metabolism. Our data were consistent with previous studies that showed massive accumulation of hepatic lipid metabolites and significant increase in plasma TG levels in CREBH KO mice after feeding an atherogenic high-fat diet.

Glucagon is a hormone that regulates blood glucose in a manner opposite to that of insulin. In the present study, we found that when HepG 2 cells were treated with glucagon, both mRNA and protein levels of CREBH increased significantly along with Insig-2a, while Insig-1 remained
unchanged. When CREBH expression was increased significantly by forskolin, there was a corresponding increase in both protein and mRNA levels of Insig-2a. Furthermore, a ChIP assay showed that there is a CREB binding site in the promoter of the Insig-2a gene, which further confirmed the physical association between CREBH and Insig-2a. Modulation of Insig-2a expression by CREBH appears to be essentially involved in regulating hepatic triglyceride metabolism. Further direct studies of Insig-2a KO mice model will be a good approach to further investigate this hypothesis.

Part 2
The impact of CREBH on hepatic VLDL assembly and secretion via increased expression of apoB

To further exploit the regulatory impact of CREBH on lipoprotein metabolism, we investigated the mechanism through which CREBH regulates apoB biosynthesis and VLDL secretion from the liver. Recent studies have also demonstrated that TNFα is able to induce ER stress which in turn activating CREBH. Specifically, TNFα can prompt CREBH mRNA expression in a time-dependent manner and increased TNFα also directly stimulate hepatic assembly and secretion of VLDL. However, whether or not TNFα regulates apoB levels by altering the expression of CREBH remains unknown. In summary, our aim is to find whether or not TNFα regulates apoB levels by altering the expression of CREBH. Firstly, we demonstrated that CREBH upregulated apoB mRNA expression. Forced expression of CREBH increases apoB biosynthesis and VLDL secretion. Depletion of CREBH compromised VLDL-apoB assembly and secretion which resulted in the accumulation of lipids in the liver of CREBH-KO mice. Our study provides novel insights into the duo-role of CREBH in regulating hepatic lipid and lipoprotein metabolism. It
may land support for developing CREBH as a nutritional target for the prevention and treatment of hyperlipidemia and obesity.

We will be able to determine whether overexpression of apoB induced by TNFα is capable of reducing hepatic lipid load to rescue CREBH-null mice from TNFα-induced CREBH expression. To investigate if overexpression of TNFα will promote the secretion of lipids from hepatocytes, we will deliver TNFα or saline (as control) into WT/CREBH KO mice via ip injection. Current in vitro studies shows that forced expression of CREBH can induce apoB expression. On the other hand, apoB expression decreases when we silence CREBH expression. In animal experiments, we found that TNFα treatment can induce triglyceride accumulation in the liver. Further research is necessary to gain more insight into the proposed mechanisms and to achieve a complete picture of them.
**Contribution to Original Knowledge**

CREBH, a recently identified transcription factor, had been shown to play a crucial role in regulating hepatic lipid homeostasis, including hepatic lipogenesis, fatty acid oxidation, and lipolysis under metabolic stress conditions\(^{50}\). The candidate’s major contributions are to demonstrate that CREBH inhibits hepatic de novo lipogenesis by regulating Insig-2a expression. The candidate further demonstrated that CREBH might be involved in VLDL secretion by influencing apoB expression. Mutation of CREBH has been reported to be closely associated with the development of hypertriglyceridemia in human subjects\(^{55}\). Previous findings indicate that CREBH is a molecular link between lipid homeostasis and inflammation\(^{53}\). This study has the potential to advance our understanding of the pathogenesis of hyperlipidemia, obesity, metabolic syndrome and NAFLD, and would provide novel insights into metabolic syndrome and potentially lead to innovative nutrient-based therapeutic strategies.
Chapter Five

References


Table 3. Primers used for qRT-PCR

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