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Role Of Crebh In Endotoxin Mediated Modulation Of Hepatic Metabolism

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ROLE OF CREBH IN ENDOTOXIN MEDIATED MODULATION OF HEPATIC METABOLISM

by

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DEDICATION

I would like to dedicate this work to my ever supporting family. This work is also a tribute to all those innocent lab animals who sacrifice their lives for research purpose.

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ABBREVIATIONS

AHF- Atherogenic High Fat Diet

CREBH- Cyclic AMP Response Element Binding protein H

FA- Fatty Acids

TG- Triglycerides

HFD- High Fat Diet

HDL- High Density Lipoproteins

LPS- Lipopolysaccharides

LDL- Low Density Lipoproteins

NAFLD- Non Alcoholic Fatty Liver Disease.

PTM- Post Translational Modifications

TLR- Toll Like Receptors

Ub- Ubiquitin

UPR- Unfolded Protein Response

ER- endoplasmic Reticulum

UPS- Ubiquitin proteasome System

VO2- Volume of Oxygen

VLDL- Very Low Density Lipopoproteins

ChIP- Chromatin Immunoprecipitation

INTRODUCTION

1.1 General introduction

Inflammation and metabolism are intrinsically linked, and chronic, systemic inflammation is an essential feature of metabolic syndrome (2). Inflammatory responses crosstalk with intracellular stress signaling pathways through a variety of professional cells of immune and metabolic features, such as adipocytes, macrophages, hepatocytes, and pancreatic β cells. Increasing evidence suggests that the integrated inflammatory stress responses modulate energy metabolism in these professional cells, contributing to the initiation and progression of metabolic diseases, such as obesity, type-2 diabetes, and atherosclerosis (3). Integrated inflammatory stress responses and their pathophysiological impact in metabolism have been a hot research topic in the past decade. However, an in-depth mechanistic understanding of the crosstalk between the intracellular stress signaling pathways and inflammatory responses, and their participation in disease progression remains to be further elucidated. Understanding the molecular networks underlying inflammation-modulated metabolism may lead to identifications of lucrative targets for pharmaceutical interventions or therapeutic benefits towards controlling diseases. This thesis research was focused on elucidating one such cross-talk between innate immunity and liver metabolism, mediated through a stress-inducible, liver-specific transcription factor, CREBH, under the endotoxin/ lipopolysaccharide (LPS) treatment.

LPS, a component of the gram-negative cell wall, is one of the potent factor capable of inducing a significant immune response in acute and chronic infection. It is a unique glycolipid located at the outer membrane of the bacteria. LPS is not found free in circulation, 80–97% of it is attached to the lipoproteins (4). The task of neutralization and clearance of LPS is mediated by HDLs in circulation (5). Circulating bacterial endotoxins are capable of causing acute as

well as systemic infections. The primary route of endotoxin contribution is through translocation of endotoxins from gut microbiota. The significant role of endotoxin in metabolic and cardiovascular disease has been proved through studies on germ free animals (6, 7). Endotoxins are capable of activating both adaptive and innate immune systems leading to the release of antibodies, cytokines, and other inflammatory mediators, which causes hepatic insulin resistance (8, 9). Treating rats with an antibiotic specifically targeted against gram-negative bacteria reduces the production of TNFα by macrophages, thereby reducing steatosis (10).

In humans, high-fat diet increases body weight and induces insulin resistance. The primary cause of the high-fat diet-induced metabolic syndrome associated with endotoxin originated from gut microbiota (11). These types of diets cause compromised intestinal permeability by altering the tight junctions in cells (12). Altered permeability is one of the main underlying reasons behind the phenomenon termed "metabolic endotoxemia". The severity of inflammation may depend on a complex interplay between specific proteins, receptors, and lipoproteins that mediate the endotoxin bioactivity and metabolic fate (13).

The richness of fat in western food makes the western countries more susceptible to different metabolic outbreaks. Etiology of obesity is closely associated with the intricate interaction between genetic and environmental factors (14). Metabolic syndromes like obesity and diabetes are the classical example of the influence of diet on human health. The significant effect of high-fat diet is through impairment of insulin signaling and the signaling associated with body weight maintenance (15). In addition, it has been recently determined that obesity and insulin resistance are associated with low-grade chronic systemic inflammation (16). In the diet-induced models for obesity, obese animals displayed increased levels of proinflammatory cytokines, such as tumor necrosis factor TNF-α, interleukin IL-1, and IL-6 were detected (17).

Production of such proinflammatory cytokines has harmful effect on action of insulin; for example, TNF-α mediated insulin resistance is through increasing serine phosphorylation on insulin receptor substrate-1, leading to its unresponsiveness to insulin signaling (18-20). The consequence of such impaired insulin signaling will cause hyperinsulinemia and increase in hepatic and adipose tissue lipid storage. However, all the research focus has been dedicated to the effect of an inflammatory reaction in body metabolism; the actual triggering factors connecting inflammatory stress to metabolic syndrome is still elusive (21). Recently, it has been demonstrated that fatty acids from the diet can induce activation of Toll-Like Receptor 4 (TLR4) signaling pathways in adipocytes and macrophages (22). Besides regular ligands, the metabolites generated during lipolysis in adipose tissues also serve as potential TLR4 ligands (23).

A new paradigm has recently been proposed that points out to the importance of human microflora in influencing the energy homeostasis in metabolic disease. The primary hypothesis was backed up by the evidence that obese individuals were associated with gut microbiota, and aberrant condition of gut microbiota promotes metabolic disease occurrence (24). A recent study has reported that the treatment of diabetes susceptible rats with antibiotics protect them from insulitis (25). The possible explanations behind this are that treatment with antibiotics changes the composition of gut microbiota, subsequently reducing the load of potential TLR ligands. Since, there is a decrease in the ligand, it leads to a reduction in proinflammatory cytokine production. From this viewpoint, we have been investigating a mechanism originating from the microbial component that trigger hepatic metabolic changes. We hypothesized that the bacterial LPS from the gram-negative intestinal microbiota would fulfill all the prerequisites to be an eligible ligand for our study. It has been established that endogenous LPS is: 1) continuously produced in the gut by the death of gram-negative bacteria and physiologically translocated into intestinal capillaries through a TLR4-dependent mechanism;

2) able to reach from the intestine toward target tissues, i.e., liver mediated by lipoproteins, notably chylomicrons freshly synthesized by epithelial intestinal cells in response to a high-fat diet; and 3) eliciting the production of proinflammatory cytokines when it binds to the complex of mCD14 and the TLR4 at the surface of innate immune cells. In my thesis research, I aimed to demonstrate a novel molecular link through which LPS could modulate lipid metabolism under metabolic and/or inflammatory condition.

Among the stressors, microbial components, such as peptidoglycan and LPS can affect the growth performance and also modulate the metabolism. Additionally, recent biomedical evidence suggests that the low-grade inflammation caused by intestine- derived LPS is linked to metabolic diseases, such as Type II diabetes, atherosclerosis, and cardiovascular diseases (26). Importantly, the intact mucosa from the gastrointestinal tract acts as a mechanical barrier for bacteria in the intestinal lumen; a primary source of LPS (27). LPS in mammals is recognized by various cells expressing the pattern recognition receptor and other proteins including LPS binding protein (LBP), a cluster of differentiation 14 (CD14), and MD-2. These proteins and receptors are shown to be present in most of the cells from the liver and have been ascribed role in the permeability of luminal LPS into circulation (28). Once in the systemic circulation, detoxification of LPS can be carried by immune cells, such as macrophages, Kupffer cells, and splenic cells, or even by binding to plasma proteins. However, if there is failure in detecting and deactivating the circulating LPS, increased intestinal permeability may increase circulating LPS concentrations that eventually lead to systemic inflammation, endotoxemia, multi-organ failure, and even death (29).

In human health, presence of LPS in the circulation have been shown to contribute to the development of chronic inflammatory processes that promote the development of dysregulated metabolism which results in many metabolic diseases such as type II diabetes and non-alcoholic fatty liver disease (NAFLD) through the stimulation of TLR4. Interestingly, the

permeability of LPS from the intestine has been shown to be regulated by various factors such as dietary factors, and different stressors, such as heat, infection, and malnutrition. The major dietary factor that appears to modulate the permeability of luminal LPS is dietary fats. As the percentage of dietary fats increases, so does the concentration of circulating LPS (30). Further studies are warranted to investigate the relationship between LPS and metabolic changes.

1.2 Structure of bacterial LPS

The structure of LPS consist of three main part; a hydrophobic domain, lipid A, through which it is anchored into the outer leaflet of the outer membrane of the bacterial cell wall, a core oligosaccharide, and a distal oligosaccharide (31). The hydrophobic lipid A domain is the immunogenic part of the LPS molecule, and it is often used as 'endotoxin' because of its ability to stimulate the innate immune cells. In a wild-type Escherichia coli, lipid A contains the following structural properties: 1) the backbone of the lipid A contains di-glucosamine, which is phosphorylated at positions 1 and 4', 2) two 3-hydroxymyristate molecules are directly attached to each glucosamine, and 3) at positions 2' and 3', the hydroxyl groups of the fatty acids are substituted by laurate and myristate, and they form an acyloxyacyl bond with the primary fatty acid chains (32).

1.3 Microbiota and human metabolism

Gut microbiota primarily inherited from the intestinal bacteria that are acquired from the mother first and subsequently modified by surrounding environment immediately after birth. Establishment of microbiota takes about few months and tend to remain stable for a lifetime with minimal variation. The microflora in a different segment of gastrointestinal tracts, however, displays variability in both density and species. Tiny numbers of microorganisms colonize in the stomach and the duodenum, whereas the lower gastrointestinal tract harbors

progressively increased number of microbes. Despite up to 1,000 species of microorganisms colonize the whole intestinal tract, the gut immunity fitness is maintained over the time of life. Pathological stimuli, such as immunosuppressive drugs, radiations, and chemicals, can lead to alteration in the gut microbiota.

Microorganism in the digestive tract also offers much beneficiary functions for harboring hosts. For instance, intestine microorganisms contribute to the synthesis of short chain fatty acids, the number of which serves substrate in energy metabolism process. Propionate that is a substrate for both gluconeogenesis and de novo lipogenesis in hepatocytes is produced by microbiota. Obesity-associated microbiota modulation has been reported in both animal and human studies (33). In another animal study, two dominant bacterial divisions in the gut microbial community were reported to have distinct abundance in genetically obese ob/ob mice in comparison to their lean littermates: relatively fewer Firmicutes while more Bacteroidetes (34). Furthermore, microbiota from ob/ob mice showed an efficient ability to harvest energy from the indigestible diet in the intestine than microbiota from their lean littermates. In humans, it is reported that the ratio of Firmicutes to Bacteroidetes was notably higher in obese subjects than lean people. Furthermore, increased abundance of Bacteroidetes correlates with a degree of weight-loss following fat restricted or carbohydrate-restricted dietary interventions. Taken together, these findings suggest that the alteration of microbiota plays an important role in terms of outcomes associated with obesity and its related metabolic disorders in both animals and humans.

1.4 Endotoxemia in metabolic disorders

One of the significant contributions of altered gut microbiota in metabolic disorders is the elevation of circulating LPS. As mentioned before, high-fat diet (HFD) results in an increased amount of the LPS-containing microbiota in the intestine and a subsequently causes

elevation of circulating LPS levels. Confirming these results are the observation from human studies. Blood endotoxin (LPS) levels in healthy human subjects were significantly high after a high-fat meal (35). Several mechanisms have been proposed to explain why high-fat diet causes endotoxemia. Genetically obese ob/ob and db/db mice had increased portal endotoxin levels. This leads to enhanced intestinal permeability, and eventually increased circulating cytokine levels relative to lean control mice (36). New research provide an evidence that lipid facilitate absorption of LPS in intestine In vitro incubation of human intestinal epithelial Caco-2 cells with oleic acid, a long-chain fatty acid that can induce chylomicron formation, resulted in more release of cell-derived LPS, whereas this effect of oleic acid is abolished after the blockade of chylomicron formation by Pluronic L-81 (36). Consistently, in vivo study also showed that administration of LPS with oil induces significant effect than the mixture with water (9). The circulating LPS is an important player in the pathogenesis of obesity-related metabolic disorders. In mice on normal chow, continuous LPS infusion caused a cluster of metabolic disorders, including hyperglycemia, hyperinsulinemia, as well as increased body weight (37). Most strikingly, these LPS-induced metabolic alterations in normal chow-fed mice were comparable to those in mice upon high-fat diet feeding. Not only endotoxemia affects the local adipose tissue and muscles they also exert profound influences on energy metabolism in the liver. Chronically subcutaneous infusion of LPS in vivo leads to hepatic insulin resistance and excessive hepatic triglyceride accumulation. Intriguingly, LPS-stimulated hepatic insulin resistance and steatosis are almost entirely suppressed in CD14-deficient mice (38). Overall, these data demonstrated the potential link between low-grade endotoxemia and metabolic disorders.

Infection and inflammation can cause significant changes in lipid and lipoprotein metabolism that result in increased circulating free fatty acids (FAs), hypertriglyceridemia, and altered plasma HDL levels. These changes are mediated by cytokines, such as interleukins

(IL6, IL1β), tumor necrosis factors (TNF-α and TNF-β), and interferons (IFN-α, IFN-β, and IFN-γ) (39). These mediators are also increased in several low-degree inflammation disorders like atherosclerosis, obesity, metabolic syndrome and diabetes that show abnormalities in lipid metabolism similar to those found during infection and inflammation (39, 40).

There are several lines of evidence supporting the idea of an intricate network of relationships between inflammatory responses and lipid biology, including that of lipid droplets (LDs). It has been shown that administration of TNF-α, induce modifications in the surface of LDs and also promote an increase in lipolysis in adipocytes (41). On the other hand, clinical samples from sepsis patient tend to show accumulation of LDs and association of inflammatory proteins with LDs (42). Injection of endotoxins in rats displayed increased levels of serum VLDL-ApoB (10-fold), -triglyceride (2-fold), and -cholesterol (2-fold). Similarly, the hepatocytes isolated from such endotoxic mice secreted more VLDL-ApoB than their comparable controls. Currently, little is known about the mechanism of crosstalk between the body's immune system and metabolism. My thesis work uncovers one of the aspects and a mechanism underlying previously noted metabolic changes associated with endotoxemia. Infection and inflammation are accompanied by similar cytokine-induced alterations in lipid and lipoprotein metabolism.

1.5 Innate immune signaling

The innate immune system provides protection against a large variety of pathogens, possible through its array of receptors called pattern recognition receptors (PRRs). These receptors recognize specific and conserved molecular patterns or domain of pathogens. The PRRs include the members of the TLRs family and the nucleotide-binding oligomerization domain proteins (NOD-like receptors, NLRs).

TLRs belong to the type I transmembrane proteins that have distinct domains, an extracellular leucine-rich domain (LRR) and an intracellular or cytoplasmic domain homologous to the interleukin-1 receptor (IL-1R) and therefore called Toll/IL-1 receptor (TIR) domain. LPS is recognized and signaled by the PRR, TLR4 (43). However, the presence of LPS is not sensed by TLR4 alone. LPS is generally present in clumps bound to other LPS molecules on which LBP leeches out a monomer that is then presented to a co-receptor called as a CD14 receptor. The CD14 receptor is present in two forms, membrane-bound (mCD14) or soluble (sCD14) (44).The CD14 protein lacks an intracellular domain so it associates with TLR4, which has a Toll-interleukin 1 receptor (TIR) intracellular domain through which it can transmit the intracellular signal. TLR4 then dimerizes with MD-2, which transmits the signal through the TIR intracellular domain through two different pathways. One is a myeloid differentiation factor 88 (Myd88)-dependent and the other one is a Myd88-independent pathway. The first pathway leads to translocation of nuclear factor kappa beta (NFκB) to the nucleus and the initiation of transcription of inflammatory mediators. Alternately, the independent pathway leads to the activation of interferon regulatory factor 3 (IRF3) as well as NFκB (45). Both pathways lead to the secretion and stimulation of pro-inflammatory cytokines and other immune mediators. TLR4 signaling is quenched by endocytosis of TLR4, along with LPS, to an endosome where it is then degraded (46). Current research indicates that apart from the signaling protein, the role of lipid rafts are essential for TLR4 signaling and permeability through the membrane to occur (47, 48). Lipid rafts have also been implicated in endocytosis of pathogens (49). Interestingly, TLR4 has been shown to localize to these membrane lipid raft domains upon LPS stimulation, and disruption of LPS signaling occurs if the lipid raft is dissociated (50). Further saturated, and unsaturated fats have been shown to modulate the TLR4 localization reciprocally into lipid raft and its signaling. Saturated fatty acids stimulate the

TLR4 to localize into rafts and start the inflammatory signaling cascade whereas n-3 PUFA's prevent the stimulation and localization into lipid raft (51).

1.6 TLR4 and ligands

TLRs sense signals derived from invading pathogens through the recognition of pathogen-associated molecular patterns (PAMPs). LPS was the first PAMP discovered, which was initially described as "endotoxin" by Richard Pfeiffer in the year 1892. Serving as a component of the outer membrane of gram-negative bacteria, LPS has been well-characterized as the most common ligand of TLR4. As mentioned previously, LPS is structurally composed of three portions, including a predominantly lipophilic region (lipid A), a hydrophilic core oligosaccharide, and a polysaccharide chain (O-polysaccharide). The lipid A part is the significant portion responsible for TLR4- agonist effect of LPS. The O-polysaccharide region shows variations in different forms of LPS. LPS in some types of bacteria does not contain O-polysaccharide portion, known as "rough LPS". Conversely, LPS containing O-polysaccharide is named as "smooth LPS". The rough and smooth form of LPS activates TLR4 in different manners. Rough LPS may activate TLR4 independently of LPS-binding protein (LBP) and CD14 whereas LBP and CD14 are required for smooth LPS-mediated TLR4 activation. On the other hand, TLR4 activation caused by smooth LPS in the presence of LBP and CD14 may be more efficient than that induced by rough LPS. (52)

Besides LPS, a cluster of molecules released after cellular damage or wound healing responses have been demonstrated to have binding ability to induce TLR4 signals, such as high mobility group protein b-1 (HMGB1) and hyaluronan. A specific term, damage-associated molecular patterns (DAMPs), has been coined to describe this class of molecules with the TLR4-agonist activity (53). HMGB1 is a 30-kDa heparin-binding protein which exerts both transcription factor-like and cytokine-like functions in cells undergoing apoptosis and necrosis

(54). Furthermore, HMGB1 can catalytically disaggregate and transfer LPS to both soluble CD14 protein and human peripheral blood monocyte (PBMCs). In addition, treatment with a mixture of HMGB1 and LPS resulted in significant production of TNF-α production in human PBMCs than LPS or HMGB1 treatment alone, suggesting that HMGB1 facilitates LPS to induce TLR4-mediated proinflammatory response (55). FAs have been also demonstrated to induce TLR4-mediated inflammatory signals. In macrophages, treatment with saturated FA led to NF-κB activation, COX-2 expression, as well as proinflammatory cytokine IL-1α production. A dominant-negative TLR4 blocked this effect, suggesting that an indispensable role of TLR4 in unsaturated FA-induced inflammatory response in macrophages. Likewise, another study reported that FAs activated TLR4 signaling in macrophages, adipose cells, and tissues (56).

1.7 Innate immunity in the liver

Out of the conventional arterial system blood supply the majority (about 80%) of blood entering the liver through a portal vein that is rich in gut content. This leads to exposure of the liver to an array of immunogenic stimuli. The enriched bacterial products and environmental toxins in the portal blood stream are transported to the liver. The immune system responds to both endogenous and foreign harmless antigens to avoid abnormal and excessive immune response causing tissue damage (57). Hence, innate immunological components are predominant in liver immunity. On the one hand, innate immune cells, including macrophages (Kupffer cells), NKT cells, and NK cells are selectively enriched in the liver. In a healthy liver, Kupffer cells constitute about 20% of mesenchymal cells (58). The number of macrophages in the liver (liver resident and circulating macrophages) is further elevated in response to several stimuli. NKT cells, accounting for up to 30% of the hepatic lymphocyte fraction, are abundant in the liver than other organs (59). On the other hand, liver cells produce several factors

participating in systemic innate immunity, such as inflammatory cytokines, chemokines, and complement components. Therefore, innate immunity plays a crucial role in maintaining the homeostasis and the defense of pathogens in the liver.

Recognition and initiation of inflammation is a key in the development of NAFLD. In NAFLD patients as well as animal models, NF-κB activation is observed in liver cells, including hepatocytes, hepatic stellate cells, and Kupffer cells. NF-kB activation elevates in NAFLD. NF-kB activation plays an important role in proinflammatory cytokine and chemokine productions. It also plays a major role in insulin resistance. It is unclear whether NF-κB activation in hepatocytes leads to steatosis. Hepatocyte-specific IKKβ overexpression induces steatosis. In contrast, NF-κB essential modulator (NEMO) deficiency in hepatocytes results in spontaneous steatohepatitis (60). NEMO deficiency completely blocks NF-κB activation, indicating that NF-κB activation in hepatocytes is not a primary cause of steatosis. One of the important function of NF-kB is activation of TNFα genes, which in turn exerts a significant effect on NAFLD outcome (61).

1.8 Inflammation, stress response, and Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD represents a spectrum of diseases ranging from simple fatty liver (steatosis) to nonalcoholic steatohepatitis (NASH), to irreversible cirrhosis. NAFLD is the most common chronic liver disease in Western countries and is considered the hepatic manifestation of metabolic disorders including visceral obesity, diabetes mellitus, dyslipidemia, and hypertension (60). The hallmark of NAFLD is characterized by excessive accumulation of hepatic lipid, mainly triglycerides, in the absence of significant ethanol consumption, viral infection, or other specific etiologies. The development of a state of non-alcoholic hepatic steatosis may be caused by an increased uptake of lipids by the liver, an increased hepatic

synthesis of fatty acids, decreased oxidation of fatty acids and/or decreased synthesis or secretion of very low density lipoproteins (VLDLs). The progression from hepatic lipid accumulation and steatosis to NASH is explained by a "two-hit" working model (62). According to this model, steatosis represents the "first hit," which increases the vulnerability of the liver to various "second hits" induced by endotoxin, oxidative stress, saturated fatty acids, or other liver injuries. The "second hits" will subsequently lead to the inflammation, fibrosis and cellular death characteristic of steatohepatitis. Consistent with this model, administration of variously proposed second hits (e.g., endotoxin and pro-oxidants) results in significantly greater liver damage and lethality in obese mice with fatty liver compared to lean mice with healthy livers. Furthermore, in humans, the severity of steatosis is one of the strongest predictors of the development of NASH. Several factors have been suggested to constitute the second hit(s), most notably oxidative stress, pro-inflammatory cytokines, and gut-derived bacterial endotoxin. The effects of the "two hits" are not mutually exclusive, but act in a coordinated and cooperative manner to hasten the development and progression of NASH. For example, excess fatty acids are associated with increased proinflammatory cytokines and oxidative stress, as well as an exaggerated inflammatory response to endotoxin administration. In environments conducive to the generation of various second hits, for example, obesity, a vicious cycle of insults may cause liver injury and culminate in NASH and end-stage liver disease. Despite this knowledge, numerous critical questions remain unanswered. For example, what are the molecular determinants that mediate the effects of "first hit" and/or "second hit" in the development of NAFLD? What metabolic signaling initiates the development of NAFLD? Investigations of these questions will have important implications for understanding the pathways that control hepatic steatosis and steatohepatitis and will be informative to prevention and treatment of human NAFLD and its associated metabolic syndrome.

1.9 Hepatic lipid metabolism

In the liver, the lipid content is regulated by dietary FA or carbohydrates uptake, hepatic FA biosynthesis, esterification, oxidation, and export. Dietary carbohydrates are partly consumed for normal bodily functions and metabolized to glycogen or FAs in the liver if in excess (63). Fatty acids are esterified to triglycerides (TG) and then either be stored in cytoplasmic lipid droplets or incorporated into VLDL particles and secreted into the blood. Additionally, TG can also be hydrolyzed, and the fatty acids are destined towards the oxidation pathway. Conversion of carbohydrates to TG involves two steps: glycolysis which generates acetyl-CoA from glucose, and lipogenesis that converts acetyl-CoA to fatty acids. Enzymes involved in glycolytic and lipogenic pathways are dynamically regulated at both transcriptional and post-translational levels by various factors such as substrate concentrations and hormones. Pancreatic hormones, insulin, and glucagon play critical roles in the transcriptional regulation of these enzymes. For example, insulin can reduce hepatic glucose production through decreasing glycogen breakdown (glycogenolysis) and increasing the rate of glucose uptake, primarily into skeletal muscle and adipose tissue. Insulin can also stimulate FA biosynthesis by activating the sterol regulatory element binding protein-1c (SREBP-1c) transcription factor that activates the expression of lipogenic genes involved in FA synthesis and TG synthesis (64, 65). During fasting, glucagon activates the protein kinase A, which phosphorylates carbohydrate response element-binding protein (ChREBP), preventing its translocation to the nucleus and the subsequent activation of its target genes involved in glycolysis and lipogenesis (66).

Transcriptional regulation plays a key role in regulating hepatic lipid homeostasis under metabolic and inflammatory conditions. Metabolic signals, such as increased levels of FAs, glucose, and insulin, can regulate the activity or abundance of key transcription factors to modulate hepatic lipid metabolism (67). Many hepatic transcription factors have been

identified as prospective targets for de novo lipogenesis and fatty acids oxidation, including sterol regulatory element binding protein-1c (SREBP-1c) (67), retinoid X receptor (RXRα) (68), liver X receptor (LXRα) (69), peroxisome proliferator-activated receptors (PPARα, β, γ1, and γ2) (70), CAMP Responsive Element Binding Protein 3-Like 3 (CREB3L3 or CREBH) (71, 72), and chREBP. These factors integrate signals from various pathways and coordinate the activity of the metabolic machinery necessary for hepatic lipid metabolism with the supply of energy and fatty acids.

1.9 Understanding Cyclic AMP Response Element Binding Protein H (CREBH)

CREBH, A novel liver-specific cell stress sensor, has been demonstrated to be involved in NAFLD (73). CREBH is a basic leucine zipper transcription factor of CREB/ATF family. The CREB family is a group of a bZIP transcription factor in mammals consisting of CREB3L1, CREB3L2 CREB3L3, and CREB3L4 (74). CREB3L3 or CREBH was first reported to be a liver-specific transcription factor (75, 76). The entire CREB family transcription factor shares significant homology within their bZIP domain that mediates DNA binding and dimerization. They all have a transmembrane domain at the C-terminal side of the bZIP region. The N-terminus faces the cytoplasm while the C-terminal is anchored to the ER membrane into ER lumen. All the CREB3 families of the transcription factor are activated by a mechanism called Regulated Intramembrane Proteolysis (RIP) (77).

It has been shown that the CREBH is richly expressed in liver hepatocytes. Low levels of CREBH can be detected in the pyloric stomach and small intestine tissues. Full-length CREBH protein is localized in the ER membrane of liver hepatocytes and bears a similarity in structure with an ER stress sensor, Activation Transcription Factor 6 (ATF6). Transcription of the CrebH gene is highly induced by pro-inflammatory cytokines including TNFα, IL-6, IL1β,

as well as bacterial endotoxin LPS. This was evident that expression and activation of CREBH were induced by LPS or TNFα challenge (1).

The current model for activation of CREBH under ER stress is elucidated by Zhang et al (78). Under ER stress and inflammatory challenge, CREBH is released from ER membrane and translocate to Golgi. At Golgi, it undergoes proteolytic cleavage by S1P and S2P protease to release its functional, cytosolic (N-terminus) fragment that functions as a transcription factor. Interestingly, rather than activating the transcription of UPR genes, it activates the expression of acute phase inflammatory factors, C-reactive protein, serum amyloid component P (SAP), and serum amyloid A (SAA). Hence, it has been propose that CREBH represents a molecular link between ER stress and hepatic inflammation. Additionally, it has found that CREBH can induce expression of hepcidin, a novel liver acute-phase protein that plays a central regulatory role in iron homeostasis under inflammatory stress conditions (79).

RIP can be triggered by ER stress, inflammatory stress or metabolic stress (80, 81). After RIP process, the cleaved CREBH N-terminal fragment then transits into the nucleus to function as an active bZIP transcription factor (1).

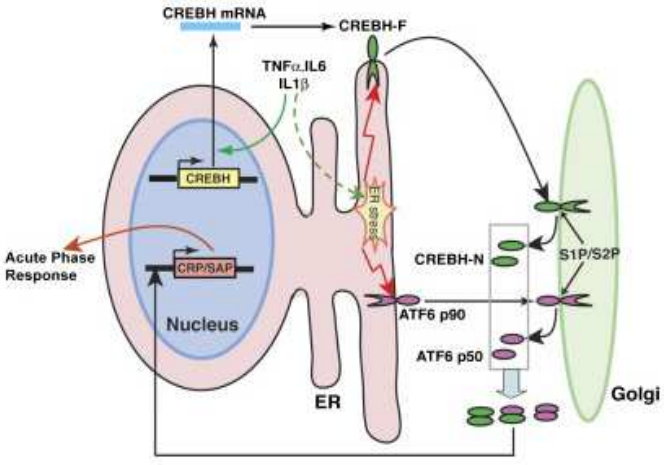

Image adapted from Cell Press.(1)

Figure 1: Schematic model for CREBH location and its activation mechanism.
ER stress leads to cleavage of CREBH to release precursor CREBH that translocates to Golgi to undergo proteolytic cleavage by S1P and S2P proteases. The N-terminal cleaved form then goes to the nucleus to activate transcription. CREBH does not contribute to the classical UPR induction but is required for the APR by regulating transcription of the *CRP* and *SAP* genes.

Recently, we have shown that CREBH plays important roles in regulating lipid metabolism in the liver. Fasting condition, high-fat diet, insulin signals, or saturated fatty acids, such as palmitate, can activate CREBH (73). Pro-inflammatory cytokines including TNFα, IL-6, and IL1 β, as well as bacterial endotoxin LPS, can induce CREBH cleavage in the liver in vivo. It has been demonstrated that administration of TNFα, IL-6 plus IL1 β, or LPS into the animals can induce physiological ER stress in the live (1). Upon activation, CREBH acts as a potent transactivator to induce expression of the genes encoding key regulators or enzymes involved in gluconeogenesis, lipogenesis, FA oxidation, ketogenesis, and lipolysis (71, 73). Notably, CREBH interacts with PPARα to synergistically activate the metabolic hormone FGF21 to regulate lipolysis, FA oxidation, and ketogenesis upon fasting or under an atherogenic high-fat (AHF) diet (71). The overall role of CREBH is to maintain energy homeostasis under metabolic stress. A defect in CREBH leads to non-alcoholic steatohepatitis (NASH) and hyperlipidemia under the AHF diet or fasting (71, 73, 82). Bioinformatics analysis indicated that human patients with hyperlipidemia had higher rates of CrebH gene mutations (82).

1.10 Hypothesis, objectives, and project overview

Invasion by pathogens can cause a variety of physiological responses in the host. These include various metabolic changes in local tissues. Components of bacterial cell walls are key molecules involved in triggering a particular metabolic responses in the host. Hyperlipidemia usually accompanies bacterial infection as well as inflammation (76). The association between the increase in TG and infection is partially due to increased in lipoprotein production as well as defective clearance of lipoproteins from circulation. Currently, little is known about the mechanism underlying the crosstalk between the body's immune system and metabolism. My thesis research uncovers a mechanism behind the previously noted metabolic changes

associated with endotoxemia. LPS, a potent ligand of TLR4 and a major bacterial endotoxin, is produced during the metabolic process. Therefore, we utilized LPS stimulation as model endotoxin for our studies.

In light of the central role of TLR4 in innate immunity and inflammation, a major hypothesis of my study is that the inflammatory pathway mediated through TLR4 is a key regulator of cleavage and activation of CREBH. We have hypothesized that the adaptors of TLR4 signaling cross-talk with CREBH and leads to CREBH cleavage and activation through post-translational modifications. Subsequently, TLR4 signaling pathway modulates hepatic lipid metabolism through activation of CREBH-mediated transcriptional program. I hypothesized that some of the metabolic hallmarks of endotoxemia were partially regulated by the TLR4-CREBH signaling axis.

To test this hypothesis, I comprehensively evaluated the suitability of in vitro cell culture system as well as in vivo low-grade inflammation model. To this end, I employed strategies to induce typical low-grade inflammation in C57BL/6J mice by administration of low levels of LPS administration. The dosage of 2μg/gm body weight of mouse has been previously used as low dosage to understand the metabolic effects of LPS challenge on body glucose metabolism and mitochondrial biology (83, 84). Liver inflammatory and metabolic alterations in these animal models were systematically evaluated using both pathological parameters and biomarkers. After this evaluation, we found that intraperitoneal injection of LPS under normal mouse chow diet is a suitable animal model for studying endotoxemia-induced inflammatory metabolic responses in the liver and to explain some of the key mechanisms underlying endotoxin-mediated modulation of hepatic metabolism.

Chapter 1: Defining the role of hepatocyte-specific TLR4 signaling in CREBH cleavage and subsequent activation. In this chapter, I investigate LPS-induced cleavage of CREBH protein, involvement of TLR signaling in LPS-induced CREBH cleavage, and effects of other stimuli on CREBH activation. I also provide new light on ambiguity associated with the presence of TLR4 in hepatocytes.

Chapter 2: Elucidating the cross talk between TLR4 signaling pathway and activation of CREBH. Under this chapter, I demonstrated the molecular basis through which TLR4 signaling interacts with CREBH transcription factor and the components of the TLR4 signaling pathway that are essential to mediate the cross talk. I determined the possible location of CREBH interaction with TLR4 signaling components under the acute endotoxin challenge.

Chapter 3: Determining the post-translation modifications involved in CREBH cleavage and activation. I have discussed the role of ubiquitination in CREBH cleavage and activation. I also briefly evaluated the potential role of phosphorylation in CREBH cleavage and activation.

Chapter 4: Determining the downstream targets of CREBH under the bacterial endotoxin challenges. In this chapter, I identified CREBH-target genes under endotoxin by gene expression analyzes, Chromatin Immunoprecipitation (ChIP) analysis, and gene expression reporter analysis.

Chapter 5: **Delineating the pathophysiological effects of CREBH deficiency on metabolism and inflammation.** In this chapter, I determined the hepatic and serum TG levels, serum cholesterol (HDL, LDL, and total cholesterol) levels in CREBH knockout and wild-type

control mice under LPS challenge. I also evaluated the impact of CREBH deficiency in energy consumption, food intake, and body weight under the LPS challenge.

Chapter 6: **Conclusions and Significance**

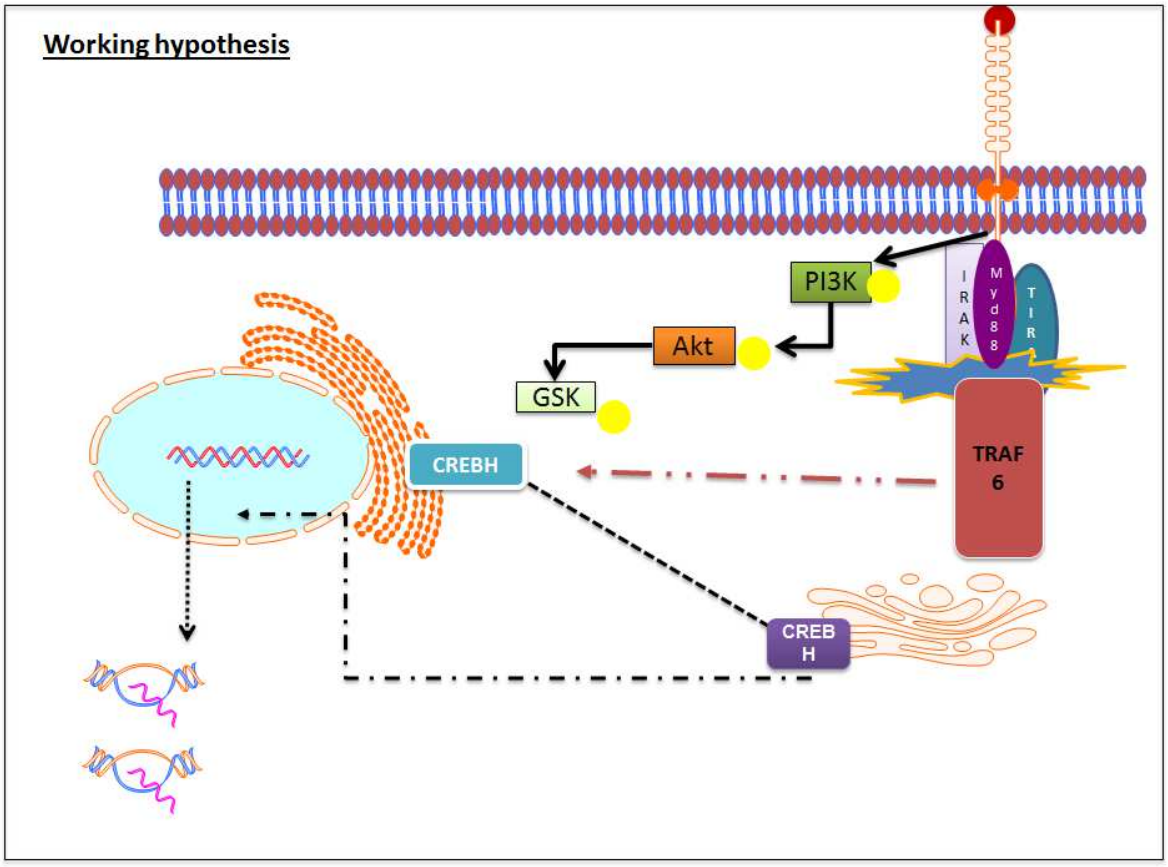

Figure 2: Working hypothesis for TLR4 mediated cleavage of CREBH under LPS treatment

Working model of our hypothesis. LPS upon binding to TLR4 receptor induce activation of TL4 receptor signaling pathway. Component of TLR4 signaling pathway are involved in direct cross talk with CREBH for its subsequent activation. PI3K-Akt axis pathway triggered by TLR4 receptors also plays crucial role in CREBH cleavage and activation. These signaling pathways leads to CREBH translocation and cleavage into active form of CREBH. Active CREBH translocates to nucleus inducing transcription of specific genes involved in response to LPS stimulation.

CHAPTER 1: Delineating the role of TLR4 signaling in CREBH cleavage and activation in hepatocytes

Summary

As described above, TLR4 is one of the mammalian pattern recognition receptors, recognizing pathogen-associated molecules and playing pivotal roles in the innate immune response. A recent study reported that saturated FA-mediated TLR4 signaling activation induces changes in expression of metabolic genes in hepatocytes (85). In addition, TLR4 activation is also associated with insulin resistance and high-fat diet-induced obesity and diabetes (86). Our previous study suggested a role for inflammatory stress in cleavage and activation for the CREBH hepatocytes specific transcription factor. Intraperitoneal injection of LPS can cause cleavage and activation of CREBH (87). A direct effect of LPS on CREBH activation in hepatocytes has not been well studied.

To address this question, we first assayed for the presence of TLR4 in hepatocytes. Healthy liver express low levels of TLR4 mRNA (88). Since there is ambiguity about the presence of TLR4 receptors in hepatocytes, we assessed whether CREBH is cleaved in primary hepatocytes challenged with LPS along with TLR4-specific agonist UT12 and antagonist RP105 (89). Under the alcoholic liver disease condition, hepatocytes and Kupffer cells have been shown to interact closely to induce steatosis and cirrhosis (90). We used UT12 , which enables us to verify the TLR4-specific effect on CREBH cleavage mechanism. I also evaluated the direct and indirect effects of LPS on CREBH activation in primary hepatocytes. Since LPS can stimulate cytokine production in Kupffer cells (resident macrophages in the liver), which may in turn lead to CREBH cleavage through TNFα production (indirect mechanism), I investigated the potential role and contribution of this indirect mechanism to CREBH activation in hepatocytes. I co-cultured hepatocytes and Kupffer cells to reveal the cross-talk between two cell types under LPS stimulation.

Analyzing all these aspects helps establish the system for my further mechanistic studies. Meanwhile, these approaches also reveal important aspects of endotoxin-mediated CREBH cleavage in hepatocytes. In this chapter 1) assessed the presence of TLR4 receptors in primary hepatocytes, 2) confirmed the direct role for TLR4 signaling pathway in primary hepatocytes, 3) addressed the contribution of the indirect pathway under endotoxin challenge, and 4) established the cell culture and animal model system for our future studies.

Material and Methods

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. All animal use and procedures were approved by the Wayne State University Animal Care and Use Committee.

Animal experiments

CrebH-null mice with exons 4–7 of the CrebH gene deleted were previously described (91). CREBH-null and wild-type control mice on a C57Bl/6J background of approximately 3-month-old were used for the experiments. The normal chow diet was from Harlan Laboratories. All the animal experiments were approved by the Wayne State University IACUC committee and carried out under the institutional guidelines for ethical animal use. For the LPS injection experiment, CREBH-null and wild-type control male mice under the normal chow diet were injected intraperitoneally with LPS (2 μg/gm body weight) or vehicle PBS. After 18 hr post injection, mice were sacrificed for tissue collection.

Western blotting analysis

Protein from liver tissues or cultured cells was solubilized by disrupting and homogenizing with NP40 lysis buffer (1%Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and freshly added protease inhibitor cocktails) in the presence of the protease inhibitor cocktail (Roche Applied Science). After homogenization, tissue or cell lysate was centrifuged at a speed of 14,000g for 20 min. at 4°C. The clear supernatant was carefully aspired and transferred to a new microcentrifuge tube. Protein concentrations were determined with bicinchoninic (BCA) protein assay kit according to manufacturer's instruction (Pierce Biotechnology Inc., Rockford, U.S.A.). Thirty μg of protein s were mixed with protein loading

buffer, heated at 95°C for 10 min, subjected to SDS-PAGE, and then transferred onto PVDF membrane. Following the washing with TBS-T buffer for 5 min, the membranes were incubated with 5% (w/v) non-fat milk in TBS-T buffer at room temperature for 1 hr to block non-specific binding. The membranes were then probed with specific primary antibodies at 4°C overnight. The membranes were washed with 1x TBS-T for 4 times (15 min per time), followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hr. After washing with 4 changes of TBS-T buffer for a total of 60 min, the protein bands were visualized with enhanced chemiluminescence reagents (Thermo Fischer, USA). Primary antibodies used including CREBH (Thermo Fischer, USA), GAPDH, β-Actin (Sigma Aldrich, MO, USA)

Isolation and culture of mouse primary hepatocytes and Kupffer cells

Primary hepatocytes from wild-type C57BL/6J mice were prepared as described previously. Briefly, in situ liver perfusion was performed with 0.02% collagenase type IV (Sigma Aldrich, USA) in Hank's balanced salt solution through a portal vein at a rate of 8 ml/min. After complete perfusion, liver cells were dispersed in DMEM medium. Cell suspensions were then filtered through 100 μm nylon cell strainer (BD Falcon, USA) to remove tissue debris and cellular aggregates. The filtrates were centrifuged at 50g for 2 min in an Eppendorf centrifuge 5810/R and subsequently divided into two layers, hepatocytes-enriched pellet and non-parenchymal cells (NPC)-enriched supernatant. Hepatocytes were collected after cell pellet was washed with PBS.

Cell culture

Isolated primary hepatocytes were seeded in culture flasks and maintained in Dulbecco's Modification of Eagle's Medium (Thermo Fischer, Rockford,MA, USA) containing 10% fetal calf serum (Thermo Fischer, Rockford,MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Human hepatoma cell line Huh7 was a generous gift from Dr. Charles Rice Rockefeller University. Huh7 cells, mouse macrophage cell line Raw264.7, and primary hepatocyte Cells were incubated in an incubator at 37°C with humidified atmosphere with 5% CO2 and 95% air. Cells were seeded at a number of 1 million/ well of 6 well plate. Various treatments were performed at about 80% confluence. When the treatments were finished, cells were harvested by incubating with 1 ml of 0.1% trypsin/EDTA (0.02%) for 3 min to allow the detachment of cells. Then, pelleted cells were re-suspended in NP40 lysis buffer for protein analysis.

Reverse transcription (RT)-PCR

Total RNA was extracted from primary hepatocytes using TRIZol (Life Technologies, Gaithersburg, Md) extraction method. First-strand cDNA was synthesized from 0.5 µg of RNA using reverse transcriptase (SuperScript III RT; Life Technologies, Gaithersburg, Md.) and pool of 20 random primer (Life Technologies, Gaithersburg, Md) in 20-µl reaction mixtures, according to the manufacturer's instructions. The synthesized first-strand cDNA was diluted to a total volume of 200 µl with distilled water. An aliquot of first-strand cDNA was amplified by PCR master mix (Promega, Cat No: M7501) in a total volume of 50 µl including of 2X super mix, primers (1µM) and 100ng template cDNA. PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 0.5 min. Before the first cycle, an initial denaturation step of 3 min at 94°C was included, and for 30 cycles, the

PCR products were separated by electrophoresis on 2% agarose gels. The sequence of primers used has been listed in Appendix B.

Result

LPS treatment induces CREBH cleavage, at a level comparable to ER stress and metabolic stress.

CREBH cleavage and activation is induced by ER stress and metabolic stress conditions like fasting and high-fat diet (1, 71, 72, 92). We investigated the levels of CREBH cleavage and activation under LPS treatment and compared the levels of CREBH cleavage between LPS and previously defined stressors (71, 87). Huh7 cells infected with adenovirus expressing full-length CREBH were treated with glucagon (fasting stress), Tunicamycine (Tm), Thapsigargin (Tg), and LPS for 4hr, 8hr, and 12hr. LPS significantly induced cleavage of CREBH precursor, at a level comparable to that of glucagon, Tm, or Tg (Fig 3A). Interestingly, LPS or glucagon treatment increased levels of CREBH precursor protein, compared to vehicle, Tg, or Tm treatment (Fig 3A). Indeed, treatment with pharmacological ER stress inducer Tm or Tg led to a reduction in CREBH precursor protein levels, presumably due to the translational attenuation mediated through the classic UPR signaling (Fig3A). These results help us to evaluate the effect of LPS on CREBH activation in comparison to the effects of the other defined stressors.

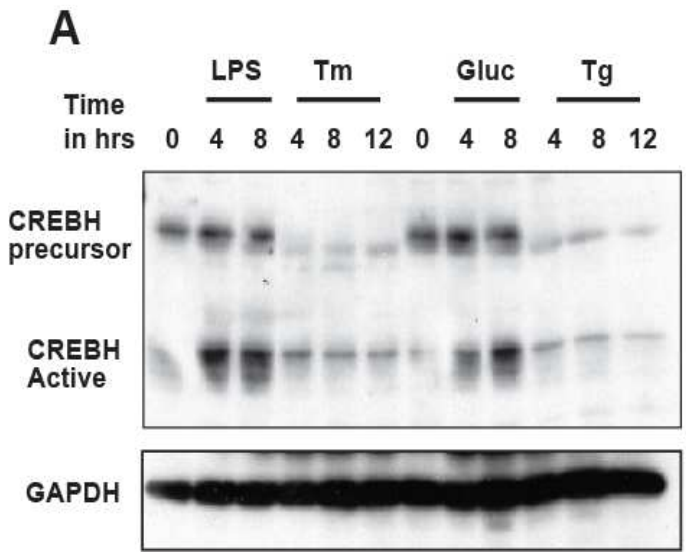

Figure 3 CREBH cleavage and activation with inflammatory, metabolic and ER stress inducers

(**A**) Huh7 cells were infected with adenovirus expressing human full-length CREBH for 72 hr, and then treated with LPS (1μg/ml), tunicamycin (5μg/ml), glucagon (500nM/ml), or thapsigargin (10μM/ml) the non-transfected cells were included as negative controls. Western blot analysis was performed to detect CREBH cleavage by using CREBH polyclonal antibodies. Levels of GAPDH were determined as loading control.

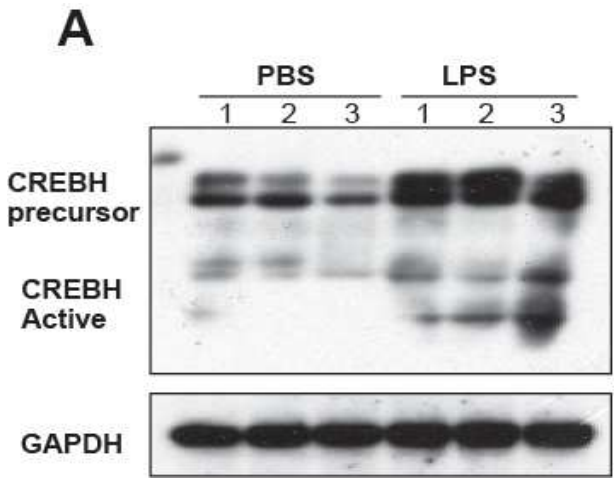

Figure 4. CREBH cleavage and activation with inflammatory stress inducers

(A) Western blot analysis of CREBH cleavage in the liver tissue samples from wild-type mice under the normal chow (NC) injected with either PBS (vehicle control) or LPS (2 μg/gm body weight) for 18 hr. Western blot analysis was performed to detect CREBH cleavage by using CREBH polyclonal antibodies with GAPDH as loading control.

C57/BL6 mice upon LPS injection displayed low-grade inflammation and CREBH cleavage.

As mentioned in the methods, the animals were injected intraperitoneally with LPS (2μg/gm of body weight) for inducing acute endotoxemia. This dosage of LPS has been defined as low dosage of endotoxins (84) and has been implicated in acute phase response associated with LPS (93, 94). Upon LPS challenge, levels of both CREBH precursor and activated form of CREBH proteins were elevated in the liver of C57/BL6 mice (Fig 4A).

TLR4 receptors are present in a low amount in primary hepatocytes.

Because of uncertainty surrounding presence of TLR4 on primary hepatocytes, I evaluated the relative abundance of the TLR4 receptor mRNA, in comparision to other common TLR receptors, including TLR2 and TLR3. Semi-quantitative RT-PCR analysis of total mRNA from primary hepatocytes showed that TLR4 mRNA are present on mouse hepatocytes, although in low levels (Fig 4A). The relative abundance of TLR4 on primary hepatocytes, compared to RAW cells, is low, nonetheless our studies confirmed the low-level presence of TLR4 mRNA in primary hepatocytes, in accordance with some of the previous literature (95).

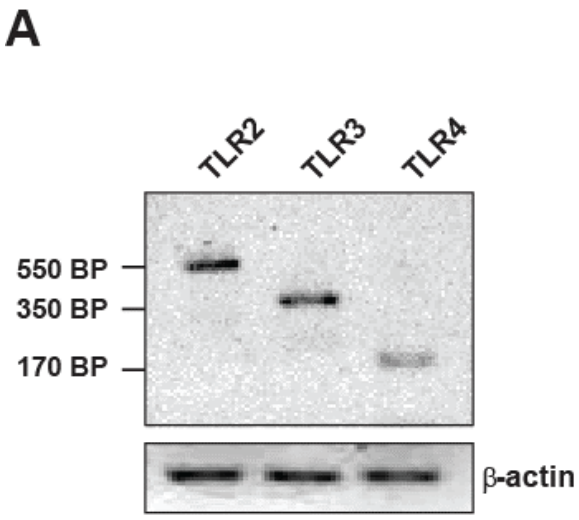

Figure 5. TLR expression levels in primary hepatocytes.

(A) Semi-quantitative RT-PCR analysis of the expression levels of mRNAs for TLR2, TLR3, and TLR4 in primary hepatocytes isolated from wild type mice. β-actin mRNA was used as a loading control.

TLR4 signaling in primary hepatocytes induces cleavage of CREBH transcription factor.

To determine the involvement of TLR4 signaling in CREBH cleavage, I examined CREBH cleavage in primary hepatocyte in the presence of specific TLR4 agonist and antagonist. In response to LPS treatment, CREBH cleavage was increased in primary hepatocytes isolated from C57/BL6 mice (Fig 5A). Treatment of UT12, a specific agonist for TLR4 (96), led to a modest increase in CREBH cleavage, compared to the PBS treatment control. As a control, treatment with RP105, a specific inhibitor of TLR4 signaling (96), caused a marginal change in CREBH cleavage in primary hepatocytes, compared to the PBS treatment (Fig 6A).

Since liver-resident macrophages, Kupffer cells, are the major inflammatory resource in the liver, I next evaluated the potential involvement of Kupffer cells-mediated cytokine production in CREBH cleavage in primary hepatocytes. Primary hepatocytes isolated from wild-type C57/BL6 mice were subjected to the treatment with conditioned medium from mouse macrophage cell line RAW264.7 treated with LPS (100ng/ml). Condition medium treatment resulted in increased CREBH cleavage, compares to the primary hepatocytes directly treated with LPS. However, this increase was not tremendous. Hepatocytes directly treated with LPS, as well as hepatocytes treated with LPS-stimulated conditioned medium from macrophages displayed increased in CREBH expression and cleavage (Fig 6B).

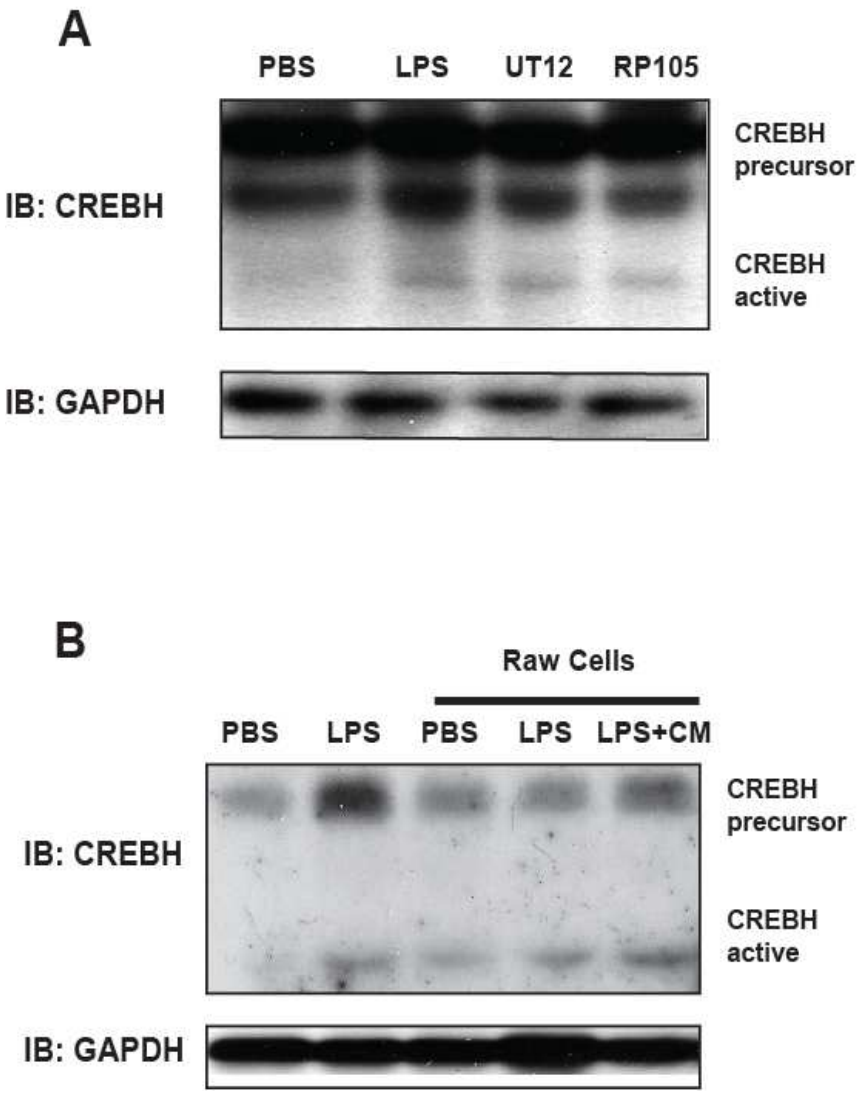

Figure 6. Activation and cleavage of CREBH is a direct effect of LPS through TLR4 signaling in hepatocytes.

Primary hepatocytes isolated from wild-type mice were treated with LPS (100ng/ml) with condition medium from Raw cells treated with PBS or LPS (100ng/ml) for 4hr **(B)** and **(A)** UT12 (5μg/ml), RP105 (5μg/ml) for 4 hr. Western blot analysis was performed to detect endogenous CREBH cleavage using a CREBH polyclonal antibodies with GAPDH as loading control.

Discussion

The liver is the primary metabolic and detoxification organ in the body. Upon bacterial or viral infection, the liver plays an important role in mounting a defense response to pathogens (97). Liver immune cells and hepatocytes produce both inflammatory cytokines and chemokines as well as acute phase response proteins. Mounting of such response not only remove pathogens but also influence the liver homeostasis. A consensus opinion is that the response of hepatocytes to LPS is complicated, because it involves cell to cell interactions between hepatocytes, Kupffer cells, and stellate cells (98). Previous studies suggested that hepatocytes can respond independently to LPS (99). Accordingly, we undertook the present study to assess the LPS-responsive pathway in hepatocytes. We demonstrated that hepatocytes expressed low levels of transcripts for TLR4, suggesting the existence of TLR4 signaling in hepatocytes.

Studies over the past few years indicated that hepatocytes can respond directly to microbial products. The effect of LPS In vivo on hepatic function is also well known (100). LPS increases liver mass and hepatocyte volume, as well as modulating the synthesis of acute-phase proteins through the release of cytokines (TNFα and IL-6) (101). We showed that not only TLR4 are present on hepatocytes but also that TLR4 signaling is actively involved in cleavage and activation of CREBH transcription factor. Kupffer cells also play a significant role in CREBH expression, cleavage, and activation, but hepatocytes also respond to LPS response. Inhibition of theTLR4 response signaling lead to less CREBH cleavage under LPS stress conditions. My study suggested that hepatocytes can directly respond to LPS challenge in cleavage and activating CREBH. *In vivo* microenvironment, signaling from Kupffer cells can also contribute to CREBH cleavage and activation in hepatocytes, possibly through the production of pro-inflammatory cytokines, such as TNFα and IL-6. Indeed, this is consistent with our previous study showing that TNFα and IL-6 can induce CREBH cleavage (1).

Nevertheless, the direct effect of LPS on hepatocytes in triggering CREBH cleavage was significant, suggesting the inflammatory pathway in hepatocytes plays a major role in CREBH activation upon bacterial endotoxin challenge.

In summary, the studies described in this chapter indicated a prominent role of TLR4 on hepatocytes in CREBH cleavage and activation. The experimental results also confirmed the participation of Kupffer cells in CREBH cleavage and activation in the liver in vivo. Involvement of TLR4 signaling-mediated CREBH cleavage and activation laid a foundation for further study on the proposed role of endotoxin in modulating hepatic metabolism through activation of CREBH.

CHAPTER 2: TRAF6-mediated ubiquitination of CREBH promotes CREBH cleavage and activation

Summary

As we showed previously, LPS or TNFα stimulation triggered CREBH cleavage in hepatocytes. Additionally, treatment with UT12, a TLR4-MD2 agonist, can also induce CREBH cleavage. Therefore, the component of TLR4 signaling likely plays a crucial role in CREBH cleavage and activation. TNFα- or LPS- induced inflammatory signaling is mediated through TNFα receptor-associated factors (TRAF's). Member of TRAFs are a family of conserved adaptor proteins that, through their association with cytoplasmic domains of different receptors, mediate the activation of various intracellular signaling pathways. TRAF family of adaptor proteins shares considerable homology in their structures. While the TRAF factors, TRAF2, and TRAF6, are ubiquitou, TRAF6 functions as a key mediator in TLR signaling. Hence, I hypothesized that TRAF6 may interact with CREBH to regulate CREBH cleavage and activation under LPS challenge.

Distinct from other TRAFs, TRAF6 is an E3 ubiquitin ligase, and through association with the dimeric ubiquitin-conjugating enzyme Ubc13/Uev1A, it catalyzes lysine 63 (K63)-linked polyubiquitination of several target proteins. Recent studies suggested that TRAF6 functions as a key regulator in multiple signaling pathways, such as NF-κB, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt, in response to various cytokines and microbial products (102). Although it remains a mystery if the E3 ubiquitin ligase activity of TRAF6 is crucial for its participation and function in signaling pathway, recent studies have shown that TRAF6 functions as a central regulator in multiple signaling pathways, such as NF-κB, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/Akt, in response to various cytokines and microbial products (103). In addition to its

association with cytoplasmic domains of various cell surface receptors, such as Toll-like receptors and the interleukin-1 receptor (IL-1R), TRAF6 has also been found to interact with multiple components of the ubiquitin proteosomal system (UPS) and involved in autophagosome formation in skeletal muscles (103).

Under TLR4 signaling pathway, TRAF6 undergoes self-polyubiquitination and helps in docking of different downstream kinases at the ternary complex of Myd88-IRAK4-TRAF6. It has been shown that TRAF6 is involved in insulin signaling and phosphorylation of Akt. Our group recently demonstrated that TRAF6 interacts with IRE1α, an ER-anchored protein mediating unfolded protein response (UPR). Interaction of TRAF6 with IRE1α induces IRE1α ubiquitination and prevents its dephosphorylation by the phosphatase PP2A.(104).

Protein ubiquitination is an important PTM that regulates various biological functions. Although most of ubiquitination processes lead to protein degradation, a particular type of ubiquitination, namely the lysin 63 (K63)-lined ubiquitination, is essential for signaling activation and protein trafficking. It has been demonstrated that K63-linked auto ubiquitination of TRAF6 is a process essential to its regulatory role in starvation-induced autophagy (105).

In this chapter, my studies confirmed that TRAF6 functionally interacts with CREBH. For the first time, my results demonstrated that TRAF6 positively regulates the activation of the TLR4-CREBH signaling pathway by binding to CREBH, a novel mechanism by which an ubiquitin E3 ligase promotes CREBH cleavage and subsequent activation.

Materials and Methods

Animal experiments

CrebH null and wild-type control mice on a C57Bl/6J background of approximately 3-month-old were used for the experiments. The *Myd88*-null mice were from Dr. Ashok Kumar at Kresge Eye Institute, Wayne State University. All the animal experiments were approved by the Wayne State University IACUC committee and carried out under the institutional guidelines for ethical animal use. The intraperitoneal administration of LPS was carried out as described previously in the Material and Methods of Chapter 1.

Cells and reagents

Human endothelial kidney (HEK) 293T cells were maintained in Dulbecco's Modification of Eagle's Medium (Thermo Fischer, Rockford,MA, USA) containing 10% fetal calf serum (Thermo Fischer, Rockford,MA, USA). Transfection in HEK 293T cells was performed with Lipofectamine™ 2000 protocol (Invitrogen, Grand Island, NE, USA). Plasmids expressing CREBH and its truncated mutants were generated by PCR using linker primers and sub-cloned into a pCMV-Flag vector (Sigma-Aldrich, St. Louis, MO, USA). Myc-tagged TRAF6 expression plasmid and its C70A mutant were used as previously reported (Yang et al, 2009). The truncated mutants were generated by PCR with linker primers followed by sub-cloning into a pCMV-Myc vector (Invitrogen, Grand Island, NE, USA). LPS was purchased from Sigma-Aldrich.

Transfection, co-immunoprecipitation, and Western blotting analysis

Transient transfection of HEK293T cells was performed by using Lipofectamine 2000 (Invitrogen, Grand Island, NE, USA) or Transit 2020 (Mirus Biotech, NJ, USA) according to the manufacturer's instructions. 24 hr after transfection, cells were lysed in NP-40 lysis buffer (1%Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and freshly added protease inhibitor cocktails). For co-immunoprecipitation, cell lysates were incubated with an antibody (1 μg) for overnight at 4^0C, followed by the addition of 30μl recombinant protein G-Sepharose beads (Invitrogen, Grand Island, NE, USA) for 4 hr at room temperature. For co-immunoprecipitation with anti-Flag pull down, cell lysates were incubated with M2 flag agarose beads (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4^0C. Immunoprecipitates were washed four times with NP-40 lysis buffer and boiled in 40 μL of 2.5× Laemmli buffer. Samples were subjected to 10% or 12% SDS-PAGE analysis and electrotransferred onto nitrocellulose membranes (0.45μM; EMD Millipore). Membranes were probed with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with an enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA,USA). When necessary, membranes were stripped by incubation in stripping buffer (Thermo Fischer, Rockford, MA, USA), washed, and then re-probed with other antibodies. For IP-Western blot analysis, total protein lysates from in vitro cultured lysate or were immune-precipitated with anti-FlagM2 antibody beads (Sigma Aldrich), followed by Western blot analysis using the anti-Flag (Sigma-Aldrich, St. Louis, MO, USA), anti-myc (EMD Millipore) antibody to detect. Antibodies used in this chapter include polyclonal CREBH Polyclonal anti-CREBH antibody was raised by immunizing rabbits with a mouse CREBH protein fragment spanning N-terminal amino acids 75–250 of mouse CREBH protein, TRAF6 (Santa Cruz Biotech, CA,USA), and Myd88 (Cell signaling technologies, CA USA) antibodies.

Immunofluorescence staining

Immunofluorescence analysis of protein subcellular localization was performed as described. Briefly, Huh7 cells transfected with the plasmid vector expressing full-length CREBH and myc- tagged TRAF6. At 48 hr after the transfection, the cells were fixed with 4% (v/v) paraformaldehyde (Sigma, St. Louis, MO) for 10 minutes at room temperature followed by cell permeabilization with 0.5% Triton-X 100 (Fisher) for 2 min. Cells were blocked with 5% horse serum overnight at 4°C, followed by incubation with a 1:1000 dilution of an appropriate primary antibody, anti-Flag (Sigma Aldrich) or anti-Myc (EMD Millipore), for 1 hr at room temperature. After antibody incubation, the cells were washed by washing buffer (0.5% Triton in PBS) for 5 min for a total of 3 times, followed by treatment with the secondary antibodies, anti-rabbit Alexa545 (Abcam, MA, USA) or anti-mouse Alexa 695 (Abcam, MA, USA). Images were analyzed using Zeiss LSM Alpha Imager Browser v4.0 software.

Results

TLR signaling is involved in the interaction of CREBH and TRAF6 in mouse liver tissue under LPS challenge.

We utilized *Myd88*-null mice to elucidate the molecular mechanism underlying TLR4 signaling-mediated CREBH cleavage and activation. Myd88 is an upstream adaptor protein that is involved in TLR4 signaling pathway (Fig 7B). We first analyzed the responsiveness of *MyD88* null mice to LPS challenge. *MyD88* null and wild-type control mice were intraperitoneally injected with low dose of LPS (2 μg/gm body weight). After the challenge, the *Myd88* null and wild-type control mice had comparable levels of weight loss and food intake. The levels of cleaved CREBH in the wild-type control mice were comparable to those in Myd88 null mice in the absence of LPS treatment (Fig 7A). However, less CREBH cleavage was observed in *Myd88* null livers upon LPS challenge. We further investigated the mechanism underlying the reduced amounts of cleaved CREBH protein. We examined the interaction between endogenous CREBH and TRAF6 in the liver tissues of wild-type control and *Myd88* null mice upon LPS challenge. The interaction between endogenous CREBH and TRAF6 was barely detected in the *Myd88* null mouse livers under the LPS challenge (Fig 7A). Taken together, these results suggested that the absence of MyD88, a key component of TLR4 signaling, diminished the effect of LPS in activating CREBH in the liver.

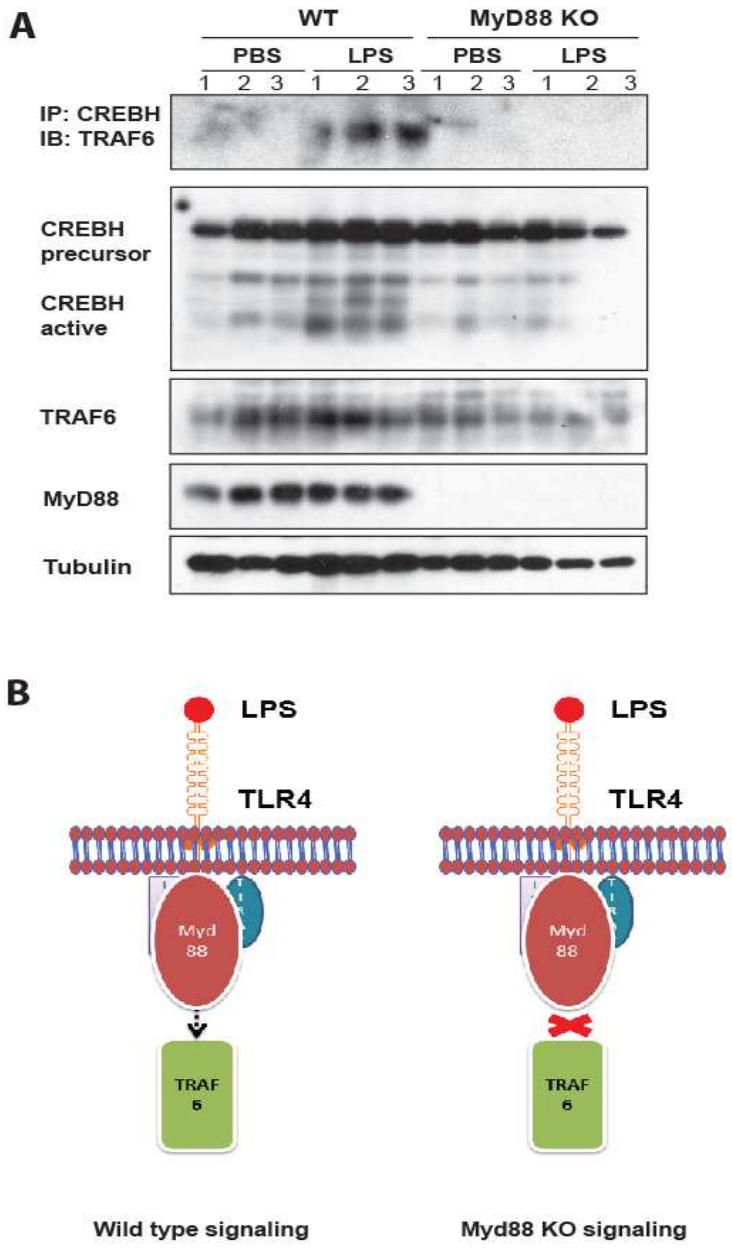

Figure 7. Intact TLR4 signaling is necessary for CREBH cleavage and for its interaction with TRAF6

(**A**) Immunoprecipitation and Western blot analysis of CREBH, TRAF6 and Myd88 protein levels in liver tissue samples from wild-type mice and Myd88 null mice under the normal chow (NC) injected with either PBS (vehicle control) or LPS (2 µg/gm body weight). Top panel shows the interaction between CREBH and TRAF6 in wild type and *Myd88* null mice injected with LPS. CREBH protein in liver lysates was immunoprecipitated with the polyclonal CREBH antibody. (**B**) Schematic diagram shows the role of Myd88 adaptor molecule in TLR4 signaling pathway.

MATH domain of TRAF6 interacts with the linker domain of CREBH protein.

To further delineate the molecular mechanism by which TLR4 signaling regulates CREBH cleavage, we investigated the molecular basis underlying the interaction between TRAF6 and CREBH. First, we tested the interaction between CREBH and TRAF6 in the presence or absence of endotoxin stimuli (Fig 7A). Immunoprecipitation (IP)–Western blot analysis with the HEK293T cells expressing exogenous CREBH and TRAF6 showed that CREBH protein can interact with TRAF6 (Fig 8B). Further, we demonstrated the interaction between endogenous TRAF6 and CREBH in LPS-injected mouse liver. Importantly, LPS stimulation significantly enhanced TRAF6-CREBH interaction in mouse liver.

To gain insights into the mechanism underlying CREBH and TRAF6 interaction, we generated truncated mutations for both TRAF6 and CREBH proteins to map their interaction domains. IP–Western blot analysis revealed that the C-terminal meprin-associated TRAF homology (MATH) domain of TRAF6 is required for its interaction with CREBH (Fig 8B). Moreover, we defined that the linker region between the transmembrane domain and the kinase domain of CREBH is required for CREBH interaction with TRAF6 (Fig 9B). Importantly, deletion of E3 ligase domain of TRAF6 protein significantly affects the cleavage of CREBH protein, as revealed by Western blot analysis (Fig 8B). Interaction of TRAF6 (MATH domain mutant) with CREBH was correlated with a reduction in CREBH cleavage, these results suggest that CREBH and TRAF6 interactions may contribute to the CREBH activation process (Fig 8B).

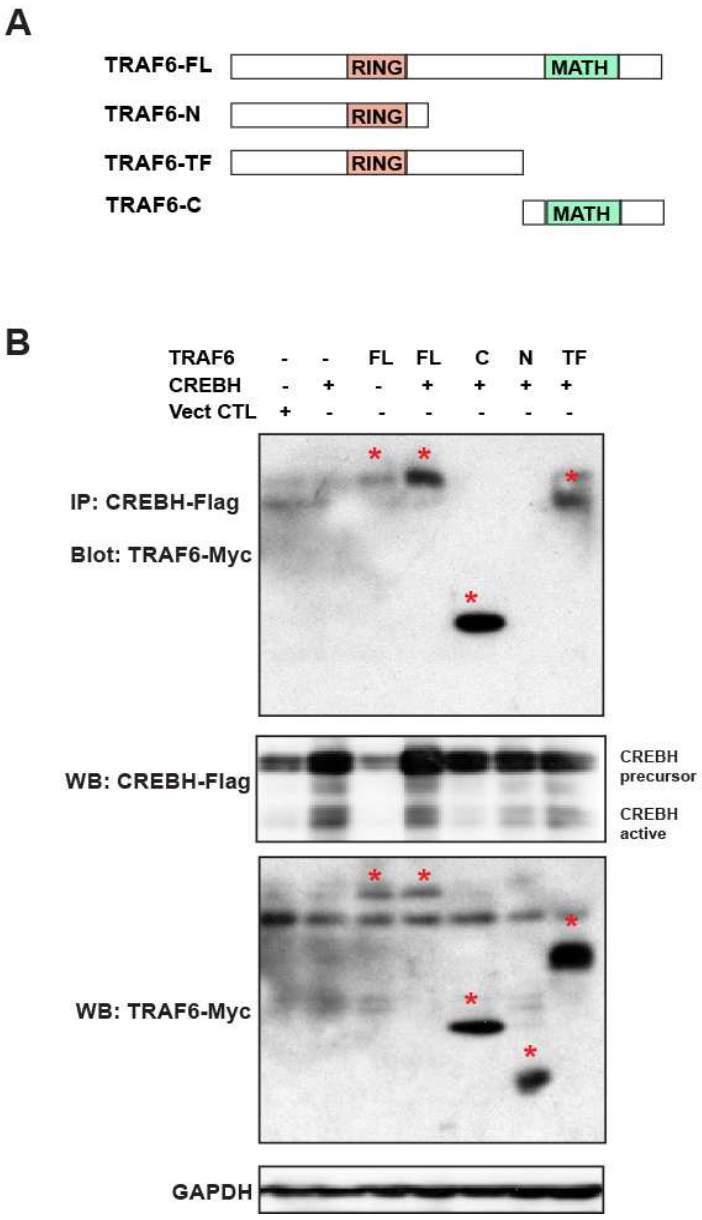

Figure 8. MATH domain of TRAF6 interacts with CREBH for its cleavage and activation.

(A) Schematic representation of TRAF6 and its truncated mutants. TRAF6 carries an N-terminal RING finger domain and a C-terminal MATH domain (top panel). FL: full-length structure, N: N-terminal RING finger domain, TF: trans-membrane domain, C: C-terminal MATH domain. **(B)** IP –western blot analysis showing CREBH and TRAF6 interaction. Flag-tagged CREBH and Myc-tagged TRAF6 plasmids were co-transfected into HEK293T cells. CREBH protein in the lysates of transfected cells was immunoprecipitated with an anti-Flag antibody. The bound TRAF6 was determined by western blotting using an anti-Myc antibody, The expression of the full-length and truncated TRAF6 protein was indicated by the symbol '*'. GAPDH was detected as a loading control.

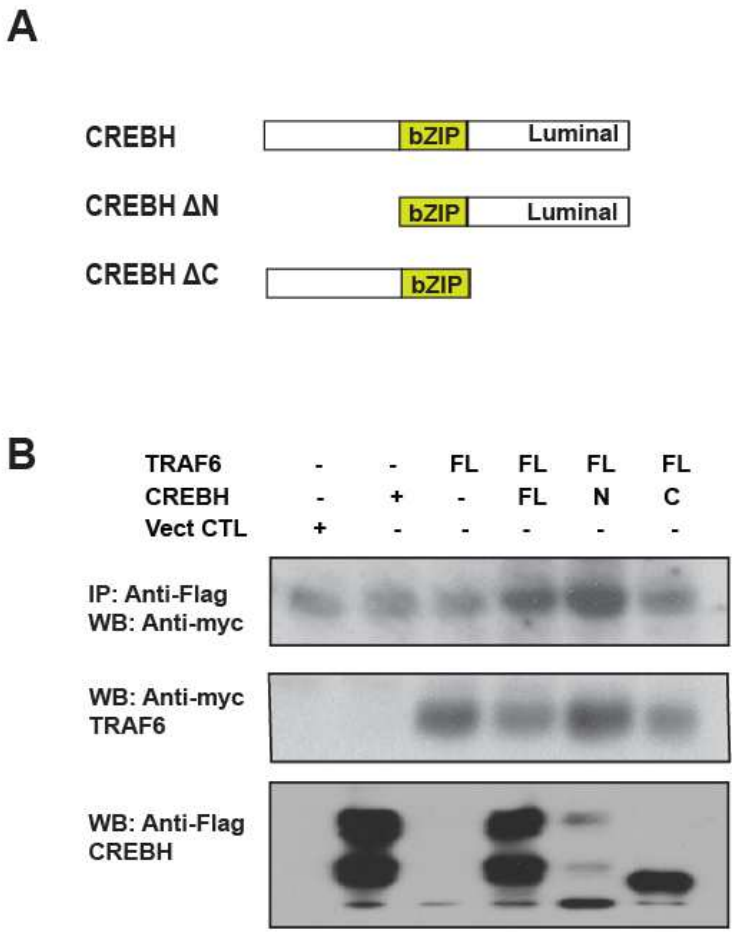

Figure 9. Linker domain of CREBH interacts with TRAF6 for its cleavage and activation.

(A) Schematic representation of CREBH and its truncated mutants. CREBH carries an N-terminal domain, B-Zip domain, and a C-terminal region (top panel). FL: full-length structure; Δ N, N-terminal deletion mutant; Δ C, C-terminal deletion mutant. **(B)** IP-Western blotting showing CREBH and TRAF6 interaction. Flag-tagged CREBH and Myc-tagged TRAF6 plasmids were co-transfected into HEK293T cells. CREBH protein in the lysates of transfected cells was immunoprecipitated with an anti-Flag antibody. The bound TRAF6 was determined by western blotting using an anti-myc antibody.

CREBH and TRAF6 interactions take place at ER membrane.

To further investigate and confirm the interaction between CREBH and TRAF6, we examined the subcellular distribution of the full-length CREBH and TRAF6 protein via immunofluorescence analysis. To elucidate and confirm the interaction, we transiently overexpress full-length CREBH and wild-type TRAF6 protein in Huh7 cells. As shown in Fig 10A, the full-length CREBH protein is localized in the ER membrane. We confirmed the ER localization of CREBH by co-staining the cells with PDI (Fig 10C), an ER retention marker, as the green fluorescence of CREBH co-localized with red fluorescence of PDI. Co-localization of CREBH and TRAF6 was also confirmed with Huh7 cells transiently expressing full-length CREBH and TRAF6 proteins (Fig 10D). These results confirmed the interaction between the ER anchored full-length CREBH protein and TRAF6 at ER membrane of the cells.

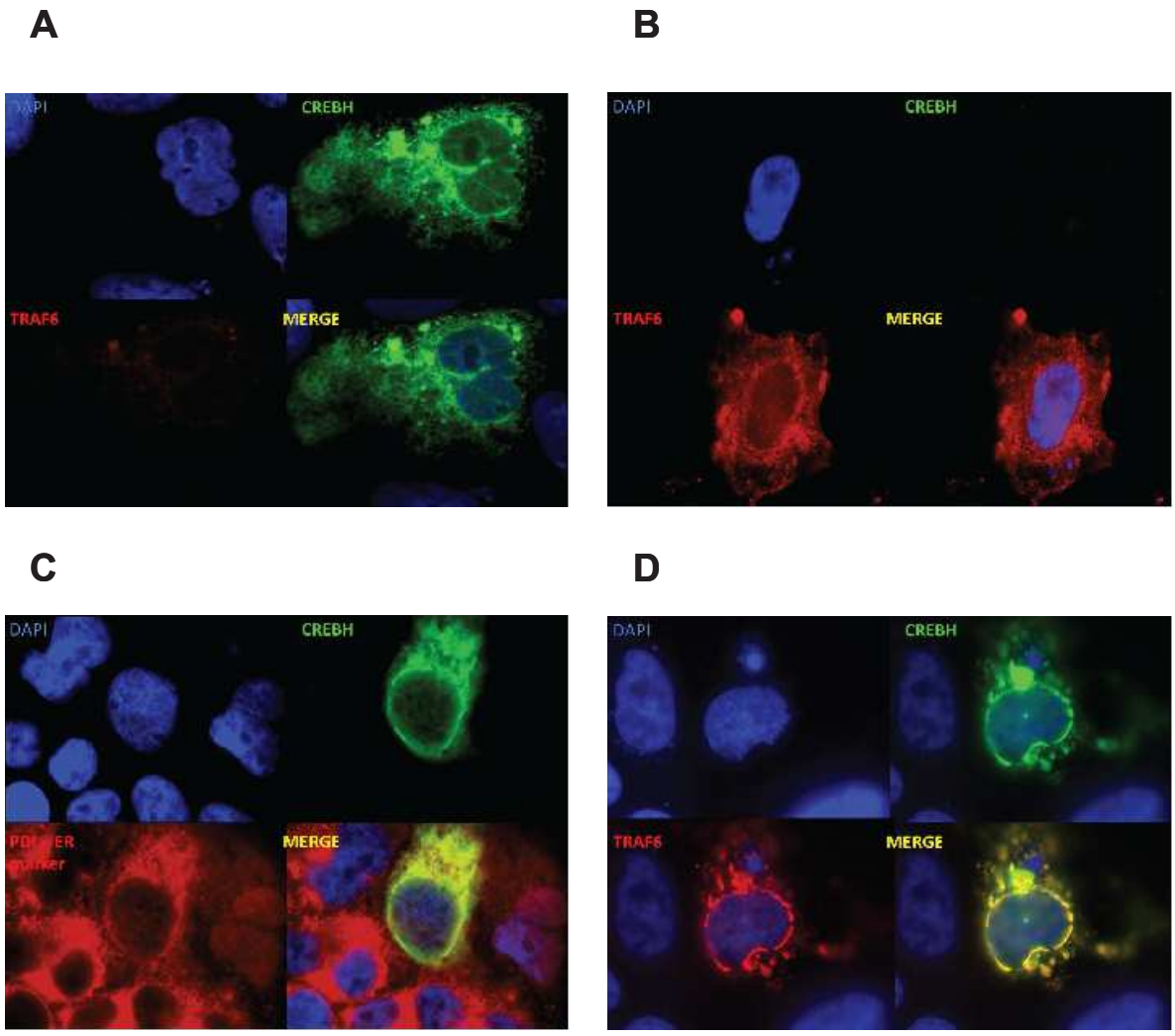

Figure 10. Interaction between CREBH and TRAF6 takes place at ER membrane.

Immunofluorescence analysis of Huh7 cells co-transfected with full-length Flag tagged human CREBH and Myc tagged TRAF6. **(A)** CREBH only **(B)** TRAF6 only **(C)** CREBH + PDI (ER marker) **(D)** Co-transfection showing CREBH and TRAF6 overlaps in Huh7 cells stained with anti-flag FITC (CREBH) and anti-myc ALEXA (TRAF6). Images were analyzed using Zeiss LSM Alpha Imager Browser v4.0 software (Zeiss). Magnification: 600X

Discussion

This chapter demonstrated one of the most important aspects of our hypothesis, the interface of innate immunity and metabolism through the interaction between CREBH and TRAF6. This provided important insights into the molecular mechanisms underlying CREBH cleavage and activation under LPS treatment.

First, my study indicated that TRAF6 is essential for CREBH cleavage under LPS challenge. Co-expression of TRAF6 functional mutants (TRAF6 DC, DN, or Math domain deletion) with full-length CREBH significantly decreased CREBH cleavage, compared to co-expression of wild-type TRAF6 with full-length CREBH (Fig 8B). Our finding also demonstrated that TRAF6 interacts with CREBH through its MATH domain of TRAF6 and linker domain of CREBH. Note that without the overexpression of TRAF6 in the cell culture system, the cells still exhibited CREBH cleavage and activation, which might be contributed by endogenous TRAF6 molecules. Additionally, ectopic expression of CREBH and TRAF6 can induce cell stress that stimulates the CREBH-TRAF6 interaction without the TLR4 stimulation. It has been reported that MATH domain of TRAF6 is involved in interacting with upstream kinases, as well as adaptor molecules, to transmit the signals (106). TLR4 signaling needs to be intact, a decrease in interaction between CREBH and TRAF6 was observed in Myd88 null mice. Myd88 null mice also exhibited lower levels of CREBH precursor proteins, in part due to the role of inflammatory signaling in the up-regulating expression of CREBH. Deletion of the E3 ligase RING domain of TRAF6, led to less CREBH cleavage, indicating a role of E3 ligase activity of TRAF6 in CREBH cleavage and activation. There are reports indicating the role of TRAF6 as an E3 ligase in insulin signaling and inflammatory responses (107, 108). The TRAF6 mutation study leads us to think about the potential of ubiquitination mechanism being involved in CREBH activation, as the deletion of MATH domain, where the E3 ligase activity resides, led to decreased CREBH cleavage (Fig 8B). Additionally, the

CREBH mutants exhibited lower levels of interaction between CREBH and TRAF6 due to the deletion of the linker region between b-Zip and transmembrane domain. The ΔN mutant displayed elevated levels of interaction due to its exposed b-Zip and linker domain of protein compared to WT and the ΔC mutant.

Immunofluorescence microscopic analysis revealed the ER membrane location for the interaction of CREBH and TRAF6. My study confirmed that TRAF6 is relocated to the ER membrane for the interaction with full-length CREBH. This is consistent with a report suggesting that TRAF6 translocate and interacts with target molecules for the post-translational modification activities of Akt (107). In summary, this chapter revealed the mechanism and location of interaction between TLR4 signaling and CREBH, an important event for CREBH cleavage and activation under LPS challenge.

CHAPTER 3: Posttranslational ubiquitination of CREBH is necessary for CREBH cleavage and activation

Summary

Post-translational modifications (PTMs) can restrict the inflated number of transcription factors, expanding the functional repertoire of genetic regulatory elements to cover the diverse metabolic requirements (109). Transcription factors are subjected to protein turnover and targeted for degradation by the ubiquitin-proteasome system. Increasing evidences pointed towards the close relation between the ubiquitin proteasomal degradation system and transcriptional activation (110). Protein sequences are associated with proteolysis of some activators overlap with their transcriptional activation domains and that components of the proteasome can be recruited to gene promoters through interactions with transcriptional regulators. It was demonstrated that ubiquitination can potentially enhance the activity of specific transcription factors (109). For example, ranscriptionally active forms of SREBPs are degraded by the proteasome in an ubiquitination-dependent manner (111). It has been suggested that nuclear SREBP molecules are, at least in part, ubiquitinated and degraded as a functional consequence of their transcriptional activity. However, the mechanistic link between activation of transcription factors and their degradation remains elusive.

Protein turnover in the cells is controlled by the rate of protein synthesis and the rate of protein degradation. There are two major paths of protein degradation: the first is ubiquitin-mediated proteasome pathway, and the other one is lysosomal degradation. Ubiquitin is a small, but highly conserved protein consists 76 amino acids. To mark a protein for degradation, a ubiquitin tag is ligated to the substrate protein. Ubiquitin tagging is carried out by the sequential action of three enzymes: E1, a ubiquitin-activating enzyme; E2, a ubiquitin-conjugating enzyme; and E3, a ubiquitin-protein ligase. The ubiquitinated proteins typically

contain multiple chains of branched ubiquitin molecules that enable recognition by the 26S proteasome, which degradesof the ubiquitinated protein into small peptides (112).

In this chapter, I investigated the role of ubiquitination modifications, mediated through TRAF6, in CREBH cleavage and activation. Using molecular and cellular biology approaches, I demonstrated that the interaction between CREBH and TRAF6, as discussed in chapter 2, promotes CREBH ubiquitination. Additionally, I also evaluated the potential roles of phosphorylation and kinases in CREBH cleavage.

Materials and Methods

Cells and reagents

Human endothelial kidney (HEK) 293T cells were maintained in Dulbecco's Modification of Eagle's Medium (Thermo Fischer, Rockford,MA, USA) containing 10% fetal calf serum (Thermo Fischer, Rockford,MA, USA). Transfection in HEK 293T cells was performed with Lipofectamine™ 2000 protocol (Invitrogen, Grand Island, NE, USA). Myc-tagged TRAF6 expression plasmid and its C70A mutant were as reported (Yang et al., 2009). HA-tagged ubiquitin plasmids K33 only (K33O), K48 only (K48O), K63 only (K63O), K33, K48, and K63 mutants (K33R, K48R, K63R) were from Dr. Fei Sun, Department of Physiology Wayne State University . The resources of antibodies were: Flag, β-actin, and Tubulin from Sigma-Aldrich (St.Loius, MO, USA); c-Myc and HA, TRAF6 were from Santa Cruz Biotech (CA, USA). Huh-7 cells were maintained in DMEM/High Glucose media containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The cells at about 60% confluence were infected with adenovirus-expressing full-length CREBH. At 72hr post infection, cells were treated with LPS for 4 hr, and harvested for IP-Western blot analysis. Okadaic acid was purchased from Sigma-Aldrich, Lys294002 was from Cell Signaling technology, and lithium chloride was purcahsed from Fisher Scientific.

Transfection, co-immunoprecipitation and western blotting analysis

Transient transfection of HEK293T cells as well as immunoprecipitation of cell culture lysates were carried out with Flag-tagged M2 beads from Sigma-Aldrich, MO, USA as described previously. Western blot analysis was carried out with anti-HA, anti-CREBH, and anti-TRAF6 (Santa Cruz Biotech, CA , USA).

Cell fractionation and nuclei isolation for cell culture

Transfected HEK293T cells were collected at 80-90% confluence. Isolation of nuclei was achieved by using the hypotonic/Nonidet P-40 lysis method (113). Briefly, cultures were rinsed twice with ice-cold PBS and collected with cell scrapers. Cells and were suspended in 0.5 ml of hypotonic/Nonidet P-40 buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) and incubated on ice for 5 min. After centrifuging at 500g for 5 min, nuclear pellets were washed twice with 0.5 ml of hypotonic/Nonidet P-40 buffer. The morphological integrity of isolated nuclei (> 90%) was assessed by DAPI staining under immunofluorescence microscopy at 100X. The purity of subcellular fractions was verified by immunoblotting with antibodies specific for markers of cytosolic and nuclear fractions.

Cycloheximide half-life experiment

Huh7 cells were infected with the adenovirus expressing full-length human CREBH for 48hr. After 48hr, cells were treated with LPS (1μg/ml) for 4hr. Aftert LPS treatment, the cells were rinsed with warm PBS twice followed by treatment with media containing cycloheximide (100 μM/ml) purchased from Abcam (MA,USA). Cells were collected at 0, 30, 60, 90, and 120 min after the cycloheximide treatment.

Results

TRAF6 mediates the ubiquitination of CREBH upon LPS stimulation.

We determined the molecular basis by which TRAF6 regulates CREBH cleavage and subsequent activation of CREBH. We recently showed that TRAF6 interacts with an ER anchored signal transducer IRE1α in macrophages (104). Interaction between IRE1α and TRAF6 induces ubiquitination of IRE1α, thereby allowing the phosphorylation and subsequent endonuclease activity of IRE1α. Since CREBH is an ER anchored stress associated protein similar to IRE1α, we hypothesized that the interaction between CREBH and TRAF6 may induce CREBH ubiquitination event and subsequently contribute to CREBH cleavage and activation. This hypothesis is supported by the truncated mutant studies, where the RING domain deletion in TRAF6 decreased the cleaved form of CREBH protein (Fig 8B). Through IP-Western blot analysis, we demonstrated that upon LPS treatment CREBH undergoes ubiquitination (Fig 11B). This was further confirmed by the observation that CREBH undergoes ubiquitination in the livers of mice challenged with LPS (Fig 12A). Furthermore, ubiquitination of CREBH in Huh7 cells transiently expressing Flag-tagged CREBH was markedly increased in response to LPS treatment. However, in HEK 293T cells, the presence of the E3 ligase catalytic-inactive C70A mutant of TRAF6 failed to induce CREBH cleavage (Fig 12B). These results indicate that the E3 ligase ubiquitin activity of TRAF6 is required for CREBH ubiquitination and subsequent cleavage.

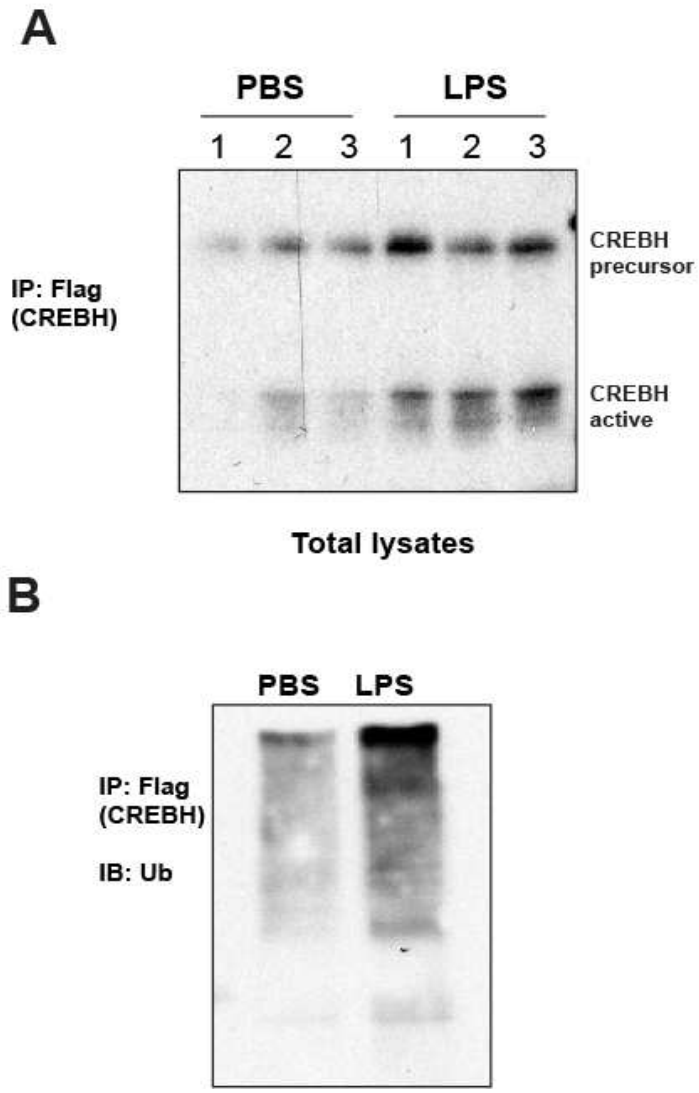

Figure 11. LPS treatment induces CREBH ubiquitination

(A) Western blot analysis showing protein levels of CREBH in Huh7 cells infected with adenovirus expressing human full-length CREBH for 72 hr. Post 72 hr. cells were treated with LPS (1μg/ml) for 4hr. **(B)** CREBH ubiquitination was determined by immunoprecipitation using the anti-Flag antibody and western blotting using the anti-Ub antibody. Huh7 cells infected with adenovirus were pooled together to analyses CREBH ubiquitination.

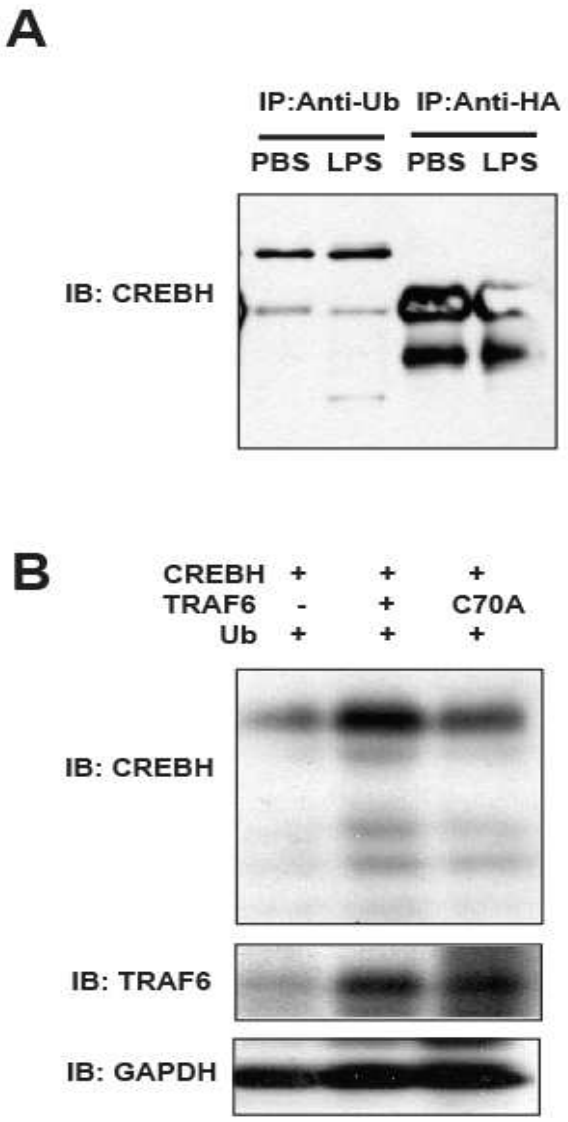

Figure 12. TRAF6 is an E3 ligase inducing CREBH ubiquitination upon LPS treatment

(A) Western blot analysis showing CREBH ubiquitination in mouse liver injected with either PBS or LPS (2μg/ml) for 18 hr. CREBH ubiquitination was determined by immunoprecipitation using the anti-Ub antibody and Western blotting using the anti-CREBH polyclonal antibody. Last 2 lane represent CREBH immunoprecipitation with HA antibodies as controls. **(B)** HEK293T cells were co-transfected with CREBH, TRAF6, and TRAF7 C70A expression plasmids. Levels of CREBH protein were analyzed by Western blot analysis using CREBH polyclonal antibodies, and GAPDH as loading control.

TRAF6 promotes K63-linked ubiquitination of CREBH.

E3 ubiquitin ligases are known to promote ubiquitination of their binding proteins. TRAF6 often catalyzes K63-linked polyubiquitin conjugation onto its substrates. To characterize TRAF6-mediated ubiquitination of CREBH, we co-expressed CREBH with a mutant ubiquitin isoform that carries a single lysine residue at position 63 (K63O) or 48 (K48O) or 33 (K33O) in HEK293T cells. K63O and K48O ubiquitin mutants carry a single lysine residue, residues 33, 48 and 63, respectively, which allows us to determine the topology of polyubiquitin chains. When Ub/K63O mutant is expressed, CREBH ubiquitination was detected (Fig 13A). In contrast, when the Ub/K48O mutant was co-transfected, only a low levels of CREBH ubiquitination were detected (Fig 13A). Surprisingly, K33O mutant also displayed a comparable level of ubiquitination of CREBH. To further delineate the type of polyubiquitination type occurring on CREBH, we used different ubiquitination mutant that won't allow a particular type of ubiquitination. To investigate this, we overexpressed K33R, K48R and K63R Ub mutants in HEK293T cells along with transient overexpression of CREBH. Based on this approach, we demonstrated that K63R mutant failed to mediate CREBH ubiquitination while expression of K48R led to a comparable level of CREBH ubiquitination (Fig 13A). To solve the conundrum of K33, we observed that expression of K33R led to an elevated level of CREBH ubiquitination, compared to expression of K33O mutant (Fig 13A). Therefore, the poly-ubiquitin chain conjugated to CREBH protein requires the lysine residue 63 (K63), but not the K48, indicating that TRAF6 catalyzes K63-linked, but not K48-linked polyubiquitination of CREBH. The weak K33-linked ubiquitination might have been catalyzed by other endogenous E3 ubiquitin ligases or a time dependent CREBH ubiquitination may be involved.

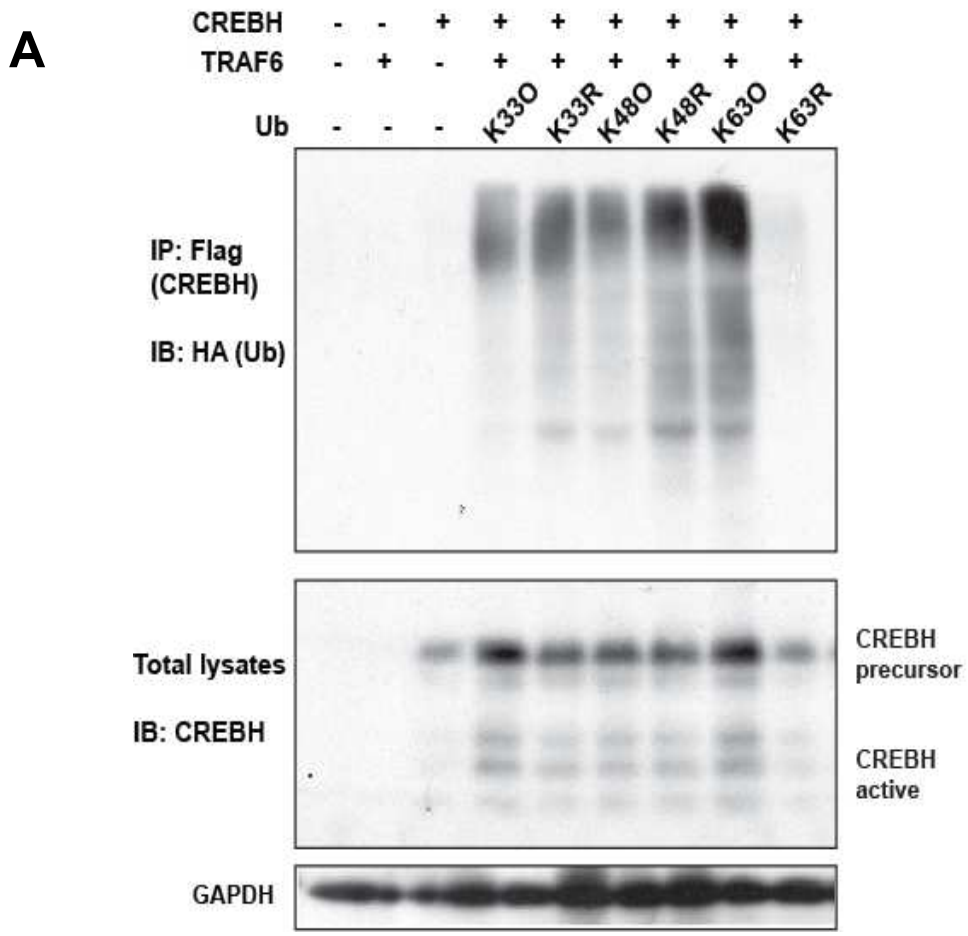

Figure 13. CREBH undergoes TRAF6 mediated K63 polyubiquitination upon interaction

A) Western blot analysis of K63-linked ubiquitination of CREBH. HEK 293 T cells were co-transfected with Flag-tagged human full-length CREBH, TRAF6, and specific ubiquitin expression plasmids, including K33 only (K33O), K63 only (K63O), K48 only (K48O), K33 mutant (K33R), K48 mutant (K48R), and K63 mutant (K63R). Ubiquitination was determined by immunoprecipitation using the anti-Flag antibody and Western blotting using the anti-HA antibody. Total lysate was detected for CREBH using polyclonal CREBH antibodies. Level of GAPDH was used as loading control.

LPS treatment induces TRAF6 mediated K63 ubiquitination that increase the stability of CREBH protein.

K48-linked polyubiquitination usually mediates protein degradation, whereas K63-linked polyubiquitination regulates the activation and functions of target proteins (112). As TRAF6 enhances K63-linked polyubiquitination of CREBH, we tested whether TRAF6-mediated ubiquitination is involved in CREBH protein stability. As shown in Fig 14A, the stability of CREBH under the LPS treatment is enhanced at the early time points, from 30 to 60 min after the cycloheximide treatment, compared to the PBS treatment. However, at the late time points, from 90 to 120 min after the cycloheximide treatment, CREBH protein in the LPS-stimulated cells was quickly degraded (Fig 14A). Importantly, at the early time window after the cycloheximide treatment (0, 30, and 60 min), the levels of cleaved/activated CREBH protein in the LPS-treated cells were significantly higher than those in PBS-treated cells (Fig 14A). These data suggested that LPS treatment may stabilize CREBH precursor, possibly through K63-linked ubiquitination, and lead to production of cleaved/activated CREBH at the early time points.

Since TLR stimulation enhances CREBH andTRAF6 interaction, we asked whether ubiquitination promotes CREBH stability and subsequent translocation to the nucleus. To address that question, we transiently overexpressed CREBH, TRAF6, and different ubiquitination mutants in Huh7 cells. Western blot analysis with cellular protein fractionations showed that expression of K63O led to the highest levels of translocation of CREBH into the nucleus, compared to expression of the other ubiquitin isoforms (Fig 14A). However, expression of K48O led to more CREBH localized to the ER and cytosolic fractions than that localized to nucleus (Fig 14A). When K63R mutant was expressed, the trend reversed, as more CREBH protein was present in the nuclear fraction. Additionally, expression of K33O also led to more CREBH protein was present in the nuclear fraction (Fig 14A). Although the role of

K33-linked ubiquitination in protein stability is not well defined, it is possible that K33-linked ubiquitination, like K63-linked ubiquitination, can stabilize protein, an interesting question to be elucidated in the future. Taken together, my studies indicated that K63-linked ubiquitination of CREBH, mediated through TRAF6, can stabilize CREBH protein and facilitate the translocation of cleaved CREBH into the nucleus, a part of CREBH activation process.

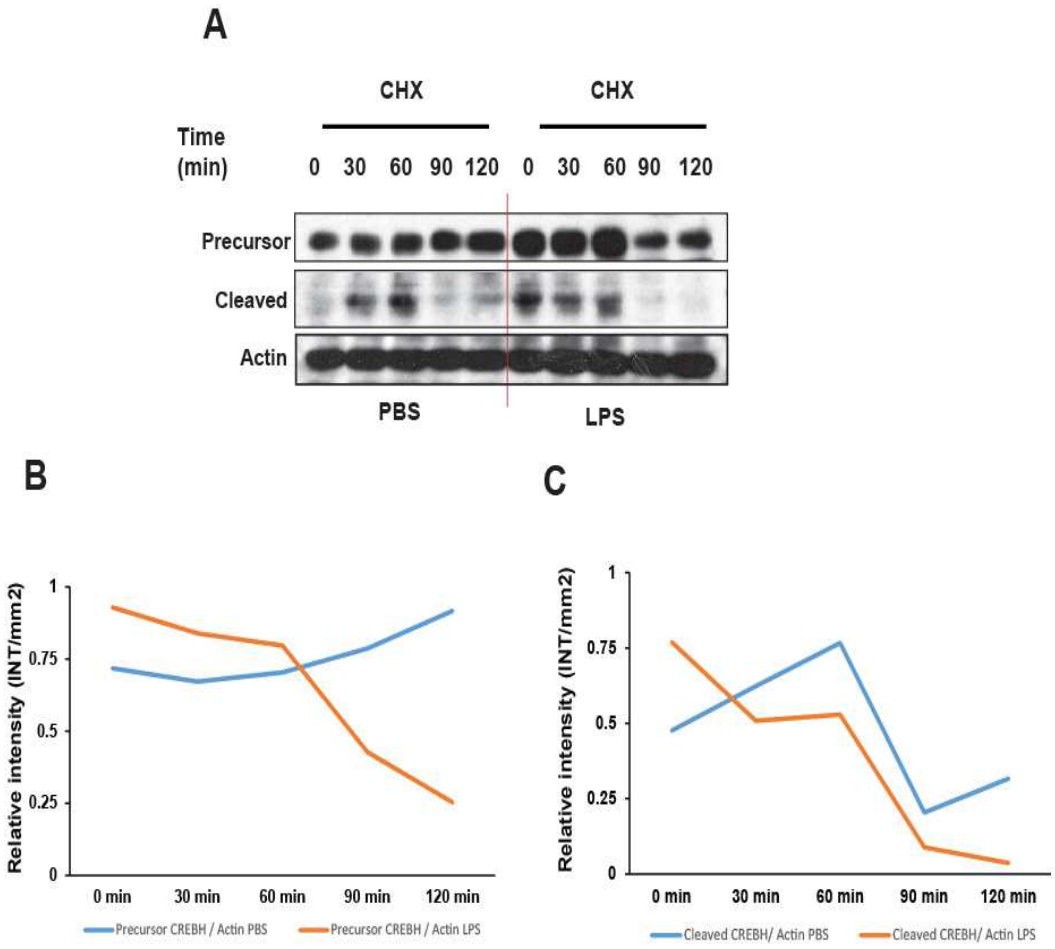

Figure 14. LPS treatment enhance the stability of CREBH protein at early time points after cycloheximide treatment.

(A) Effect of LPS on CREBH protein stability. Protein stability of CREBH was determined by the protein half-life examination after cycloheximide (100μM) was added. Top right panel: Huh7 cells infected with Flag-tagged CREBH adenovirus after 72hr treatment with LPS for 4hr, cycloheximide was added and the CREBH protein levels were assessed by Western blot analysis. Huh7 cells were incubated with cycloheximide for the indicated times periods and the cell lysates was harvested for Western blot analysis. Densitometry analysis of CREBH precursor protein **(B)** and cleaved CREBH protein **(C)** with β-actin as loading controls. Densitometry analysis was done with ImageJ software.

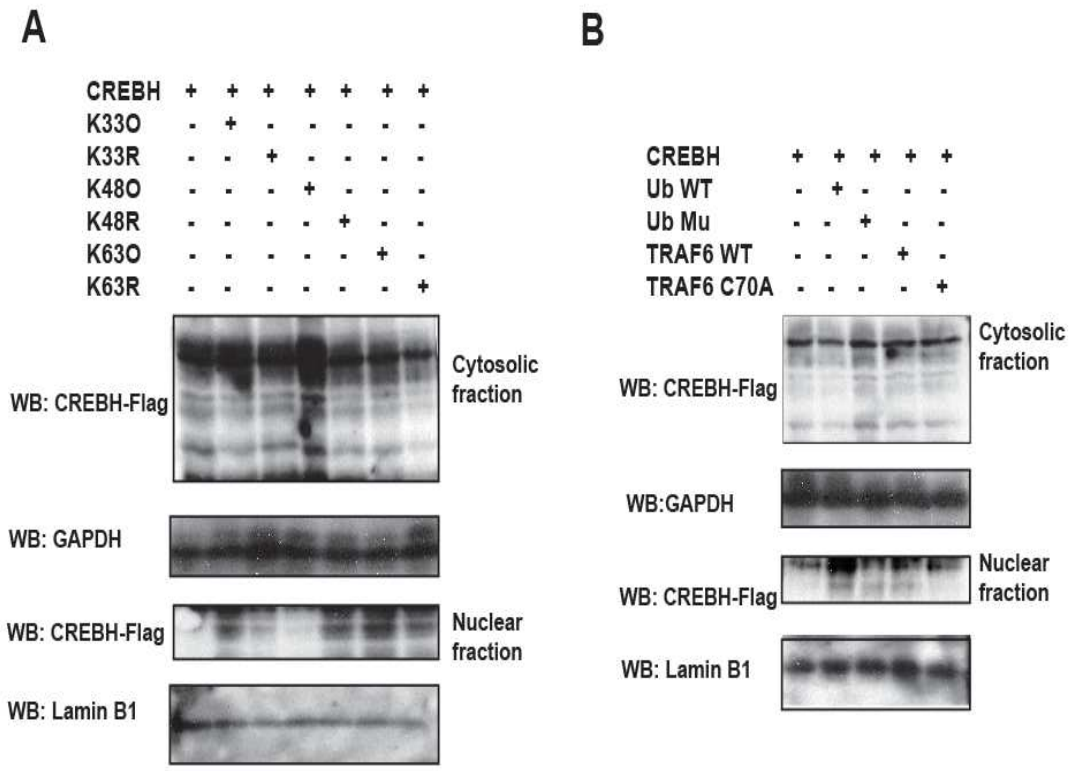

Figure 15. K63-linked ubiquitination enhances nuclear localization of CREBH

Western blot analysis of enrichment of CREBH in nuclear and cytosolic/ER fractions isolated from HEK293T cells transfected with Flag-tagged human full-length CREBH along with different ubiquitin mutant plasmids as indicated in panel **(A)** and with mutant TRAF6 and mutant Ub **(B)**. CREBH, GAPDH (cytosolic marker), and Lamin B1 (nuclear marker) protein signals were detected by Western blot analysis using the polyclonal anti-CREBH, anti GAPDH, and anti-Lamin-B1 antibody.

CREBH cleavage and activation involves the PI3K-AKT-GSK3 axis pathway.

LPS treatment in macrophages triggers activation of PI3K-AKT signaling pathway (114). To test whether the PI3K-Akt-GSK3 regulatory axis is involved in CREBH activation, we isolated primary hepatocytes from wild-type mice and pretreated them with PI3K inhibitor LYS294002 (50μM/ml, 1hr before treatment) and LiCl (30mM/ml,2hr pretreatment). After the pre-treatment, the primary hepatocytes were treated with LPS (100ng/ml) for 4hr. Western blot analysis showed that the pre-treatment with PI3K inhibitor reduced the levels of cleaved CREBH protein, compared to the vehicle pre-treatment, in response to LPS challenge (Fig 16A). Notably, the pre-treatment with LiCl, the GSK3 inhibitor increased the levels of cleaved CREBH (Fig 16A). These results suggest that the PI3K-Akt-GSK3 regulatory axis may be involved in CREBH cleavage and activation.

PI3K and GSK3 are all connected to the Akt-mediated signaling pathway. Our previous study showed that insulin signal can activate CREBH in the liver and primary hepatocytes (72). Together with my study with PI3K and PP2A inhibitors, all these results suggest that Akt pathway may be an upstream regulator of CREBH activation. To further validate this hypothesis, I over-expressed wild-type and dominant negative Akt in Huh7 cells. While overexpression of wild-type Akt elevated the levels of cleaved CREBH, expression of the dominant negative Akt reduced the levels of cleaved CREBH in Huh7 cells (Fig 16B). This result confirmed the role of Akt in CREBH cleavage and activation.

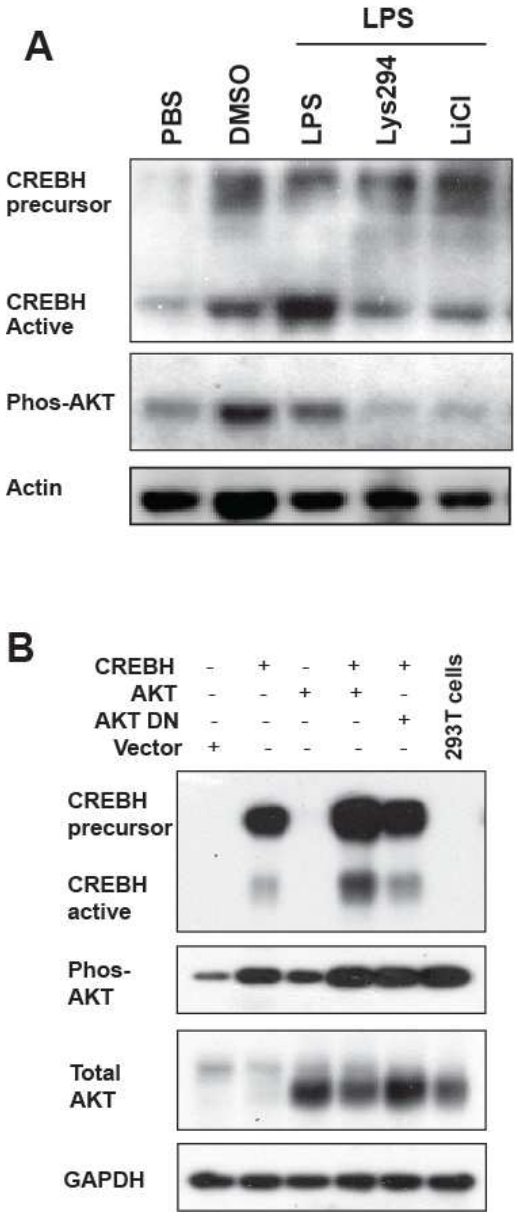

Figure 16. PI3K- Akt-GSK3 axis pathway is involved in CREBH cleavage and activation.

(A) Primary hepatocytes isolated from wild-type mice were treated with LPS (100ng/ml) for 4 hr. along with pretreatment with Lys294 for 30 min and LiCl for 2hr. Western blot analysis was performed to detect endogenous CREBH cleavage using a polyclonal anti-CREBH antibody with Actin as loading control. **(B)** Western Blot analysis showing CREBH cleavage in Huh7 cells co-transfected with full length human CREBH and Wild type Akt or dominant negative Akt.

Discussion

In this chapter, I showed that the interact to induce ubiquitination of CREBH under inflammatory stress. This confirmed the role of the E3 ligase activity of TRAF6 in CREBH activation under TLR4 signaling stimulation. K63-linked polyubiquitination is associated with programing the molecules for downstream signaling (115). This is the first demonstration that CREBH undergoes K63 ubiquitination upon TRAF6 interaction. The ubiquitination is due to the E3 ligase activity of TRAF6 since TRAF6 C70A mutant failed to show ubiquitination of CREBH protein. Expression of the K63O mutant led to CREBH cleavage comparable to wild-type Ub. In my study, K33- and K63-linked ubiquitination had similar effects on CREBH cleavage. This ubiquitination appears to be necessary to increase the stability of the CREBH precursor proteins since LPS treatment increases the protein half-life. A soluble factors ESCRTO protects the K63-ubiquitinated proteins from proteasomal degradation (116). We concluded that ubiquitination of CREBH primes it for subsequent CREBH cleavage and activation process. My study also revealed the involvement of another PTM, Akt-mediated phosphorylation, in CREBH cleavage. This modification may be correlated with TRAF6-mediated ubiquitination process since TRAF6-mediated ubiquitination can enhance phosphorylation and membrane recruitment of Akt (107).

Ubiquitination of CREBH likely facilitates its nuclear transport, although the exact role of ubiquitination in CREBH cleavage and activation needs to be investigated further. My findings suggest that K63-linked ubiquitination of CREBH is a critical event that regulates CREBH cleavage in response to TLR4 activation. However, I also detected low levels of CREBH ubiquitination that may be due to basal homoeostatic activity of CREBH. Additionally, Akt-mediated phosphorylation of CREBH appeared to be important to CREBH cleavage. However, Akt may not directly target on CREBH for its phosphorylation. Instead, Akt may be involved in cargo assembly and vesicular transport for CREBH, as transport and

activation of SREBP1c, an ER-anchored transcriptional factor similar to CREBH, are mediated through COPII vesicles that involve Akt-mediated phosphorylation through Akt (117). The similar mechanism involving Akt-mediated phosphorylation might exist in the case of CREBH translocation from ER to Golgi under the endotoxin challenge. In short, in this chapter, I demonstrated that the posttranslational modifications, namely ubiquitination and phosphorylation, are critical for CREBH activation under LPS treatment. I also provided a detailed molecular mechanism for TRAF6-mediated ubiquitination of CREBH and its role in CREBH cleavage and activation.

CHAPTER 4: DETERMINING THE CREBH TARGETS UNDER INFLAMMATORY STRESS

Summary

In this chapter, I evaluated the transcriptional targets of CREBH. Specifically, I determined inflammatory stress-specific genes targeted by CREBH. CREBH, as a liver-specific transcription factor, has already been shown to be involved in transcription of acute phase response proteins, such as cis-reactive protein (CRP) and serum amyloid component P (SAP). Previously, Zhang et al proved that CRBEH is a master regulator of hepatic lipid metabolism (87). Additionally, research in our lab showed that CREBH regulates transcription of genes associated with lipid metabolism. Microarray and qRT-PCR studies by Zhang et al have shown that deletion of *CrebH* in the liver decreased the expression of five groups of genes encoding functions critical for lipid metabolism

Phenotypically, deletion of CREBH increases serum TG levels and hepatic TG contents (118). In a bacterial sepsis model, CREBH mRNA levels were reduced due to bacterial sepsis.Protective treatment with melatonin, seems to restore and elevate the expression of *CrebH* mRNA (119). Hepatitis B virus (HBV) seems to exert its oncogenic effect through CREBH. One of the protein Hepatitis B virus protein X (HBx) showed to interact with CREBH to induce activation of critical transcription factors like c-Jun and AP1 (120). CREBH is shown to be synergistically involved in the oncogenic effect of HBV. The interaction between CREBH and HBx is necessary for proliferation of Hepatocellular carcinoma cells and mouse primary hepatocytes. Additionally, Varicella zoster virus infection also modulates CREBH expression for its successful infection in host cells. In a microarray study, VZV infection upregulates CREBH expression by 64-fold, compare to mock-infected cells. Besides, VZV

mediated up-regulation is more than the up-regulation induced by ER stress inducer tunicamycin (121).

CREBH is linked to many upstream receptors for its pathophysiological effect through stress-induced CREBH cleavage and CREBH transcriptional activation of target genes. HCV exerts its effect on glucose metabolism through the endocannaboid receptor 1. Treatment with endocanaboid receptor agonist upregulates CREBH expression which in turn cause up-regulation of PEPCK and G6Pase expression levels (122). In this chapter, I evaluated the inflammatory stress-induced transcriptional regulation through CREBH. I identified the specific gene targets of CREBH under LPS treatment and the capacity CREBH in regulating transcription of these genes.

Material and Methods

Quantitative real-time PCR

For real-time PCR analysis, reaction mixtures containing cDNA template, primers and SYBR Green PCR Master Mix (Applied Bio systems)were analyd with 7500 Fast Real-time PCR System (Applied Bio systems, Carlsbad, CA). Fold changes of mRNA levels were determined after normalization to internal control Rplpo or β-actin mRNA Levels. The sequences of real-time PCR primers used in this study are shown in Appendix B

Luciferase reporter analysis

For luciferase reporter analysis, we used the Dual-Luciferase Reporter Assay System (Promega Inc). Huh7 cells were co-transfected with the reporter vectors and control reporter vector, and the vector expressing full-length CREBH. Luciferase assay was performed at 24, 36, 48 hr after transfection/infection. Data graphs were presented as normalization of *Firefly,* luciferase reporter activities to the control *Renille* luciferase activities.

Chromatin immunoprecipitation (ChIP) Assay

Mouse liver tissues from LPS injected animals were isolated, homogenized in 10ml Wheaton tissue grinder, and suspended in NP-40 lysis buffer (HEPES 20mM,ND40 0.5%, NaCl 10mM, $MgCl_2$ 3mM, $Na_4P_2O_7$ 10mM, NaF 1mM, sodium butyrate 10mM, sodium vanadate 10mM, DTT 1mM, spermidine 0.5mM, spermine 0.15mM). The nuclear pellets were isolated using two-step gradients of 1M and 0.5M sucrose. The morphological integrity of isolated nuclear fractions was assessed with DAPI staining. For the crosslinking procedure, formaldehyde was added directly to the pellet and the reaction was stopped with 200mM Tris-HCL. Purified nuclear fractions were first sonicated 10 times for 10s and then subjected to

immunoprecipitation with the anti-CREBH antibody-coated recombinant protein G beads (Invitrogen, Grand Island, NE, USA). The amount of chromatin used was 3μg/μg antibody. Eluted DNA was subjected to quantitative RT-PCR as well as semi-quantitative PCR analysis.

Quantitative Real-time PCR for ChIP

Real-time PCR was carried out with SYBR-Green-based reagents (Invitrogen, express SYBR Green ER) using immunoprecipitated DNA-protein complex on an Applied Biosystems 7500 Fast protocol. The relative quantities of immunoprecipitated DNA fragments were calculated using the comparative C_T method. Resulting quantitation was determined after normalizing to antibody control. Results were compared to a standard curve generated by serial dilutions of input DNA. Data were derived from three independent amplifications. Error bars represent standard error of the mean.

Statistics

Experimental results are shown as mean ± SEM (for variation between animals or experiments). All in vitro experiments were repeated with biological triplicates at least three times independently. Mean values for biochemical data from the experimental groups were compared by paired or unpaired, 2-tailed Student's t-tests. Multiple comparisons were compared with ANOVA and proceeded to ad hoc statistical test when necessary. Statistical tests with $P < 0.05$ were considered significant.

Results

CREBH regulates transcription of the genes involved in TG metabolism under the endotoxin challenge.

Previously, we demonstrated that *Crebh* null mice displayed elevated levels of serum and hepatic TG under the atherogenic high-fat diet (72). To understand the genetic basis underlying the lipid phenotypes observed with the *CrebH* null mice, we performed quantitative real-time PCR (qRT-PCR) with total liver mRNA from the *CrebH* null and wild-type control mice fed on normal chow but injected with either vehicle PBS or LPS. Through the qRT-PCR analysis, we have identified a group of lipid metabolism-associated genes were upregulated by LPS treatment (Fig 17A and 17B). Most of the metabolic genes, such as *ApoA-IV*, *ApoA5*, *ApoC2*, and *ApoB*, we investigated showed changes in expression upon LPS treatment. However, expression levels of these genes, except *ApoA-IV*, were only marginally affected by CREBH deletion. In case of Apo A-IV, the mRNA levels under *CrebH* null condition were significantly lower, compared to the wild-type mice (Fig 18A). At the protein level, hepatic Apo A-IV levels were elevated in wild type mice injected with LPS. In contrast, levels of Apo A-IV in the livers of *CrebH* null mice were lower than that of wild-type control mice (Fig 18B). Moreover, the levels of serum ApoA-IV in the *CrebH* null mice were significantly lower than those in the wild-type control mice (Fig 18C).

CREBH regulates expression of the genes involved in the pro-inflammatory response to endotoxin challenge.

Previously, the CREBH transcription factor has been shown to be associated with transcription of the genes encoding acute phase response proteins and hepcidin, a gene associated with iron metabolism (123). Bearing this in mind, I investigated the role of CREBH in regulating expression of the genes involved in acute inflammation under the LPS challenge. qRT-PCR analysis with the total RNA from the *CrebH* null and wild-type control mouse livers showed that levels of *IL-6* mRNA were significantly decreased in the liver of *CrebH* null mice, compared to that of the control mice, upon LPS challenge (Fig 17C). Moreover, expression of the genes encoding the chemokines CCL2 and RANTES were also decreased in the *CrebH* null mice in the presence or absence of LPS challenge (Fig 17C).

In the liver, *IL-6* mRNA levels can be contributed by different liver cell populations, such as Kupffer cells, hepatocytes, and hepatic stellate cells. We tested whether CREBH, a hepatocyte-specific transcription factor, directly regulates *IL-6* transcription in hepatocytes. In order to answer this key question, we evaluated *IL-6* mRNA levels in the primary hepatocytes isolated from *CrebH* null and wild-type mice in response to LPS challenge. As shown in (Fig 19B), *IL-6* mRNA levels were abrogated in *CrebH* null mice (Fig 19B). This observation is consistent with our analysis with whole liver mRNA. Hence, our data suggested that CREBH transcription factor has a major role in transcription of *IL-6 gene* in the liver hepatocytes under LPS treatment.

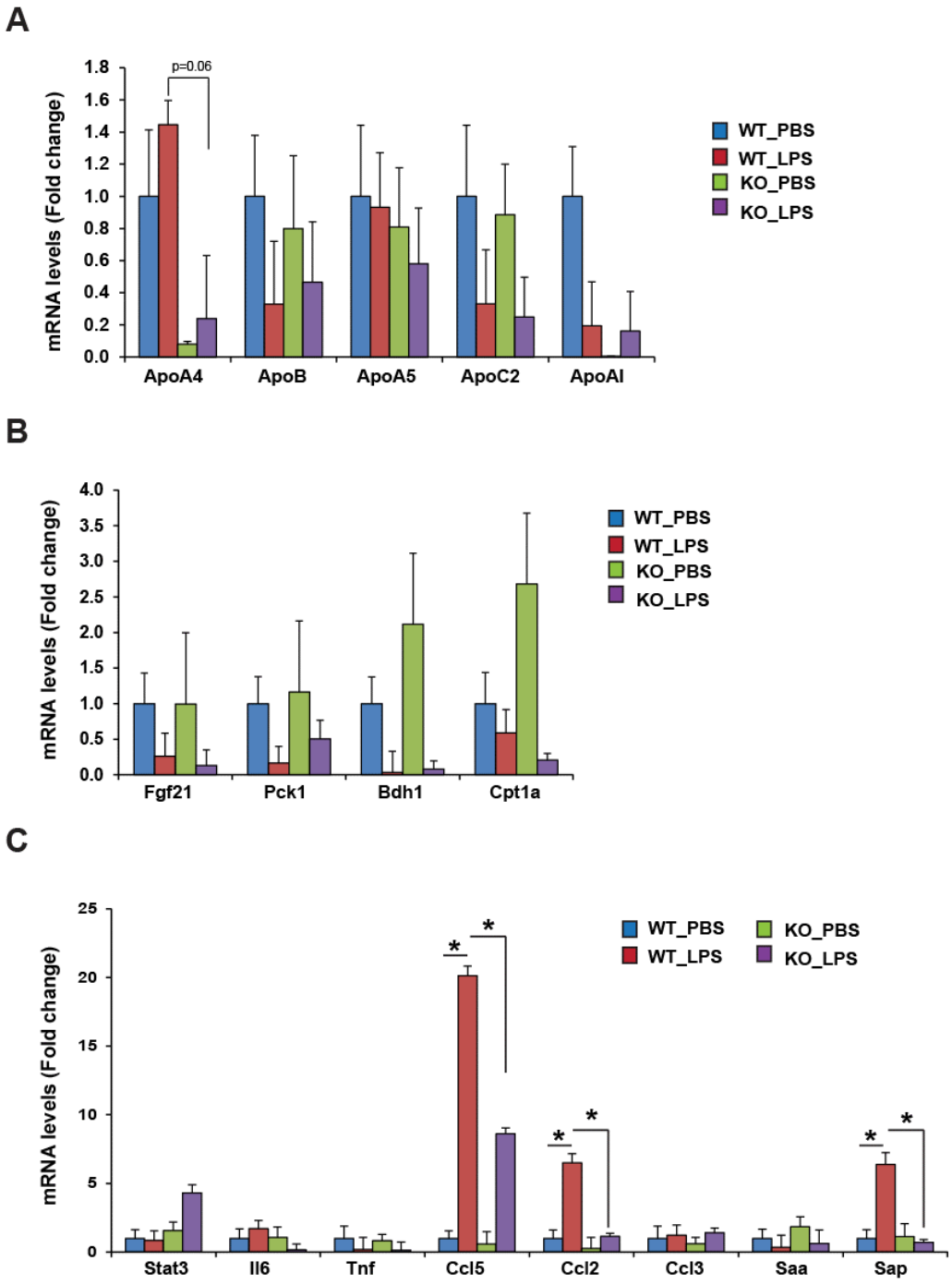

Figure 17. CREBH transcription profile under LPS treatment.
Total RNAs were isolated from liver tissues of the *CrebH* null and wild-type control mice under the normal chow diet after LPS (2μg/gm body weight) injection and subjected to quantitative real-time RT-PCR analysis of expression of the genes involved in apolipoproteins **(A)**, lipid and glucose metabolism **(B)**, and inflammation **(C)**. Expression values were normalized to *Rplpo* or *β-actin* mRNA levels. Fold changes of mRNA levels are shown by comparing to one of the control mouse under the normal chow diet. Each bar denotes the mean ± SEM (n=3). * P<0.05; ** P<0.01

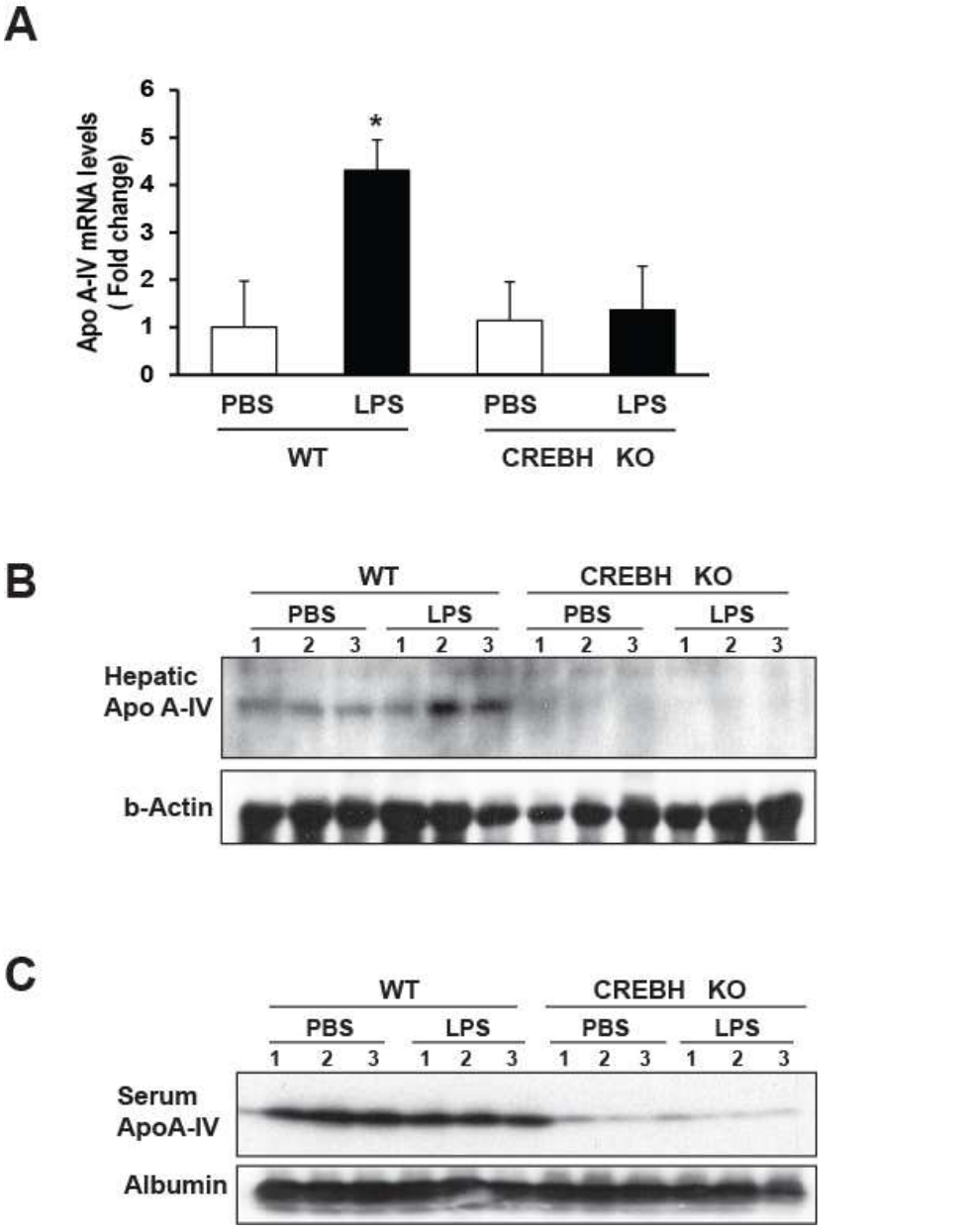

Figure 18. LPS injection increases hepatic Apo A-IV levels in liver but not in serum. (A) mRNA levels of *Apo A-IV* gene transcription from primary hepatocytes isolated from wild type and *CrebH* null mice under the normal chow diet treated with LPS (100ng//ml).Western blot analysis of wild type and *CrebH* null mice injected with LPS (2μg/gm body weight) for 18hr displaying Apo A-IV protein levels in liver (B) and in serum (C) Each bar denotes the mean ± SEM (n=3). * $P<0.05$; ** $P<0.01$

CREBH binds to the *Apo A-IV* and *IL-6* gene promoters and is involved in transcription of these genes.

To determine whether the transcriptional down-regulation observed for *IL-6* and *Apo A-IV* in our qRT PCR studies were direct effects of CREBH binding, we performed ChIP experiments to test the potential of CREBH in binding to the promoter regions of the *IL-6* and *Apo A-IV* genes. We performed endogenous ChIP analysis on whole liver tissues challenged with LPS. The ChIP analysis indicated that CREBH can bind to the promoter regions of the genes encoding *Apo A-IV* (Fig 19A and B). Further, we confirm that under *Myd88* null condition binding of CREBH was diminished with LPS treatment, thus further confirming that TLR4-mediated CREBH activation is necessary for Apo A-IV transcriptional activation (Fig 19D and 19E). Similarly, treatment with LPS leads to elevated binding of CREBH to the *IL-6* promoter region (Fig 20E)

Next, we confirmed the transcriptional role of CREBH in gene transcription of *Apo A-IV* and *IL-6* by gene expression reporter assays Huh7 cells were co-transfected with the vector expressing full-length CREBH and the vector expressing *Apo A-IV* or *IL-6* luciferase reporter. Expression of CREBH increased transcription of *Apo A-IV* genes, as indicated by high levels of luciferase activity driven by the *Apo A-IV* gene promoter (Fig 19C). This increase was almost 8-10 folds, compared to reporter control. Further, I determined the effects of CREBH transcription activity on *Apo A-IV* gene promoter at different time points ranging from 24, 36 to 48hr. Apparently, CREBH exerted its strongest transcriptional activity in driving the *Apo A-IV* promoter at 36 hr post the plasmid transfection (Fig 19C).

In case of IL-6 reporter assay, we observed the considerable basal level of *IL-6* gene transcription without CREBH overexpression (Fig 20C-D). The basal level of IL-6 reporter activity may be due to the contribution of endogenous NF-κB transcription activity triggered

by plasmid transfection and expression. Consistently, overexpression of CREBH and IL-6 reporter displayed 1.5-2 fold increase in IL6 reporter activity over the endogenous IL-6 reporter activity. In order to determine the exact effect of CREBH on *IL-6* transcription without any contribution from NF-κB, I used IL-6 reporter defective for NF-κB transcription factor binding site (Fig 20C). Under NF-κB defective IL-6 reporter overexpression, CREBH overexpression displayed a higher level of activity in increasing IL6 reporter activity (Fig 20C-D). This result confirmed the direct role of CREBH transcription factor in the *IL-6* promoter. Additionally, I also tested whether CREBH interacts with NF-kB transcription factor for *IL-6* transcription. I observed that co-expression of CREBH and P65 subunit of NF-κB led to a reduction in IL-6 reporter activity, compared to P65 subunit alone (Fig 20C), suggesting that CREBH may compete NF-κB in activating IL6 promoter, an interesting question to be evaluated in the future.

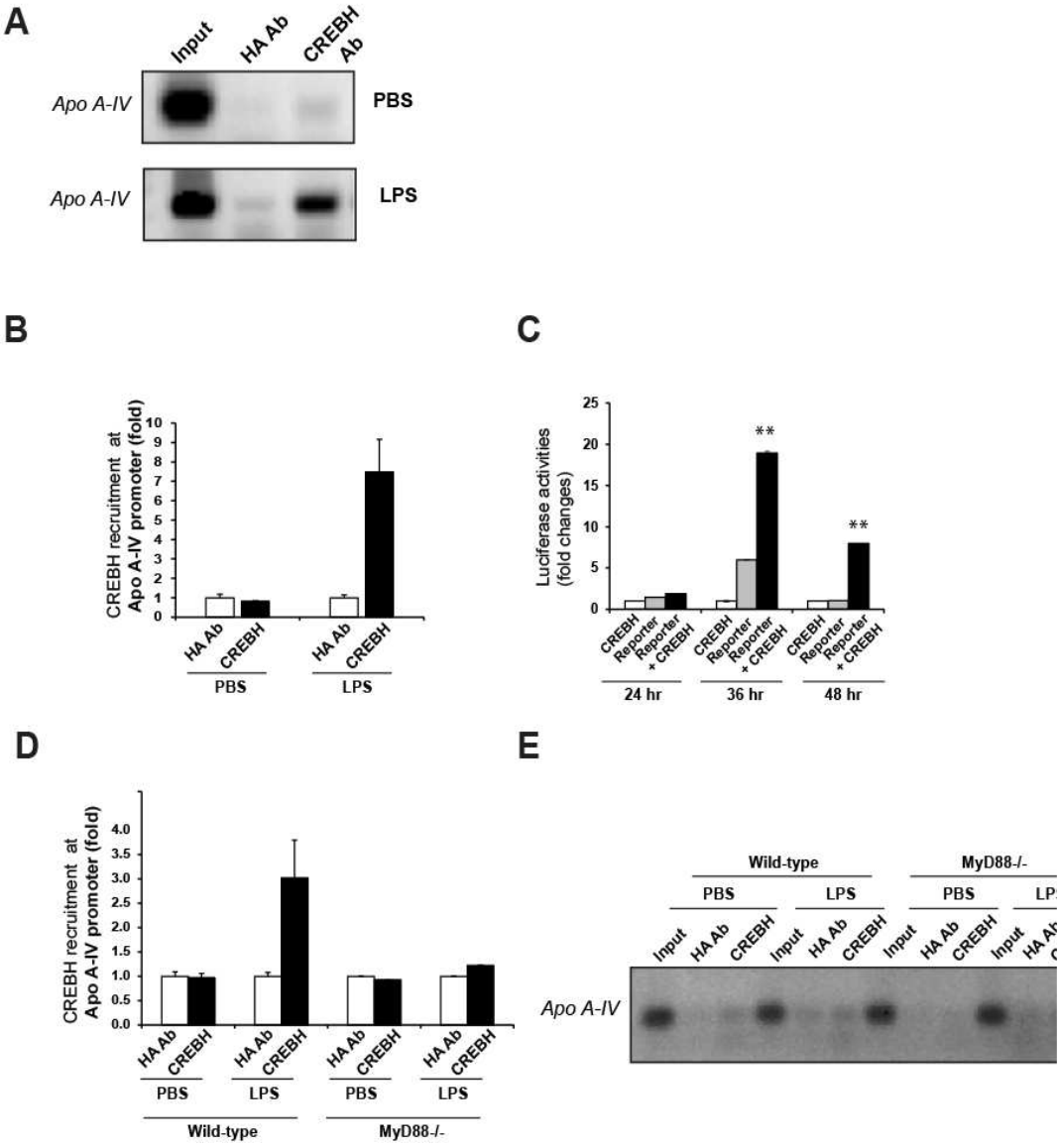

Figure 18. CREBH binds to Apo A-IV promoter under LPS treatment and this binding is TLR4 signaling dependent.

ChIP analysis of CREBH-binding activity to the promoter regions of the Apo A-IV. Chromatin isolated from the wild type mouse liver were subjected to immuoprecipitation. PCR was performed to identify potential CREBH-binding regions in the Apo A-IV promoter. Mock ChIP with control antibody was included as a control (HA). The PCR reactions with the genomic DNA isolated from sonicated cell lysates were included as positive controls. **(A)** Semi-quantitative ChIP analysis of wild type mouse injected with LPS (2µg/gm body weight) **(B)** quantitative PCR analysis of LPS injected liver tissue **C)** Luciferase activity of CREBH at *Apo A-IV* gene promoter in Huh7 cells infected with full length human CREBH plasmid along with Apo A-IV reporter. **(D)** ChiP analysis showing CREBH binding to Apo A-IV in wild type and *Myd88* null mice. **(E)** Semi-quantitative analysis of wild type and *Myd88* null liver samples. Each bar denotes the mean ± SEM (n=3). * $P<0.05$; ** $P<0.01$

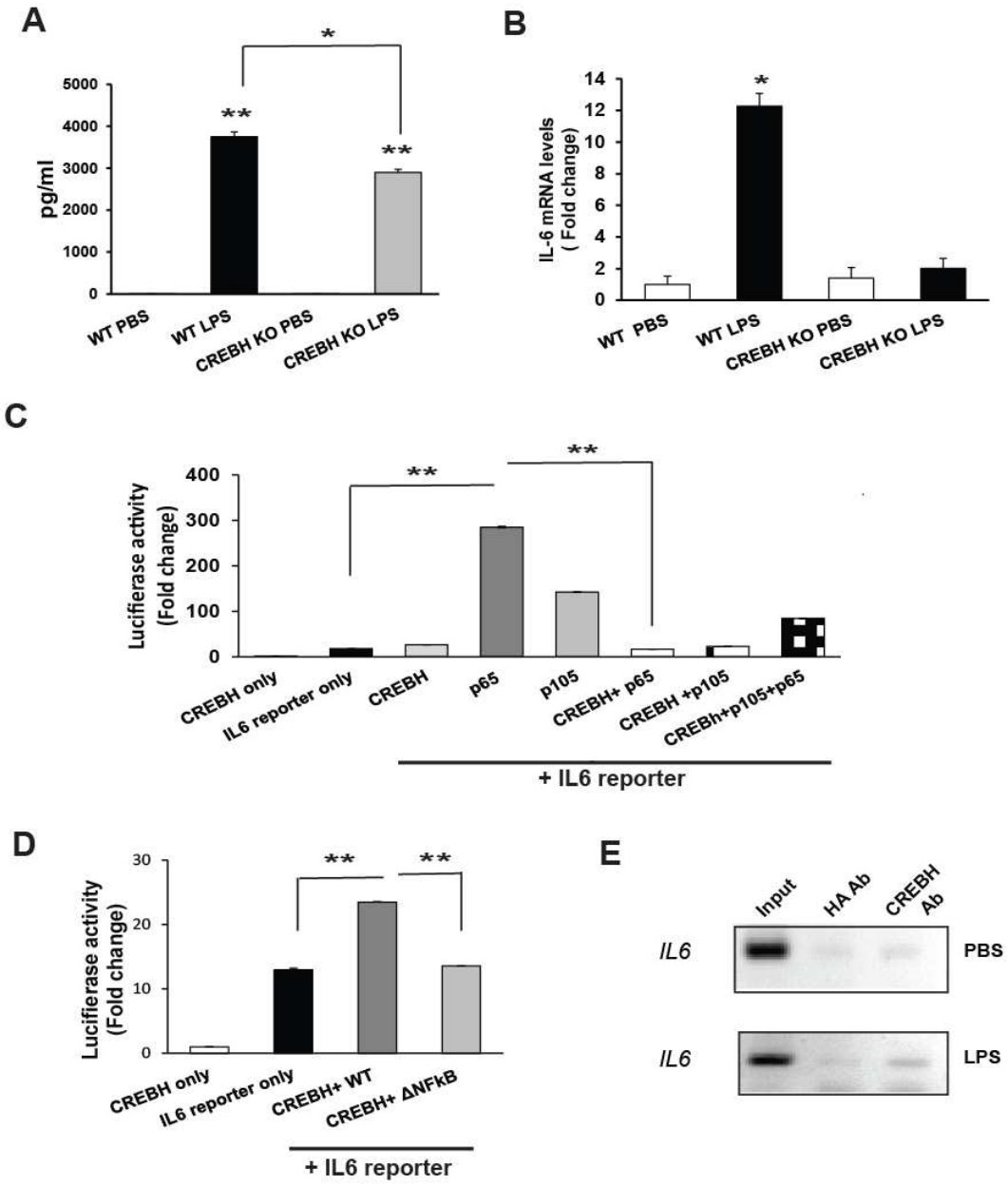

Figure 19. CREBH binds to IL-6 promoter under LPS treatment and this binding is TLR4 signaling dependent.

Levels of IL-6 in wild type and CREBH null mice injected with LPS (2µg/gm body weight) in serum **(A)** and mRNA levels of IL-6 in primary hepatocytes**(B).** ChIP analysis of CREBH-binding activity to the promoter regions of the Apo A4. Luciferase activity of CREBH transcription factor at IL-6 promoter **(D)** transcriptional activity of CREBH at IL-6 promoter along with NF-κB transcription factor. **(C). (E)** ChIP analysis of CREBH binding activity to the promoter region of IL-6. Chromatin isolated from the wild type mouse liver were subjected to immuoprecipitation . PCR was performed to identify potential CREBH-binding regions in the *IL-6* gene promoter. Mock ChIP with control antibody was included as a negative control (HA). The PCR reactions with the genomic DNA isolated from sonicated cell lysates were included as positive controls. Each bar denotes the mean ± SEM (n=3). * $P<0.05$; ** $P<0.01$

Discussion

As a liver-specific transcription factor, CREBH regulates an array of genes associated with TG and lipoprotein metabolism (72). The impact of CREBH on apolipoprotein had been proved previously (124). To further exploit the regulatory impact of CREBH on apolipoprotein metabolism, we have investigated the mechanism through which endotoxins and innate immunity regulate Apo A-IV biosynthesis. Hepatic levels of Apo A-IV elevated upon LPS injection, and it was abrogated with the loss of CREBH from mouse liver. It has been suggested that there are two cis-acting elements on the *Apo A-IV* promoter bound by CREBH transcription factor that controls the transcription of *Apo A-IV* (124). Previously it had been described that Apo A-IV and Apo A-V are acute phase proteins in mouse HDL (125). The increase in apolipoproteins level to inflammation is a well-documented response to inflammatory stimuli.

There is a prevailing theory that immune cell produced IL-6 can stimulate hepatocytes to produce acute phase response proteins. CREBH in hepatocytes was previously reported to be involved in acute phase response gene transcriptions (87). Production and secretion of IL-6 are paramount in injuries. Any damage will elicit immune reaction resulting in IL-6 secretion in circulation, which eventually reach the liver and trigger an acute phase response. In case of liver injuries, it is thought that the endogenous immune cells in the liver are responsible for IL-6 production (126). It has been demonstrated that hepatocytes play an important role in IL-6 production since selective inactivation of NF-κB in hepatocytes caused abrogated production of IL-6 (127). Our study has shown for the first time that CREBH transcription factor is involved in IL-6 production from hepatocytes. Bioinformatics data points at two potential CREBH binding sites at IL-6 gene promoter, exact location of these sites are yet to be confirmed Surprisingly potential binding sites of CREBH overlaps with another transcription factor binding site CEBP/β. CEBP/β has been shown to be involved in inflammatory gene transcription along with NF-κB. This finding perhaps explains the decrease in IL-6 reporter

activity when CREBH co-transfected with the P65 subunit of NF-κB, since NF-κB, factor requires CEBP/β for its transcription activity of IL-6. This mechanism can be a potential way to control the excessive inflammatory response to incoming endotoxins in the liver. My reporter assay with CREBH and *IL6* promoter revealed some of the interesting facts about the regulation of inflammation by hepatocytes. Since hepatocytes are the first responder in liver against exposure to antigens from portal blood flow, they need to maintain low levels of inflammatory activity in order maintain the tolerance to those antigens. My study suggested that CREBH is involved in basal or low levels of inflammatory activity. Exposure to high levels of endotoxins or inflammatory stimuli may lead to CREBH competition with classical inflammatory transcription factors like NF-κB, CEBP/β, and AP1, an intriguing question to be elucidated in the future.

Another important observation from my experiments is the differential behavior of CREBH on activating its target genes. My study unveiled how CREBH transcription activity was modulated based on the types of stress. Under endotoxin-mediated CREBH activation, unlike its previously enlisted genes under the atherogenic high-fat diet (AHF), CREBH selectively activates transcription of *Apo A-IV* and *IL-6*. Notably, under the metabolic stress, activation of CREBH has no effect on driving expression of IL-6 (data not shown). My study suggested that CREBH has an ability to respond to various stress and control an array of gene expression as per the stress requirement.

CHAPTER 5: Delineating the animal phenotypes caused by CREBH deletion

Summary

IL-6 is considered as pro-inflammatory cytokine and one of the immediate responders to inflammation. Recent studies suggested that IL-6 might be involved in dampening the immune response through suppressing the production of TNFα and IL-1β (128). A population study of obese and insulin resistant individuals pointed at the correlation between increase serum levels of IL-6, obesity, and insulin resistance (129). Common polymorphism associated with IL-6 includes SNP in the *IL-6* gene 174G to C substitution, has been independently associated with type-2 diabetes (130). IL-6 not only affects insulin signaling but also exerts its effect through manipulating the lipid metabolism in the body. IL-6 transgenic mice have low total cholesterol and TG levels (131). Treatment of Hep3B cells with recombinant IL-6 for 24hr increased levels of the nuclear receptor PPARα while decreased levels of SREBP-1c. IL-6 can increase the FA oxidation in rat muscles (132), and is targeted on hepatocytes to modulate apolipoprotein levels (133).

Apo A-IV, as discussed in previous chapter, is a component of chylomicrons and HDL. The level of Apo A-IV can serve as a surrogate marker of lipid absorption and secretion (134). Approximately 25% of Apo A-IV is attached to HDL particles while the rest of is found as a free fraction of plasma. Apo A-IV synthesis can be triggered by glucocorticoids as well as insulin (135). Functions of Apo A-IV include: 1) activating lecithin: cholesterol acyltransferase, 2) modulating the activities lipoprotein lipase, and 3) cholesterol ester transfer protein, and 4) facilitating cholesterol removal from peripheral cells.

Interestingly, recent studies suggested that Apo A-IV inhibits gastric emptying and serves as a satiety factor in response to ingestion of dietary fat (136), Two mutations in Apo A-IV protein, including Gln_{360}→His and Thr_{347}→Ser associated with lipid and lipoprotein

metabolism (137). Given the evidence that Apo A-IV may be involved in the inhibition of food intake following consumption of a high-fat meal, we examined the potential effects of these Apo A-IV defects on indices of body weight and food consumption.

Previously, we demonstrated that *CrebH* null mice displayed reduced body weights, increase hepatic steatosis, reduction in abdominal fat, and hypertriglyceridemia under the atherogenic high-fat diet (137). Since in the previous chapter I described the roles of CREBH in transcriptional regulation of Apo A-IV and IL-6, here I explored the impact of CREBH deficiency in hepatic and serum TG, serum cholesterol, and energy consumption under the LPS challenge.

Material and Methods

Measurement of mouse lipid metabolites

Liver tissue and blood plasma samples were isolated from the mice under normal chow diet after LPS (2μg/gm body weight) challenge for 18hr. To determine hepatic TG levels, approximately 100 mg liver tissue was homogenized in 500 μl PBS followed by centrifugation at 10000g for 5 min. The supernatant was mixed with 500 μl 10% Triton-100 in PBS for TG measurement using a commercial kit (Bioassay Systems,CA). Mouse hepatic TG levels were determined by normalization of liver tissue mass used for TG measurement. Mouse blood plasma samples were subjected to quantitative analyses of TG using a commercial kit (Bioassay Systems, CA).

Indirect calorimetry

Each mouse was monitored individually in the computer-controlled OxyScan open circuit indirect calorimetry system (AccuScan Instruments, Columbus, OH) (Bishop and Walker 2004) with free access to food and water. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 18 hr after PBS or LPS (2mg/kg body weight) challenge. Gas analyzers were calibrated to room air drawn through each chamber at a rate of 0.5 L/minute.

Steady-state measurements of plasma glucose after LPS injection

Basal plasma glucose levels were measured at the termination of the experiment. Blood was sampled from the tail tip using a cut below the vertebrae. Glucose was measured by a hand-held glucose meter (One-Touch Ultra; Johnson and Johnson).

Body weight measurement and food consumption

Wild-type and *CrebH* null mice were subjected to food intake measurement under the normal chow diet before LPS injection and 18hr after injection. Body weights were measured similarly before and after LPS injection.

Results

Under LPS challenge, absence of CREBH causes elevation in serum TG levels.

Since CREBH have been shown to be involved in manipulating levels of Apo A-IV, I tested the effect of CREBH on modulation Apo A-IV under LPS challenge. In order to reveal the pathophysiological effect of CREBH on TG levels, we measured hepatic, and serum TG levels in LPS-injected mice (Fig 22A). We found a comparable increase in TG levels post LPS injections in wild-type and *CrebH* null mice. We thus inferred that TLR4-CREBH- Apo A-IV axis pathway does not affect hepatic TG levels. Interestingly, *CrebH* null mice displayed elevated basal levels of serum TG, which were increased further after LPS injection (Fig 22B).

CREBH plays an important role in endotoxin-mediated cholesterol modification.

Apo A-IV is a component of chylomicrons as well as HDL cholesterols (138, 139). Transportation of TG from the intestine to tissues takes place through chylomicrons. Hence chylomicron, being an intestinal cargo transport, cannot be a good indicator for the function of CREBH- Apo A-IV regulatory axis. In order to reveal the impact of abrogated production of Apo A-IV in the absence of CREBH, we tested the serum cholesterol levels of *CrebH null* mice after LPS challenge. We observed that *CrebH null* mice had a slight increase in total cholesterol, HDL, and LDL upon LPS treatment (Fig 22C), while wild-type mice treated with LPS displayed a significant increase in total cholesterol and HDL levels, compared to control mice treated with vehicle (Fig 22D). *CrebH* null mice did not display such elevation upon LPS treatment. HDL levels of LPS-injected *CrebH* null mice were significantly lower than those of LPS-injected wild type mice. There was no significant difference in LDL/VLDL levels between *CrebH* null and their corresponding control mice (Fig 22E).

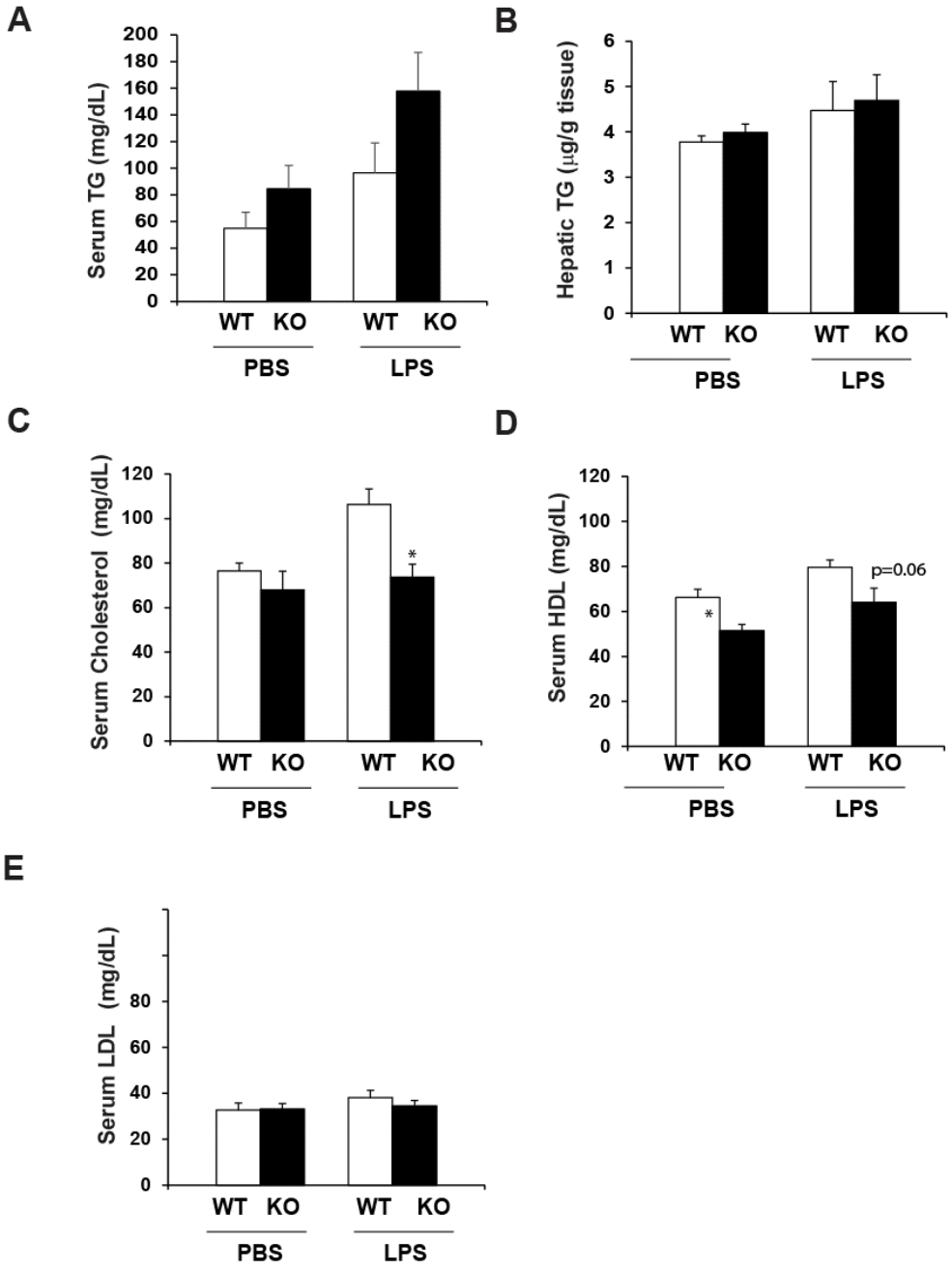

Figure 20. LPS mediated HDL cholesterol changes are dependent on TLR4-CREBH axis pathway.

Serum and hepatic lipid profiles of mice challenged with LPS (2µg/gm body weight) for 18hr **(A)** Serum TG levels in LPS injected mice **(B)** Hepatic TG levels in LPS injected mice. Serum levels of **(C)** Total cholesterol **(D)** HDL **(E)** LDL/VLDL in LPS injected mice. Each bar denotes the mean ± SEM (n=5). * P<0.05; ** P<0.01

LPS-challenged *CrebH* null mice displayed increased oxygen consumption and reduced blood glucose levels.

Since we have observed a change in lipid and cholesterol profile of *CrebH* null mice after LPS challenge, we investigated the physiological processes that may contribute towards this phenotype. LPS injected mice displayed loss of body weight post LPS injection while PBS injected mice did not lose significant body weight post-treatment (Fig 23C). Even the food consumption is consistent with the body weight loss; LPS injected mice consumed less food while the PBS injected mice consumed about 2-3 gm more food over 18hr (Fig 23B).

We also investigated the energy expenditure through indirect colorimetric analysis. The oxygen consumption for LPS-injected mice displayed lower energy expenditure compared to the PBS-injected mice (Fig 20A). The decrease in oxygen consumption levels in LPS injected mice is in part due to the inflammation induced caused by LPS action. Compared to the wild-type animals, *CrebH* null mice exhibited relatively higher oxygen consumption. This may reflect a feedback regulation of impaired lipid profile in the *CrebH* null mice. Additionally, we measured plasma glucose levels of *CrebH* null and wild-type control mice upon LPS challenge. LPS injected mice *CrebH* null mice displayed low levels of blood glucose compared to wild-type mice (Fig 23D).

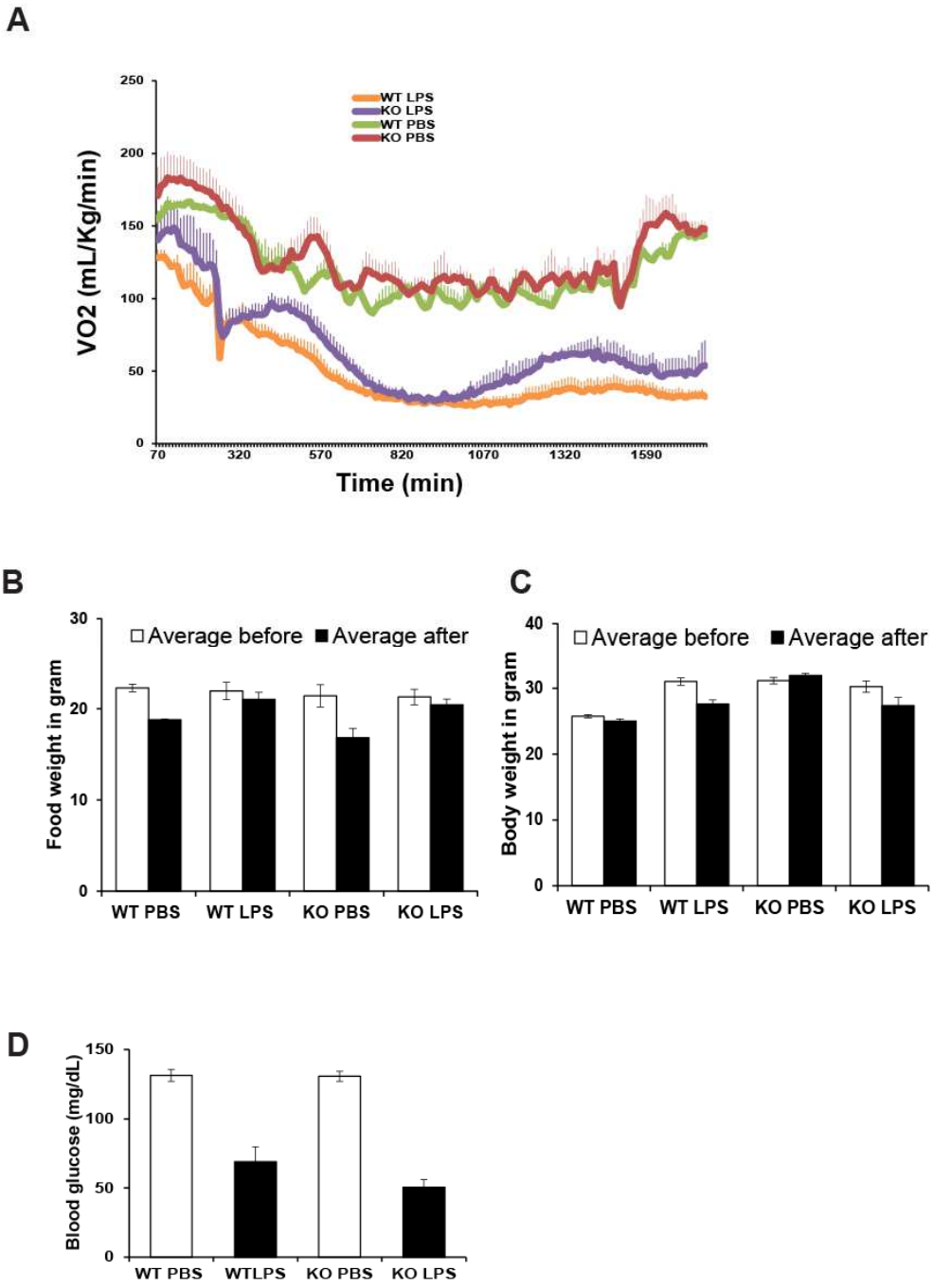

Figure 21. LPS mediated physiological changes are comparable in wild type and CREBH null mice.

Physiological parameter analysis in LPS (2µg/gm body weight) for 18hr injected mice **(A)** Energy expenditure in wild type and CREBU null mice displayed with VO_2 levels. Food consumption **(B)** Body weights **(C)**, Blood glucose levels **(D)** of *CrebH* null and wild-type control mice under the normal chow diet after LPS injection (2µg/gm body weight).

Discussion

Lipid metabolism abnormalities are a critical issue in all sepsis patients. Plasma proteins like apolipoproteins are markedly modulated during sepsis. HDL is thought to play a protective role in sepsis and endotoxemia (140). Elevation in HDL levels is an important response mounted by the body to counter the sepsis or endotoxin levels. Elevation in HDL post LPS injections confirms the previous findings. Our study in this chapter reveals one of the underlying mechanisms explaining the role of the hepatic transcription factor CREBH in a protective response to endotoxins. TLR4 mediated activation of CREBH that in turn increases transcription of Apo A-IV, a component of HDL cholesterol, is a critical signaling pathway for against sepsis. Our study indicates that the loss of CREBH damaged the ability of the animal to mount an HDL protective response against LPS. Previously, the release of TNFα increase levels of TG and cholesterol in LPS injected mice (141). It had been shown *CrebH* null mice had lower levels of serum cholesterol and TG (72). The increase in TG and cholesterol after LPS injection is a well-documented effect known to carry through an unknown mechanism. Our study with *CrebH* null mice have shed light on the possible mechanism of elevation in serum TG levels post LPS treatment. The activities of inflammatory cytokines have been suggested to be the main cause in inhibiting energy accumulation. Inflammation induces energy expenditure and inhibits food intake. Study with the transgenic mouse has confirmed this notion (142). Injection of IL-10 shown to reduce the LPS-mediated changes in body weight and food consumption (142). Injection of LPS decreases not only glucose production but also glucose utilization. Studies showing that acute exposure to LPS inhibits glucose production, conflicting reports about the effect of LPS on glucose uptake (143, 144). We found a possible mechanism of elevation in HDL cholesterol after endotoxin treatment, which is mediated through CREBH in a TLR4-dependent manner. Our study with *CrebH* null mice showed that the null mice have elevated levels of serum TG after endotoxin treatment, which is consistent

with a previous study that *CrebH* null mice being more prone to steatosis under HFD (72). Therefore, high-fat diet may induce steatosis by increasing serum and hepatic TG through CREBH (145). Additionally, investigation of energy expenditure, food consumption, and body weights have revealed the inflammation-related effect on mouse energy metabolism. Study with physiological parameters mentioned above help us to confirm the effect of the TLR4-CREBH-Apo A-IV regulatory axis on cholesterol and TG metabolism.

Work in this chapter shows the effect of endotoxin on lipid metabolism through activation of CREBH. This effect, in presence of low endotoxin levels, could be an important response to bacterial infection.

CHAPTER 6: Conclusions and Significance

In this study, we conclude that CREBH plays a critical role in connecting innate immunity with metabolic signaling during acute endotoxin challenge. This conclusion is based on the following findings: **(1)** The endotoxin LPS is capable of inducing expression and cleavage of CREBH; **(2)** TRAF6; the E3 ligase under the TLR4 signaling pathway, is capable of interacting with full-length CREBH at ER membrane in hepatocytes; **(3)** CREBH activation requires posttranslational modification by TRAF6-medaited ubiquitination; **(4)** CREBH does not contribute to the transcroption of classical UPR or metabolic genes described previously but it is required for the APR associated genes by regulating transcription of the *ApoA IV* and *IL-6* genes; (5) CREBH binds to a promoter element in the *ApoA IV* and *IL-6* gene sequence; (6). CREBH upon binding to ApoA IV promoter regulates the levels of HDL and triglycerides. Our study sheds light on the intriguing mechanism behind the connection between immune signaling and hepatic metabolism in acute endotoxemia. We demonstrate that host response against the infection involves a highly sophisticated cross communication between innate immune signaling and transcription factor associated with hepatic metabolism. Our study confirms the widespread notion that immune signaling has a pronounced effect not only on innate immunity but also modulation of hepatic metabolism. Changes in metabolism are integral parts of the immune response against endotoxins, even bacterial infections.

Our studies also provide insight into the role of the posttranslational modification, namely ubiquitination, in CREBH cleavage and activation mechanism.The ability of the same protein to function differentially in response to the wide range of cues is attributed to post-translational modifications (146). It is known that TRAF6, an E3 ligase in the TLR4 signaling pathway, is involved in regulation and activation of downstream target molecules through ubiquitination (147). Our study expanded and confirmed the role of TRAF6

from being involved in inflammatory responses to be an essential player of metabolic signaling. The role of bacterial infection in metabolic disorders, such as atherosclerosis, is well studied. Inflammation is known to contribute significantly to the atherosclerotic process and is associated with proatherogenic changes in lipoprotein metabolism that was characterized by increased VLDL and reduced HDL levels (39). Males with lower levels of HDL are more susceptible to inflammatory stimuli against endotoxin challenge (148). Considering the protective role of HDL, administration of recombinant high-density lipoprotein (rHDL) to prevent bacterial infection-associated pathogenesis inflammatory effects (149). Indeed, rHDL showed increased capacity of anti-inflammatory and anti-oxidant functions in the approaches related to prevention and treatment of atherosclerosis (150).

The CREBH knockout mouse model provided a tool to validate the functional impact of LPS-induced, TLR4-mediated CREBH activation in the liver. *CrebH* null mice had abrogated levels of serum HDL, compare to their wild-type controls, upon relatively low-dose of LPS challenge. Our studies using the *CrebH* null mouse revealed that TLR4 mediated signaling is required to induce the acute phase response. Further studies to evaluate the response to a high dose of endotoxins or breakdown of TLR4-CREBH signaling axis under sepsis condition need to be pursued in the future. Alternatively, as we previously showed, *CrebH* null mice displayed hepatic steatosis and hyperlipidemia under the atherogenic high-fat (AHF) diet (72). Interestingly, *CrebH* null mice under the AHF diet produced low levels of serum HDL. This observation implied that the metabolic diet may also activate the same pathway mediated through TLRs, an intriguing question to be answered in the future.

Our studies at the intersection of immunity and metabolism leave out a lot of open questions. Although we can delineate the role of ubiquitination in CREBH cleavage and activation, investigating why K63-mediated polyubiquitinated CREBH imported more in the nucleus is an interesting question. Phosphorylation of CREBH should also be dogged further

since TRAF6-mediated ubiquitination is involved in kinase docking and recruitment (107). Lastly, usage of CREBH exogenous expression under sepsis or particular bacterial infections needs to be tested and validated. This study can potentially lead to a new therapeutic approaches, offering benign opportunity to treat the diseases.

To summarize, our study revealed a novel crosstalk pathway that involves TLR4 signaling and CREBH. Low levels of endotoxin induce cleavage and activation of CREBH in the liver. Activated CREBH control the expression of the apolipoprotein A IV and the inflammatory cytokine IL-6. Targeting the expression of CREBH under disease conditions for therapeutic purposes may lead to novel approaches toward alleviating sepsis-related complications.

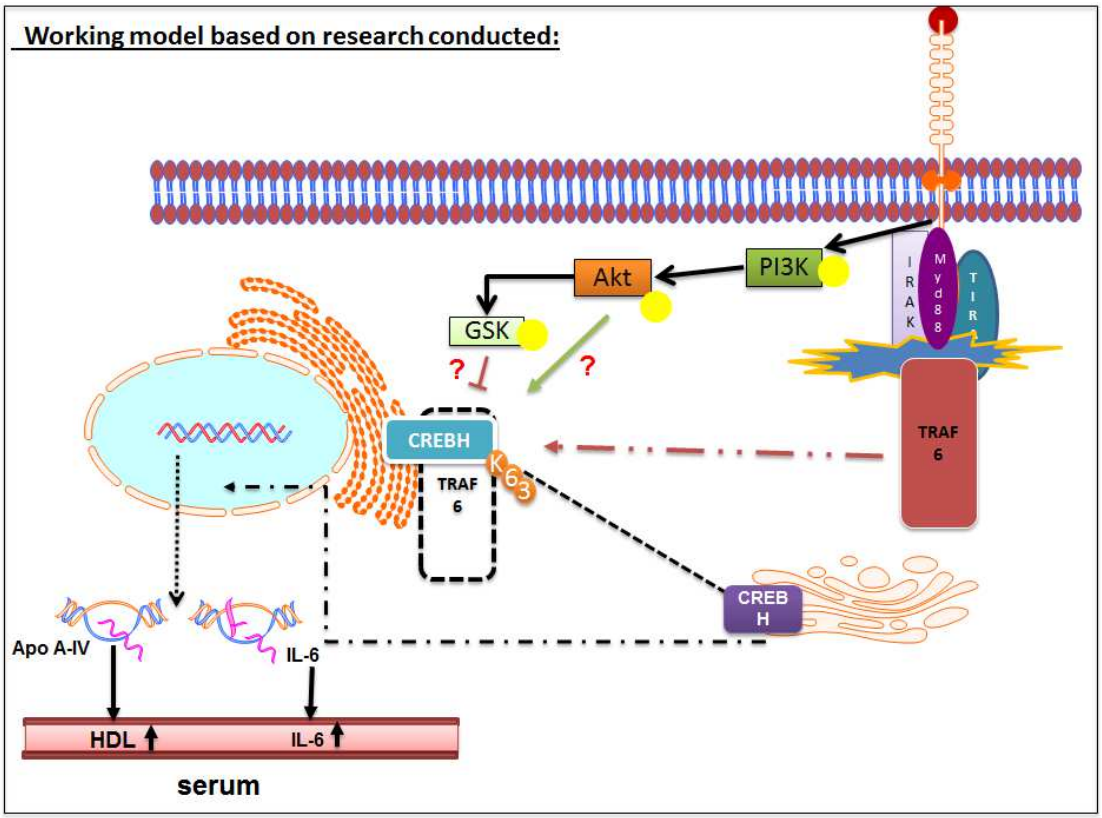

Figure 22. Working model for TLR4 mediated cleavage of CREBH and hepatic modulation under LPS treatment

Working model of our hypothesis. LPS upon binding to TLR4 receptor induce activation of TL4 receptor signaling pathway. TRAF6 of TLR4 signaling pathway are involved in direct cross talk with CREBH for its subsequent activation through K63 ubiquitination. PI3K-Akt axis pathway triggered by TLR4 receptors also plays role, but the exact mechanism is unexplained. These signaling pathways leads to CREBH translocation and cleavage into active form of CREBH. Active CREBH translocates to nucleus inducing transcription of IL-6 and Apo A-IV involved in response to LPS stimulation. Apo A-IV modulates levels of serum HDL in TLR4-CREBH dependent manner, while TLR4-CREBH also regulates low levels of IL-6 production from hepatocytes.

APPENDIX A

- **Mouse Il-6 promoter sequence:**

AGCTAGCTAAGATACAATGAGGTCCTTCTTCGATATCTTTATCTTCCATATACCATGAATCAAAGA
AACTTCAACAACATGAGGACTGCAACAGACCTTCAAGCCTCCTTGCATGACCTGGAAATGTTTTGG
GGTGTCCTGGCAGCAGTGGGATCAGCACTAACAGATAAGGGCAACTCTCACAGAGACTAAAGGTC
TTAACTAAGAAGATAGCCAAGAGACCACTGGGGAGAATGCAGAGAATAGGCTTGGACTTGGAAG
CCAAGATTGCTTGACAACAGACAGAAGATATTTCTGTACTTCACCCACTTTACCCACCTGGCAACT
CCTGGAAACAACTGCACAAAATTTGGAGGTGAACAAACCATTAGAAACAACTGGTCCTGACAAGA
CACAGGAAAAACAAGCAATATGCAACATTACTGTCTGTTGTCCAGGTTGGGTGCTGGGGGTGGGA
GAGGGAGTGTGTGTCTTTGTATGATCTGAAAAAACTCAGGTCAGAACATCTGTAGATCCTTACAGA
CATACAAAAGAATCCTAGCCTCTTATTCATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TGTGTGTGTATGTGTGTGTCGTCTGTCATGCGCGCGTGCCTGCGTTTAAATAACATCAGCTTTAGCT
TCTCTTTCTCCTTATAAAACATTGTGAATTTCAGTTTTCTTTCCCATCAAGACATGCTCAAGTGCTG
AGTCACTTTTAAAGAAAAAAAAAGAAGAGTGCTCATGCTTCTTAGGGCTAGCCTCAAGGATGACTT
AAGCACACTTTCCCCTTCCTAGTTGTGATTCTTTCGATGCTAAACGACGTCACATTGTGCAATCTTA
ATAAGGTTTCCAATCAGCCCCACCCACTCTGGCCCCACCCCCACCCTCCAACAAAGATTTTTATCA
AATGTGGGATTTTCCCATGAGTCTCAAAATTAGAGAGTTGACTCCTAATAAATATGAGACTGGGGA
TGTCTGTAGCTCATTCTGCTCTGGAGCCCACCAAGAACGATAGTCAATTCCAGAAACCGCTATGAA
GTTCCTCTCTGCAAGTAAGTGAAGGCAGTTCCTTGCCCTCTGGCGGAGCTATTGAGACTGTGAGAG
AGGAGTGTGAGGCAGAGAGCCAGCATTGTGGGTTGGCCAGCAGCCATCAGCTAGCAGCAGGCGCC
CAACTGTGCTATCTGCTCACTTGCCGGTTTTCCCTTTTCTCCACGCAGGAGACTTCCATCCAGTTGC
CTTCTTGGGACTGATGCTGGTGACAACCACGGCCTTCCCTACTTCACAAGTCCGGAGAGGAGACTT
CACAGAGGATACCACTCCCAACAGACCTGTCTATACCACTTCACAAGTCGGAGGCTTAATTACACA
TGTTCTCTGGGAAATCGTGGAAATGAGAAAAGAGGTGGGTAGGCTGTGAAACTGATGAAGACCCA
GTGTGGGCGTCCATTCATTCTCTTTGCTCTTGAATTAGAAATTCTCTGCTGGGATCTAGGGCCCTTA
GGA

- **Mouse APOA4 promoter sequence:**

GAGCTCGGGGAAGCTCGAGCCCTGTGGGGAGCCATGCAGTGCAGTGGGGCCCAGCAGAGGAGCA
CAGGTATCCAGCTGTCTTCAGTCCCATGAGACAAGCTAATCTGGACACATTTTAAAAAATGGATGG
CAACACAGCAAATCAGACTGGGCACAATCGTGGTCTATTCTAATGGCTGTCATTTCACAAATGCTG
TCTTGTGGATGGCAGTCAATGGGACAGTATGATGGATGCCCTCATCTAGTCCCTGGTGTGGTCCAC
TGAGGCTCCACACTGACCACAGCCTGGCATCTTGCCTGTGGATATCTGCTGCAATTGTATGTGTGG
ACACATGTGGAGTCTCAGTAGGAGACCTCAAAAAACTCACTTTCCACAGCAGTGTCTGTCACCTTC
TGTGGGGGGGGGGGGGGTGGGAAGAGAGAGAGAGGGAGAGAGGGAGAGAGAGAGGGAGAGAG
AGAGAGGAGTCACTCTGCATGGCTCTTGCATATGGCTGAGAACAGTGGGGCAGCAATCAAGCCTT
AGCCAGCCCTGCTCTCTCACTGTTGCCTCTAGCCCACTTGGTGACCCTCTGAGGGAAAGGGTGGCT
CTCCCTCTGCCACTGTCAGGAGAGGATCAGGTTCTCTCCTTCCTTCCTGTGCTGATGCACACAGAA
AATCATTGTCATTAATTTCAGCCCTTACTCTGGGCTAAGCTCCCTGCAGCCATCTCACAAGTACCAC
CTAATTTAATGTAACAAACTACACATTGTTCAAAAGAGAAACTTGAAGCTTCATGATAACTGGACG
GAGGTGAGCCAGCTTGACAGTCATGAGATACAAAGCCCACTATGATTAACTCCTTTGATCCTGGGT
TCTGATCCTCTCCTGACCAAGGGTATCACAGACACCTCAACTGAGGCTCACTGTCTGCTGCAGCCC
TATGCCATCTCTGGGCCTGGTACCATCTCTGTAGCTGATGTTCTGAGACAAAGTTCAGGTTGGTGG
CAGCTGTCAGACTGGTGGCTGTCTCACTGGGGTGGAAAGAGGAGACCTGGACCTTGTTCTCTCAGA
CTGGCACAGACCCAGGGCTGCCAACCGGGCCTCTGGGGCCTCAGTTCTGTTCAGGGACTCCCCTAG
ACTCCCAGGCTCATTCCTCCTGAAGTTTCTGGCTATCCTTCCCAGCCTCTTGGACAGGGTGGAGCCA
ACTCAAGAAGACTGCTTCCCTCTGCTGCCTGTGTGCTGTCAGCTTCCACGTTGTCTTAGGGCCACTA
AAGTCCAAGAGGCCTCCTGGGAGTGTGTCACCTTCCAACGTGGAGTCACACTGGGGAGGAGGCGG
GGAGAGAGGGCTGGAGGGGCTTTAAATGAGTGGCTGGCCTTGCCTGCAGTCAATCTGCACAGGGA
CACAGGTACACCGTTTCTTCTGACTCCGGGAAACATCCAGTGTAGCCGAAACTGTCCCAGCCCAGT
GAGGAGCCCAGGATGTTCCTGAAGGCTGCGGTGCTGACCCTGGCCCTGGTGGCCATCACCGGTGA

GTAGACACTGCACCTGGGAGGCAGCAAGAAAAGCCAGCTCTAGAACTGGCGGACAGCTCGGGGT
GGCCTTGTATTTGCCCAGCAGCTCATAGGAGAACAGGCCTTTGTTCTCCCTGGCACTTGTGCTCCCT
GGGTTATCCCAGGGATGGGGCAATGGTTTGGGTTATCCAAACTCCAACATTATCCAGCTCAGAGCT
GAGGCAGAGGGGCCAGGAGAGAGATGATCCTCATAAAGTTGCCTTCTGCTCTCTCTCTGCCCAGGC
ACCCGGGCTGAGGTCACTTCGGACCAGGTGGCCAATGTGGTGTGGGATTACTTTACCCAGCTAAGC
AACAATGCCAAGGAGGCTGTAGAACAG

Figure 23 Sequence of mouse il6 and APOA4 gene promoter retrieved using Genomatix software.

APPENDIX B

ChIP Primer sequence:

Negative control primer	Forward	CATGGATGTATGCTCCCGACT
	Reverse	GGAGCTCAGTCTGTGTCCAG
Il6	Forward	GGAGAGGAGTGTGTGTCTT
	Reverse	GCGCATGACAGACGACACA
ApoA4	Forward	CAGGGTCCAGCCAACTCAAG
	Reverse	CTCCACGTTCGAAGGTGACA

Quantitative RT-PCR primer sequence:

Name	Type	Sequence
Fgf21	Forward	GCTGCTGGAGGACGGTTACA
	Reverse	CACAGGTCCCCAGGATGTTG
Apoa4	Forward	AGCTTCCACGTTGTCTTAGGG
	Reverse	TGTGACTCCACGTTGGAAGG
Apob	Forward	CGTCTGGGCTCAAGATGAAGT
	Reverse	CTGGACACCGCTGGAACTTT
Bdh1	Forward	AGATGCGGCTAGTGGCAAAG
	Reverse	CAGTTCCTTGACCCCAGCAT
ApoA5	Forward	TCCTCGCAGTGTTCGCAAG
	Reverse	GAAGCTGCCTTTCAGGTTCTC
Apoc2	Forward	CTCTGCTGGGCACGGTGCA
	Reverse	GCCGCCGAGCTTTTGCTGTAC
ApoA1	Forward	AGCTGAACCTGAATCTCCTG
	Reverse	CAGAGAGTCTACGTGTGT
Pck1	Forward	CTCAGCTGCATAACGGTCTG
	Reverse	CTTCAGCTTGCGGATGACAC
Cpt1a	Forward	AGAATCTCATTGGCCACCAG
	Reverse	CAG GGTCTCACTCTCCTTGC
Stat3	Forward	AACGTCAGCGACTCAAACTG
	Reverse	CCCGTACCTGAAGACCAAGTT
Saa	Forward	CGGGACATGGAGCAGAGG
	Reverse	TTGCCACTCCGGCCC
Sap	Forward	TGTCTGGGATTGAGATCTTACAACA
	Reverse	CTGCCGCCTTGACCTCTTAC
Tlr2	Forward	CCATTGAGGGTACAGTCGTCG
	Reverse	GGCATTAAGTCTCCGGAATTATC
Tlr 3	Forward	AGCCTTATACCATAAAAG
	Reverse	CAGTTCAGAAAGAACGG
Tlr 4	Forward	GGAAGGACTATGTGATGTGAC
	Reverse	GCTCTTCTAGACCCATGAAATTGG
Ccl2	Forward	CACTCACCTGCTGCTACTCA
.	Reverse	GCTTGGTGACAAAAACTACAGC
Ccl3	Forward	CCATATGGAGCTGACACCCC
	Reverse	GTCAGGAAAATGACACCTGGC
Rplpo	Forward	AGACAAGGTGGGAGCCAGCGA
	Reverse	GCGGACACCCTCCAGAAAGCG
Actin	Forward	GATCTGGCACCACACCTTCT
	Reverse	GGGGTGTTGAAGGTCTCAAA
Il6	Forward	CCCAATTTCCAATGCTCTCCT
	Reverse	TGAATTGGATGGTCTTGGTCC
Tnfα	Forward	CCA ACG CCC TCC TGG CCA AC
	Reverse	GAG CAC GTA GTC GGG GCA GC

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ABSTRACT

ROLE OF CREBH IN ENDOTOXIN MEDIATED MODULATION OF HEPATIC METABOLISM

by

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Bacterial endotoxins can induce a variety of physiological changes in the host. This effect is not only restricted to inflammatory changes but also comprises metabolic changes in the host body. Lipopolysaccharide (LPS), one of the key components of the bacterial cell walls, is capable of triggering host metabolic changes. Hyperlipidemia usually accompanies with high endotoxin levels as well as inflammation. Lipid metabolism disorders are one of the common hallmarks of a patient with sepsis or high levels of endotoxin through diet. Previously, we have identified an endoplasmic reticulum (ER) anchored liver-specific transcription factor CREBH (cAMP-responsive element-binding protein, hepatocyte-specific), which is activated by ER stress, inflammatory stimuli, and metabolic signals. Proinflammatory cytokines TNFα, IL6, and IL1β, bacterial endotoxin lipopolysaccharide, insulin signal, saturated fatty acids, nutrient starvation, or atherogenic high-fat (AHF) feeding, can all induce expression and/or activation of CREBH in the liver. In this study, we demonstrate that CREBH acts a key player in mounting an acute phase response against endotoxemia by modulating apolipoproteins. Endotoxin LPS shock in the body induces activation of the TLR4 signaling pathway in mouse liver. Upon triggering TLR4 signaling pathway, LPS stimulates cleavage and activation of CREBH

transcription factor LPS induces the interaction between CREBH and TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase that plays a key role in mediating TLR signaling. While LPS-induced TRAF6-CREBH interaction relies on MyD88, TRAF6 mediates the ubiquitination of CREBH to facilitate CREBH activation upon LPS challenge. Functionally, CREBH directly activates expression of the gene encoding Apolipoprotein (Apo) A IV and IL6 under LPS challenge, leading to modulation of high-density lipoprotein (HDL) in animal models. In summary, my study suggested that TLR-dependent, LPS-induced CREBH activation may represent a host defense response to bacterial endotoxin by modulating apolipoproteins. Targeting the expression of CREBH under disease condition may represent a novel approach towards alleviating the sepsis-related complications.

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