INSULIN STIMULATION OF STEROL REGULATORY ELEMENT-BINDING PROTEIN-1C PROCESSING IN LIVER: LESSONS FROM TRANSGENIC RATS

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DEDICATION

To my wife, our families, and my mentors for all their help and support.

I am merely a dwarf standing on the shoulders of giants.

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INSULIN STIMULATION OF STEROL REGULATORY ELEMENT-BINDING PROTEIN-1C PROCESSING IN LIVER: LESSONS FROM TRANSGENIC RATS

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Transcriptional control of hepatic fatty acid (FA) and triglyceride (TG) synthesis is mediated by SREBP-1c, one of three sterol regulatory element-binding protein (SREBP) isoforms. SREBPs are endoplasmic reticulum (ER) membrane-bound transcription factors that require escort to the Golgi apparatus followed by proteolytic cleavage from the membrane. This process liberates the active transcription factor portion of SREBP to translocate to the nucleus to activate transcription of its target genes. SREBP-1c is unique among the SREBP isoforms since it is highly enriched in liver and is activated by insulin. Therefore the best cell culture model system to study SREBP-1c function is freshly isolated primary rat hepatocytes due to their tissue origin and insulin

sensitivity (cultured mouse hepatocytes lose insulin responsiveness). Using this model, insulin has been shown to activate the transcription of SREBP-1c, requiring mechanistic target of rapamycin complex 1 (mTORC1), but not its downstream target, p70 ribosomal protein S6 kinase (S6K). Insulin has also been implicated in the activation of proteolytic processing of SREBP-1c, but previous attempts to study this process have been confounded by the concomitant increase in SREBP-1c mRNA and precursor protein when primary hepatocytes are treated with insulin. To circumvent this problem, transgenic rats were created that express epitope-tagged human SREBP-1c under control of the apolipoprotein E (apoE) promoter/enhancer, which imparts constitutive, liver-specific expression to the transgene. Since the expression of the transgene is not regulated by insulin, the effect of insulin on the processing of SREBP-1c *per se* can now be studied. When hepatocytes isolated from transgenic rats were treated with insulin, the amount of cleaved nuclear SREBP-1c rapidly increased. Insulin-induced processing of SREBP-1c required both mTORC1 and S6K. This indicated a bifurcation in the insulin signal downstream of mTORC1, with SREBP-1c processing requiring S6K and SREBP-1c transcription bypassing this requirement. These findings have implications for the role of insulin in the control of hepatic FA and TG synthesis through SREBP-1c. This is relevant in states of insulin resistance and Type 2 diabetes where abnormally high levels of insulin cause pathologic accumulation of FAs and TGs.

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apoE – Apolipoprotein E

bHLH-Zip – Basic helix-loop-helix-leucine zipper

cAMP – 3'-5'-Cyclic adenosine monophosphate

ER – Endoplasmic reticulum

FA – Fatty acid

FFA – Free fatty acid

HA – Influenza hemagglutinin

IR – Insulin receptor

LXR – Liver X receptor

mTORC1 – Mechanistic target of rapamycin complex 1

nSREBP – Nuclear sterol regulatory element-binding protein

PEPCK – Phosphoenolpyruvate carboxykinase

RNAi – RNA interference

S1P – Site-1 protease

S2P – Site-2 protease

S6K – p70 ribosomal protein S6 kinase

SEM – Standard error of the mean

SREBP – Sterol regulatory element-binding protein

T2DM – Type 2 diabetes mellitus

TG – Triglyceride

VLDL – Very low-density lipoprotein

WT – Wild-type

CHAPTER ONE
Introduction

Insulin

Insulin, a peptide hormone produced in beta cells of the islets of Langerhans in the pancreas, is central to whole body metabolism. Insulin is secreted from the pancreas in response to high blood glucose, acting as a signal for nutritional abundance. Insulin exhibits wide-ranging systemic effects, including increasing all of the following: glucose uptake (e.g., muscle), glucose storage in the form of glycogen (e.g., liver and muscle), fatty acid (FA) and triglyceride (TG) synthesis as an alternative fuel (e.g., liver), and FA and TG storage (e.g., adipose) (Reaven, 1995).

Glucagon is a peptide hormone produced in alpha cells of the islets of Langerhans that acts in opposition to insulin. Glucagon is secreted from the pancreas in response to low blood glucose, acting as a signal for nutritional deficit. Systemic effects of glucagon include glycogen breakdown (glycogenolysis), fatty acid oxidation, and mobilization of stored FAs and TGs for catabolism (Unger and Cherrington, 2012).

Diabetes Mellitus

Diabetes mellitus (DM), the most common insulin disorder, is separated into two main types. Type 1 diabetes (about 10% of DM cases in the United States) generally exhibits sudden onset in childhood, resulting from an autoimmune destruction of pancreatic beta cells, causing hypoinsulinemia. This is life threatening unless insulin supplementation is provided. Type 2 diabetes (T2DM) is far more common (about 8% of

the United States population), largely exhibiting a gradual onset in adulthood (Gregg et al., 2009). The precise etiology of T2DM is not well understood, but it is clear that its early stages involve not a deficiency of insulin, but an "insulin resistance" characterized by insulin acting inefficiently at its target tissues (e.g., liver, muscle, adipose). Insulin resistance will initially be overcome via increased insulin secretion by pancreatic beta cells. However, beta cells eventually become exhausted and no longer produce the required levels of insulin, which may be life threatening unless insulin supplementation is provided (Reaven, 1995).

McGarry Hypothesis and the Vicious Cycle

Historically, DM has been viewed as a disorder of glucose metabolism. Researchers and clinicians have largely focused on the hyperglycemia rather than the other clinical manifestations of the disease, including alterations in lipid metabolism. This "glucocentric" view of DM was challenged by the late J. Dennis McGarry. Known as the McGarry Hypothesis (McGarry, 1992, 2002), Dr. McGarry asked the seemingly simple question, what if instead of focusing entirely on the hyperglycemia found in patients with DM, disturbances in lipid metabolism are also considered?

For instance, progression of T2DM begins with underlying target tissue insulin resistance. The precise initiating events are manifold, but include genetics, diet, and environment (Reaven, 1995). Initially, beta cells will increase insulin secretion (hyperinsulinemia) to compensate. Hyperinsulinemia subsequently leads to increased hepatic FA and TG synthesis, which are packaged and circulated in very low-density lipoprotein (VLDL) particles, and are deposited in target organs (e.g., muscle and

adipose). Increased intramuscular FAs and TGs decrease glucose uptake, augmenting muscle insulin resistance. This leads to further hyperinsulinemia by the pancreas and a decrease in muscle FA oxidation, which exacerbates the intramuscular FAs and TGs accumulation. While adipose tissue can initially expand to compensate for increased FA and TG deposition, it is eventually overwhelmed and free fatty acids (FFAs) are released into the bloodstream. Circulating FFAs will deposit in the liver (increasing hepatic glucose production), muscle (increasing intramuscular lipid content), and beta cells (initially leading to further hyperinsulinemia followed by beta cell failure). In total, these metabolic disturbances of lipid metabolism, not glucose metabolism, create a vicious cycle that, once initiated, is self-reinforcing and leads to serious DM progression and sequelae (McGarry, 1992, 2002).

Hepatic Selective Insulin Resistance

In this state of insulin resistance, the liver plays a key role in the pathogenesis of the T2DM clinical triad (hyperglycemia, hyperinsulinemia, and hypertriglyceridemia) and vicious cycle because it is not completely, but selectively insulin resistant (Brown and Goldstein, 2008). Under normal physiological conditions, insulin circulates to the liver via the portal vein, initiating two key transcriptional events. First, insulin stimulates the phosphorylation and nuclear exclusion of the transcription factor FoxO1. Since FoxO1 activates transcription of gluconeogenic genes [e.g., phosphoenolpyruvate carboxykinase (PEPCK)], hepatic glucose production will decrease (Brown and Goldstein, 2008).

The second major insulin action in liver is the activation of the transcription factor SREBP-1c, which activates transcription of genes required for FA and TG synthesis (e.g., fatty acid synthase). This increases hepatic FA and TG synthesis, which will be packaged into VLDL and delivered to adipose and muscle (Brown and Goldstein, 2008).

In patients with T2DM, the liver exhibits a selective insulin resistance: the FoxO1/gluconeogenic pathway becomes insulin resistant, leading to hyperglycemia in the face of hyperinsulinemia, a classical characteristic of DM. However, the SREBP-1c/lipogenic pathway remains sensitive to insulin (Shimomura et al., 1999a, 2000), leading to hypertriglyceridemia, the final component of the T2DM clinical triad. This selective metabolic derangement in the liver creates the vicious cycle present in T2DM.

From a molecular signaling perspective, the problem of hepatic selective insulin resistance begins at the cell surface with the insulin receptor (IR) where both signaling pathways for the repression of FoxO1 and the activation of SREBP-1c begin. Mice lacking the IR specifically in the liver exhibit a total insulin resistance: hyperglycemia in the face of hyperinsulinemia, without hypertriglyceridemia (Michael et al., 2000; Biddinger et al., 2008). Since both FoxO1 and SREBP-1c signaling pathways require the IR, at some point distal to the receptor both pathways must diverge such that only the FoxO1 pathway becomes resistant in T2DM.

SREBPs

The sterol regulatory element-binding proteins (SREBPs) are a unique class of basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors that are

synthesized as membrane-bound inactive precursors in the endoplasmic reticulum (ER) (Brown and Goldstein, 1997; Horton et al., 2002). The SREBPs (~1150 amino acids in length) have three domains: 1) a 480 amino acid N-terminal domain containing the bHLH-Zip DNA-binding region, 2) a hairpin domain consisting of two transmembrane segments and a short 30 amino acid ER luminal loop, and 3) a 590 amino acid C-terminal regulatory domain.

SREBP Pathway

Since SREBPs are transcription factors and must enter the nucleus to perform this function, they require release from the membrane—five other membrane-bound proteins coordinate this SREBP proteolytic processing. When SREBP is initially synthesized in the ER membrane, it immediately interacts with Scap, another ER membrane protein, through its C-terminal regulatory domain (Sakai et al., 1997). Scap acts as both an escort protein and a sterol sensor. When intracellular levels of cholesterol are low, the Scap/SREBP complex will bud in COPII-coated vesicles from the ER and move to fuse with the Golgi apparatus (Sun et al., 2007).

Once in the Golgi apparatus, the Scap/SREBP complex will contact two resident membrane-bound proteases and undergo a process termed regulated intramembrane proteolysis (Brown et al., 2000). Site-1 protease (S1P) first cleaves in the middle of the SREBP luminal hairpin loop, dividing the SREBP molecule in half. Site-2 protease (S2P) then cuts at a point three residues into the remaining transmembrane segment to completely release the SREBP N-terminal bHLH-Zip domain from the membrane. The SREBP bHLH-Zip domain then translocates to the nucleus where it will bind sterol

response elements to activate transcription of its target genes (Smith et al., 1990; Kim et al., 1995).

However, when intracellular sterol levels are elevated, Scap, acting as a sterol sensor, will undergo a conformational change that allows it to interact with either Insig-1 or Insig-2 (both intrinsic ER membrane proteins). Insig-1 and Insig-2 act redundantly to anchor the Scap/SREBP complex in the ER membrane, thus preventing it from being transported to the Golgi apparatus for proteolytic processing (Yabe et al., 2002; Sun et al., 2007; Brown and Goldstein, 2009).

SREBPs: Two Genes but Three Proteins

The mammalian genome encodes two SREBP genes: *srebp-1* and *srebp-2*. The *srebp-2* gene produces a single isoform that gives rise to SREBP-2 protein. However, through the use of alternative promoters, *srebp-1* produces SREBP-1a and -1c proteins that differ by the inclusion of alternative first exons (Brown and Goldstein, 1997; Horton et al., 2002).

The expression patterns and functions of the three SREBP isoforms have been studied in cell culture as well as *in vivo* using various knockout and transgenic animals. In the liver, SREBP-2 and SREBP-1c are the predominant isoforms expressed (compared to SREBP-1c, SREBP-1a is expressed 9-fold or 6-fold lower in mouse or human liver, respectively) (Shimomura et al., 1997b). By using transgenic mice that overexpress the truncated active nuclear transcription factor portion of each of the three SREBP isoforms (nSREBPs) specifically in the liver, the predominant role of each isoform was elucidated (Shimano et al., 1996; Horton et al., 1998b).

Overexpression of nSREBP-1c in mouse liver led to a 4-fold increase in FA synthesis and TG liver content, whereas there was no change in cholesterol synthesis or liver content. Overexpression of nSREBP-2 in mouse liver led not only to an increase in FA synthesis (4-fold), but predominantly to an increase in cholesterol synthesis (28-fold). Despite the fact that SREBP-1a is not expressed at high levels in liver, transgenic mice overexpressing SREBP-1a in liver were created and were shown to have increased cholesterol (5-fold) and FA (26-fold) synthesis. Taken together, it appears that SREBP-1 isoforms have a relative preference for activating FA synthesis, and SREBP-2 has a preference for activating cholesterol synthesis (Horton et al., 2002).

Transcriptional Regulation of SREBP-1c

SREBP-1c is unique among the SREBP isoforms due to its highly enriched liver expression pattern and its ability to be activated by insulin (Horton et al., 2002). SREBP-1c is regulated at both the transcriptional and post-translational levels by insulin. Three factors regulate the SREBP-1c transcriptional response: SREBP-1c itself, liver X receptors (LXRs), and the insulin-to-glucagon ratio. SREBP-1c, along with the other SREBP isoforms, can bind to its own promoter and activate its transcription in a feed-forward manner. However, SREBP-1c is the only isoform whose promoter contains LXR response elements. LXR α and β are nuclear hormone receptors that are activated by oxysterols, dimerize with retinoid X receptors on the SREBP-1c promoter, and activate SREBP-1c transcription (Chen et al., 2004).

The insulin-to-glucagon ratio also regulates SREBP-1c transcription. In isolated rat hepatocytes, insulin treatment increases both SREBP-1c mRNA and its target genes

(Foretz et al., 1999a; Shimomura et al., 1999b). The induction of SREBP-1c target genes is blocked if a dominant negative form of SREBP-1c is expressed (Foretz et al., 1999b). Acting in opposition to insulin, treatment of primary rat hepatocytes with either glucagon [which raises intracellular 3'-5'-cyclic adenosine monophosphate (cAMP) levels] or a cell-permeable cAMP analog (dibutyryl-cAMP) decreases SREBP-1c mRNA as well as its target genes (Foretz et al., 1999b; Shimomura et al., 2000).

In vivo when mice or rats are fasted, insulin levels fall, glucagon levels rise, and SREBP-1c mRNA and protein decreases. If these animals are then refed, insulin levels rise, glucagon levels fall, and SREBP-1c mRNA and protein increases. In both cases, SREBP-1c target genes follow the expression level of SREBP-1c (Horton et al., 1998a; Kim et al., 1998). In addition, when rats are treated with streptozotocin, which selectively ablates their beta cells thereby removing all insulin, SREBP-1c mRNA and protein levels fall; but when insulin is given back to the animal, SREBP-1c mRNA and protein levels return (Shimomura et al., 1999b).

Since the unique properties of SREBP-1c exist only in the liver, experimental studies on SREBP-1c must be performed in liver cells. The best known liver cell culture model system is freshly isolated primary rat hepatocytes (Berry and Friend, 1969). This system was adapted to dissect the insulin signaling cascade that leads to the induction of SREBP-1c transcription (Li et al., 2010). Using inhibitors of kinases at different points of the insulin signaling pathway, Li *et al.* determined two main findings. First, the mechanistic target of rapamycin complex 1 (mTORC1, previously referred to as the mammalian target of rapamycin complex 1) was a bifurcation point in the insulin signaling pathway in hepatocytes between insulin's suppression of gluconeogenesis and

activation of lipogenesis. When mTORC1 was inhibited by rapamycin, insulin was unable to activate the transcription of SREBP-1c mRNA but was still capable of suppressing expression of gluconeogenic genes (PEPCK).

After defining mTORC1 as an insulin signaling branch point towards lipogenesis, Li *et al.* tested the role of the next kinase immediately distal to it, p70 ribosomal protein S6 kinase (S6K). Inhibition of S6K with LYS6K2, a highly specific inhibitor of S6K, did not affect the insulin-stimulated induction of SREBP-1c mRNA. Therefore mTORC1 must phosphorylate another potentially novel kinase or non-kinase substrate that will then either continue to propagate the insulin signal or directly activate the transcription of *srebp-1c*.

Post-translational Regulation of SREBP-1c

Insulin also regulates SREBP-1c at the post-translational level of proteolytic processing, from inactive precursor to active nuclear form. As mentioned above, the regulation of SREBP processing was originally identified for cholesterol and other sterols, with high intracellular levels blocking the processing of all SREBPs. However, it has also been observed that insulin might selectively activate the proteolytic cleavage of the SREBP-1c isoform (Horton et al., 1998a; Shimomura et al., 1999b; Hegarty et al., 2005).

One of the challenges to studying insulin's effect on the processing of SREBP-1c is finding a suitable model system. As mentioned previously, primary rat hepatocytes are the best cell culture model for the study of SREBP-1c function (Berry and Friend, 1969; Li et al., 2010). Primary mouse hepatocytes are not useful for the study of SREBP-1c

function because they rapidly lose their insulin-responsiveness once removed from the whole animal (i.e., insulin is no longer capable of stimulating *srebp-1c* transcription). Others have tried cultured mouse hepatocytes (Takashima et al., 2009); hepatocellular carcinoma cells (Inoue et al., 2011; Li et al., 2011); fibroblasts, endothelial, and epithelial cells (Hannah et al., 2001; Demoulin et al., 2004; Zhou et al., 2004; Porstmann et al., 2005, 2008; Düvel et al., 2010; Lewis et al., 2011); cultured sebocytes (Smith et al., 2008); and liver and hepatocytes mostly by viral overexpression (Azzout-Marniche et al., 2000; Yellaturu et al., 2005, 2009; Takeuchi et al., 2010). All of these model systems suffered from one of the following limitations: non-hepatic cell type, required viral overexpression, or inability to dissociate transcription and processing of SREBP-1c.

This leads to the second challenge of studies on insulin and SREBP-1c processing: insulin activates both transcription and processing of SREBP-1c. Studying only the processing event in isolation from the transcriptional event has heretofore been technically difficult. Two previous approaches have been used to attempt to circumvent this problem (Hegarty et al., 2005). First, primary rat hepatocytes were pre-treated with an LXR agonist (T0901317) to induce SREBP-1c mRNA and precursor protein; then insulin was given acutely. The cells receiving the spike of insulin showed increased amount of cleaved nuclear SREBP-1 protein. The second approach was to utilize suckling (pre-weaned) mice that were shown to express SREBP-1 precursor protein while having very low levels of nuclear SREBP-1. Insulin, given to the suckling mice intraperitoneally, increased the amount of nuclear SREBP-1 compared to control injected mice. Both of these approaches have potential limitations inasmuch as the LXR agonist

may contribute to the insulin effect on SREBP-1c processing (Chen et al., 2004), and suckling rodents exhibit altered SREBP physiology (Botolin and Jump, 2003).

Transgenic Rats as a New Model for the Study of SREBP-1c Processing

In this work, another approach is taken. A line of transgenic rats was created that expresses full-length human SREBP-1c under the control of the apolipoprotein E (apoE) promoter/enhancer, imparting constitutive, liver-specific expression to the transgene. Since transgene expression is not regulated by insulin, the insulin-stimulated effects on SREBP-1c processing can be isolated from its effects on *srebp-1c* transcription. The transgene also contains an N-terminal influenza hemagglutinin (HA) tag, allowing the human SREBP-1c protein to be visualized via antibodies to the epitope. Freshly isolated primary rat hepatocytes as well as whole liver from these transgenic rats will be used to dissect the insulin-mediated signaling pathway required for the processing of SREBP-1c. These studies will help to further characterize the role of insulin in the control of hepatic FA and TG production, affecting whole body lipid metabolism in both normal and abnormal states such as insulin resistance and T2DM.

CHAPTER TWO
Insulin Stimulation of SREBP-1c Processing in Transgenic Rat Hepatocytes: Role of p70 S6 Kinase

Adapted from Owen JL, Zhang Y, Bae S-H, Farooqi MS, Liang G, Hammer RE, Goldstein JL, and Brown MS (2012), submitted to *Proceedings of the National Academy of Sciences of the USA*.

ABSTRACT

Insulin activates sterol regulatory element-binding protein-1c (SREBP-1c) in liver, thereby increasing fatty acid and triglyceride synthesis. Here, we created a line of transgenic rats that produce epitope-tagged human SREBP-1c in liver under control of the constitutive apolipoprotein E promoter/enhancer. This new system allows us to dissect the pathway by which insulin stimulates SREBP-1c processing without interference by the insulin-mediated increase in SREBP-1c mRNA. Rats are used because freshly isolated rat hepatocytes respond much more robustly to insulin than do mouse hepatocytes. The data reveal that insulin-mediated stimulation of SREBP-1c processing requires the mechanistic target of rapamycin complex 1 (mTORC1), which is also required for insulin-mediated SREBP-1c mRNA induction. However, in contrast to mRNA induction, insulin stimulation of SREBP-1c processing is blocked by an inhibitor of p70 ribosomal protein S6 kinase (S6K). The data indicate that the pathways for insulin enhancement of SREBP-1c mRNA and proteolytic processing diverge after mTORC1. Stimulation of processing requires the mTORC1 target S6K, whereas induction of mRNA bypasses this enzyme. Insulin stimulation of both processes is blocked by glucagon. The

transgenic rat system will be useful in further defining the molecular mechanism for insulin stimulation of lipid synthesis in liver in normal and diabetic states.

INTRODUCTION

The liver plays a unique role in lipid metabolism because it is the only organ that synthesizes fatty acids (FAs) and triglycerides (TGs) for export to other tissues. These synthetic processes are controlled reciprocally by insulin and glucagon, which are secreted by the pancreas and delivered directly to the liver via the portal vein. Precise control is important because excess TG synthesis leads to hypertriglyceridemia, which contributes to the peripheral insulin resistance and lipotoxicity seen in Type 2 diabetes. Excess TG synthesis also causes fatty liver, which sometimes leads to cirrhosis and liver failure (Browning and Horton, 2004; Leavens and Birnbaum, 2011).

Insulin stimulates FA synthesis in liver by increasing the mRNA and the processed nuclear form of sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that activates all of the genes needed to produce FAs and TGs in liver (Horton et al., 2002). Of the three SREBP isoforms, SREBP-1c is the one whose expression is highest in liver, and it is the only one that is primarily controlled by insulin. For this reason, definitive studies of insulin-mediated activation of SREBP-1c must be performed with liver cells.

Studies of insulin action on liver cells are difficult because none of the established hepatocyte cell lines responds to insulin with the robustness observed in the

livers of living animals. Moreover, freshly isolated hepatocytes lose their insulin responsiveness within 48-72 h after isolation. Therefore, studies must be performed in living animals or with freshly isolated hepatocytes less than 72-h old. Even more perplexing are species differences. Although mouse and rat livers manifest robust elevations in SREBP-1c mRNA when exposed to insulin *in vivo*, freshly isolated mouse hepatocytes show much less sensitivity to insulin than rat hepatocytes. Although numerous studies have dealt with regulation of SREBP mRNA and processing in various tissues (reviewed in (Krycer et al., 2010)), only a few have been carried out in freshly isolated rat hepatocytes (Foretz et al., 1999a; Shimomura et al., 1999b, 2000; Chen et al., 2004; Hegarty et al., 2005; Yellaturu et al., 2005; Li et al., 2010).

SREBP-1c, like the other two SREBP isoforms, is synthesized as a membrane-bound protein embedded in endoplasmic reticulum (ER) membranes (Brown and Goldstein, 1997). Immediately after synthesis, SREBP forms a complex with Scap, a polytopic membrane protein that facilitates incorporation of SREBP into COPII-coated vesicles that bud from the ER and fuse with the Golgi apparatus (Sun et al., 2007). There SREBP is processed by two proteases to liberate a soluble fragment that travels to the nucleus and activates transcription. Movement of the Scap/SREBP complex is inhibited by Insig proteins, intrinsic ER proteins that bind the Scap/SREBP complex and prevent its movement to the Golgi (Sun et al., 2007). The liver produces two Insig isoforms—Insig-1 and Insig-2, both of which retard the movement of Scap/SREBP complexes (Goldstein et al., 2006).

Insulin activates SREBP-1c in liver at two levels. It increases SREBP-1c processing to liberate the nuclear form, and it increases transcription of the SREBP-1c

gene, leading to increased SREBP-1c mRNA and precursor protein (Brown and Goldstein, 1997; Ferré and Foufelle, 2010). Under optimal conditions with freshly isolated rat hepatocytes in cell culture, insulin can induce SREBP-1c mRNA by as much as 40-fold within 6 h (Chen et al., 2004; Li et al., 2010). This increase is blocked by an inhibitor of phosphatidylinositol 3-kinase, wortmannin, an early enzyme in the insulin signaling cascade. The increase is also blocked by low concentrations of rapamycin, indicating that the mechanistic target of rapamycin complex 1 (mTORC1, previously referred to as the mammalian target of rapamycin complex 1) is also required. Importantly, the increase was not blocked by an inhibitor of p70 ribosomal protein S6 kinase (S6K), one of the major downstream targets of mTORC1, suggesting that another target of mTOR is involved (Li et al., 2010). Remarkably, rapamycin did not block the other major metabolic effect of insulin in liver—namely, the reduction in mRNAs encoding enzymes of gluconeogenesis. This finding indicated that the insulin signaling pathway diverges upstream of mTORC1—one pathway leading through mTORC1 to increase SREBP-1c mRNA, and the other bypassing mTORC1 and down-regulating gluconeogenic mRNAs (Li et al., 2010).

The other action of insulin on SREBP-1c—namely, the increase in proteolytic processing, has been difficult to study because the increase in SREBP-1c mRNA and precursor protein levels makes interpretation of an increase in nuclear SREBP-1c difficult to interpret. Hegarty, *et al.* attempted to circumvent this problem by first treating freshly isolated rat hepatocytes with an activator of liver X receptor (LXR), which increased SREBP-1c mRNA without stimulating proteolytic processing, and then adding insulin to stimulate processing (Hegarty et al., 2005). They found that the insulin stimulation of

processing was blocked by wortmannin. However, further advances with this approach may be limited because of the requirement for an LXR activator, which might contribute to insulin's action in stimulating SREBP-1c processing (Chen et al., 2004).

In the current study, we have taken an alternative approach. In order to eliminate transcriptional effects of insulin on the SREBP-1c gene, we generated a line of transgenic rats that produce epitope-tagged SREBP-1c driven by a human *apoE* promoter/enhancer expression cassette, which is not regulated by insulin. Inasmuch as rat hepatocytes give a dramatically more robust response to insulin than do mouse hepatocytes (unpublished data), we injected our transgene into fertilized eggs of rats instead of mice. When treated with insulin, freshly isolated hepatocytes from these transgenic rats exhibited a consistent increase in the amount of the transgene-encoded nuclear SREBP-1c, allowing further dissection of the responsible signaling mechanism.

RESULTS

Figure 2-1A shows the HA-tagged human SREBP-1c transgene that was used to generate the transgenic rats. Expression was mediated by the *apoE* promoter and its hepatic control region, which maximizes expression in hepatocytes. The transgenic rat line, hereafter designated *TgHA-hSREBP-1c*, was maintained as hemizygotes by breeding with wild-type (WT) Sprague-Dawley rats. In the liver, the amount of human SREBP-1c mRNA from the transgene was approximately 2.5 times that of the endogenous rat

SREBP-1c mRNA when the *ad lib*-fed animals were euthanized 6 h into the dark cycle (Figure 2-1B). Lower levels of transgene expression were seen in brain, spleen, and lung.

In WT rats, as observed previously (Li et al., 2010), the amount of SREBP-1c mRNA declined dramatically after a 48-h fast and increased markedly after the animals had been refed with a high carbohydrate diet for 6 h (Figure 2-2A, left panel). Nearly identical changes were observed when endogenous SREBP-1c mRNA was measured in transgenic rats. In marked contrast, the transgene mRNA did not decline significantly when the animals were fasted, nor did it rise when they were refed (Figure 2-2A, right panel). SREBP-1c protein levels were measured by immunoblots (Figure 2-2B). The precursor and nuclear forms of endogenous SREBP-1c declined dramatically during fasting and were restored by refeeding in transgenic rats as well as in WT animals (lanes 1-6). Immunoblots of the SREBP-1c precursor generated by the transgene revealed little decrease on fasting (lanes 4-6). Nevertheless, the nuclear form was dramatically reduced, suggesting a decrease in proteolytic processing. Refeeding produced an increase in the nuclear form, again without a change in the precursor, suggesting that proteolytic processing was activated.

To determine whether insulin mediates the increase in SREBP-1c processing, we used fresh hepatocytes from the transgenic rats (Figure 2-3). When the cells were exposed to 100 nM insulin, the increase in nuclear SREBP-1c was maximal within 15 min, and the nuclear protein remained elevated through 6 h (Figure 2-3A). Similar results were obtained with insulin concentrations as low as 10 nM. Addition of glucagon decreased the amount of insulin-induced nuclear SREBP-1c, as determined by densitometric staining of immunoblots (Figure 2-3B). Glucagon also decreased the basal

level of nuclear SREBP-1c in the absence of insulin. In experiments not shown, we also observed that forskolin, a direct activator of cyclic AMP, markedly inhibited insulin stimulation of SREBP-1c processing at a concentration of 10 μM.

Nuclear SREBPs are known to be degraded rapidly by proteasomal proteolysis (Wang et al., 1994). The rapidity of the insulin effect (Figure 2-3A) suggests that insulin acts by increasing the production of the nuclear form rather than blocking its degradation. To test this conclusion in another way, we made use of the proteasome inhibitor MG132 (Figures 2-3C and D). In the absence of MG132, addition of insulin to the transgenic hepatocytes produced a 6-fold increase in nuclear SREBP-1c as determined by densitometric scans. Addition of MG132 also caused an increase in nuclear SREBP-1c, presumably by preventing degradation. However, the amount of the nuclear protein rose further when insulin was added, even when the nuclear protein was stabilized by MG132, indicating that insulin acts by increasing production of the nuclear form rather than blocking degradation.

To dissect the insulin signaling pathway that mediates the increase in SREBP-1c processing in transgenic hepatocytes, we used the three inhibitors shown in Figure 2-4. In the absence of inhibitors, insulin increased the amount of nuclear SREBP-1c (lane 2). As expected, this increase was blocked by wortmannin (lane 3). It was also blocked by rapamycin (lane 4). The increase in SREBP-1c processing was inhibited by LYS6K2, an inhibitor of S6K that blocked the phosphorylation of S6 (lane 5). The result with LYS6K2 was unexpected because we reported previously that LYS6K2 did not block the insulin-induced increase of SREBP-1c mRNA (Li et al., 2010). As a control, neither rapamycin nor LYS6K2 blocked the insulin-induced phosphorylation of Akt.

Figure 2-5 shows a compilation of results from 10 independent experiments with rapamycin and 8 with LYS6K2, all of which found that both inhibitors blocked the insulin-induced processing of transgene-encoded SREBP-1c. To confirm the failure of LYS6K2 to block the mRNA induction of SREBP-1c, we used the conditions of Li, *et al.* (Li et al., 2010). Hepatocytes from the transgenic rats were pretreated for 30 min with vehicle, rapamycin, or LYS6K2. The cells were then incubated for 6 h in the absence or presence of 30 nM insulin. Cells were harvested and the amount of endogenous SREBP-1c mRNA was quantified by real-time PCR. Nuclear extracts were prepared from identically treated cells and used for measurement of nuclear SREBP-1c derived from the transgene as determined by SDS-PAGE, immunoblotting, and densitometric scanning (Figure 2-6). After 6 h, insulin increased the amount of endogenous SREBP-1c mRNA by 14-fold. Rapamycin reduced this increase by 85%, but LYS6K2 had no effect. In similarly treated cells, insulin over 6 h increased transgene-encoded nuclear SREBP-1c by 12-fold. Rapamycin blocked this increase by 64% and LYS6K2 reduced it by 74%. Thus, the LYS6K2 inhibition was specific for protein processing and not for mRNA induction.

To provide further evidence that rapamycin and LYS6K2 were blocking processing by inhibiting their respective enzymes, we performed dose response curves with the inhibitors and measured phospho-S6 and nuclear SREBP-1c in the same cells (Figure 2-7). Within the range of precision of these immunoblotting assays, the degree of inhibition of SREBP-1c processing by rapamycin and LYS6K2 was well correlated with the degree of reduction of phospho-S6, supporting the notion that the two inhibitors are

working by blocking the activity of S6K (Figures 2-7C and D). Again, insulin-induced phosphorylation of Akt was not blocked by either of the inhibitors.

Figure 2-8 shows an *in vivo* experiment designed to determine whether rapamycin blocks the increase in SREBP-1c processing in livers of rats after refeeding. Transgenic rats were fasted for 48 h and then refed for 3 h. One hour prior to refeeding, they were injected intraperitoneally with vehicle or with rapamycin. Refeeding increased the amount of nuclear SREBP-1c derived from the transgene as well as the endogenous gene (Figure 2-8A). Both of these feeding-induced increases were decreased by rapamycin. As previously reported (Li et al., 2010), rapamycin also prevented the refeeding-induced increase in endogenous SREBP-1c mRNA (Figure 2-8B). As a positive control, rapamycin prevented the increase in phospho-S6 that was induced by refeeding (Figure 2-8A). After refeeding, plasma insulin rose equally in vehicle and rapamycin-treated rats (Figure 2-8C), indicating that the rapamycin-mediated inhibition of SREBP-1c processing and transcription were not the result of decreased availability of insulin.

DISCUSSION

The pathways for insulin signaling in liver are complex. mTORC1 plays a central role, but it is embedded in an intricate web of feed-forward loops, feedback loops, bypass loops, and negative regulators of negative regulators whose interactions render definitive study difficult (for an illustration of this complexity, see Fig. 2 in Laplante and Sabatini

(Laplante and Sabatini, 2012)). Moreover, insulin signaling pathways differ in different cell types, and they are impacted by the actions of other hormones. For this reason, our laboratory (Shimomura et al., 1999b, 2000; Chen et al., 2004; Li et al., 2010) and others (Foretz et al., 1999a; Hegarty et al., 2005; Yellaturu et al., 2005) have focused on rat hepatocytes, which show robust responses to insulin both in living animals and immediately after their isolation from cell culture. In the current paper, we introduce a new system for studying insulin stimulation of SREBP-1c processing—namely, a transgenic rat expressing human SREBP-1c mRNA in liver via a promoter/enhancer that is not regulated by insulin.

Earlier studies of mRNA expression in rat hepatocytes revealed a bifurcation in the insulin pathway in liver (Li et al., 2010). One branch leads through mTORC1 and activates lipid synthesis through an increase in SREBP-1c, and the other branch bypasses mTORC1 and leads to a reduction in gluconeogenesis mediated by a reduction in the activity of Foxo1 (Lu et al., 2012). Here, we show that the branch leading to increased SREBP-1c itself bifurcates distal to mTORC1. This latter bifurcation is illustrated in Figure 2-9. One pathway leads to an increase in SREBP-1c mRNA and SREBP-1c precursor protein. This action is not inhibited by the S6K inhibitor and therefore is independent of S6K. The other pathway leads to an increase in the proteolytic processing of the SREBP-1c precursor protein. This pathway is inhibited in parallel with the decline in phospho-S6 when S6K is blocked by LYS6K2 (Figures 2-4 and 2-7). The SREBP-1c mRNA induction and the increase in SREBP-1c processing are both blocked by low concentrations of rapamycin, indicating that mTORC1 is essential for both processes.

Both pathways are also blocked by glucagon, acting through cyclic AMP (Figure 2-3B) (Shimomura et al., 2000; Ferré and Foufelle, 2010).

Although mTORC1 is essential for SREBP-1c activation in liver, it is not sufficient. This finding was reported initially by Yecies, *et al.* who found that SREBP-1c mRNA is not elevated in livers of mice with genetic ablation of TSC1, a negative regulator of mTORC1, even though these animals have markedly increased mTORC1 activity (Yecies et al., 2011). Thus, insulin must also regulate another process in addition to activation of mTORC1, and activation of both processes is required for induction of SREBP-1c mRNA.

The advantage of the current transgenic rat model is illustrated by the data in Figure 2-2B. When WT rats were fasted, their SREBP-1c mRNA declined dramatically, and as a result the SREBP-1c precursor protein was not detectable. When the animals were refed, the SREBP-1c mRNA increased and so did the precursor and nuclear forms of the protein (Figure 2-2). From such an experiment, it is impossible to tell whether insulin increases SREBP-1c processing or whether it acts only to increase the amount of the SREBP-1c precursor, whereupon processing takes place through some constitutive mechanism. A similar problem was encountered by Bae, *et al.*, who demonstrated clearly that knockdown of S6K by RNA interference in mouse liver reduced nuclear SREBP-1c after refeeding, but they could not distinguish an effect on processing versus an effect on mRNA induction (Bae et al., 2012). When encoded in rats by the transgene, SREBP-1c mRNA was present even in fasted animals, as was the precursor protein. However, there was very little nuclear form, indicating that processing was blocked. Refeeding increased

the nuclear form without increasing the amount of precursor, indicating that the processing of SREBP-1c was enhanced (Figure 2-2).

Inasmuch as nuclear SREBPs are known to be degraded rapidly by proteasomes, we considered the possibility that insulin stabilized the nuclear form rather than increasing its production. Two observations argued against this possibility. First, insulin increased the steady-state level of nuclear SREBP-1c by 4 to 5-fold within 15 min (Figure 2-3A), which is much too fast to be attributed to stabilization. Second, insulin increased the amount of nuclear SREBP-1c even when degradation was blocked by a proteasome inhibitor (Figure 2-3C).

In addition to causing a spike in plasma insulin, refeeding also causes a decline in glucagon (Unger and Cherrington, 2012). We earlier showed that glucagon blocks the insulin induction of SREBP-1c mRNA in rat hepatocytes (Shimomura et al., 2000). In the current study, we show that glucagon also prevents the insulin-mediated increase of SREBP-1c processing in transgenic hepatocytes (Figure 2-3B). Thus, in refed WT animals the dramatic increase in nuclear SREBP-1c represents the combined effects of insulin increase and glucagon reduction.

The transgenic rat model offers the promise of a system that should allow for a more careful dissection of the mechanism by which insulin activates SREBP-1c processing. In particular, it will be necessary to define the presumptive target of S6K that leads to the liberation of the Scap/SREBP-1c complex from the ER.

MATERIALS AND METHODS

Materials

We obtained powdered bovine insulin from Sigma (No. I6634) and protein kinase inhibitors from sources as previously described (Li et al., 2010). A stock solution of 0.1 mM insulin was prepared by dissolving the powder in 1% (v/v) acetic acid, after which the solution was stored in multiple aliquots at -80° C. All kinase inhibitors were prepared in dimethyl sulfoxide and stored at -20° C. Culture media, fetal calf serum, and collagen-coated dishes were obtained from sources as described (Li et al., 2010).

Generation of Transgenic Rats

To generate transgenic rats expressing full-length human SREBP-1c in the liver, we used a pLiv-11 vector that contains the constitutive human *apoE* promoter and its hepatic control region (Engelking et al., 2004). The transgenic plasmid (pLiv-11-HA-hSREBP-1c) was generated by cloning a cDNA fragment encoding the open reading frame of human *SREBP-1c* with an N-terminal 3xHA-tag into *Mlu*I-*Cla*I sites of pLiv-11. The 11-kb *Sal*I-*Spe*I fragment of pLiv-11-HA-hSREBP-1c was then isolated and injected into the pronucleus of Sprague–Dawley rat eggs as described (Young et al., 1999). Transgenic founders were identified by dot blot analysis and mated with WT Sprague–Dawley rats. To genotype transgenic rats, ear punch DNA was prepared with a direct lysis kit (Viagen Biotech Inc.) and used for PCR with the following primers:
5′ GTGCTGGGATTAGGCTGTTGCAGATAATGC-3′ and

5′ GGTACATCTTCAATGGAGTGGGTGCAGGCT-3′. Ear punch DNA of transgenic rats produced a PCR product of 527 bp. The transgenic rats, hereafter designated *TgHA-hSREBP-1c*, were maintained as hemizygotes by breeding with WT Sprague-Dawley rats. Two independent lines were established, both exhibiting a 2-3 fold overexpression of hepatic HA-hSREBP-1c mRNA relative to the endogenous SREBP-1c mRNA. All rats were housed in colony cages with a 12-h light/12-h dark cycle and fed Teklad Rodent Diet 2016 (Harlan Teklad). Prior to obtaining blood and liver, rats were anesthetized in a bell-jar atmosphere containing isoflurane. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center.

Fasting and Refeeding Studies

The rats were divided into three groups: nonfasted, fasted, and refed. The nonfasted group was fed a chow diet *ad libitum*. The fasted group was fasted for 48 h, and the refed group was fasted for 48 h and then refed a high-carbohydrate, fat-free diet (MP Biomedicals, No. 960238) for 3 or 6 h prior to study. Feeding regimens were carried out in a staggered fashion such that all rats were sacrificed at the same time. In one experiment (Figure 2-8), rats were injected intraperitoneally with either vehicle alone (14% ethanol, 5% (v/v) Tween 80, and 5% (v/v) polyethylene glycol 400) or vehicle containing 20 mg/kg rapamycin. Plasma insulin levels were measured with a commercial ELISA kit (Crystal Chem). The plasma was obtained from blood drawn from the inferior vena cava, which was collected on ice in EDTA-coated tubes, separated by centrifugation, and stored at -20° C.

Primary Rat Hepatocytes

Primary hepatocytes were isolated from nonfasted rats (male, 2-3 month-old) and cultured as previously described (Li et al., 2010) with the following minor modifications. On day 0, isolated hepatocytes were plated onto collagen-I-coated dishes ($1x10^7$ cells/10-cm dish) in 10 ml medium A (medium 199 supplemented with 5% (v/v) fetal calf serum, 100 nM dexamethasone, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After incubation for 3-4 h at 37° C in 5% CO2, the attached cells were washed once with phosphate-buffered saline and then incubated at 37° C overnight in medium B (medium A without any fetal calf serum). Unless noted otherwise, on day 1 cells were pretreated for 2 h with a 10-μl direct addition of dimethyl sulfoxide with or without the indicated protein kinase inhibitor, after which the cells were treated with a 10-μl direct addition of acidified water with or without insulin. After incubation for the indicated time at 37° C in 5% CO_2, the cells were harvested for immunoblot and RNA analysis.

Immunoblot Analysis

For each treatment, three 10-cm dishes of hepatocytes were pooled for subcellular fractionation. The cells were scraped on ice in 1 ml buffer containing 20 mM Tris-HCl at pH 7.4, 2 mM $MgCl_2$, 0.25 mM sucrose, 10 mM sodium EDTA, and 10 mM sodium EGTA. The buffer was supplemented with protease inhibitor cocktail (consisting of 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Pefabloc, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml N-acetyl-leu-leu-norleucinal, and 10 μg/ml aprotinin) and phosphatase inhibitor cocktail sets I and II (Calbiochem, No. 524624 and 524625). The scraped cells were then homogenized using a 10-mm x 105-mm saw-tooth

generator (PRO200, PRO Scientific) for 12 s at setting 2-3. The cell lysates were centrifuged at 1000g for 5 min at 4° C, after which the resulting supernatant was centrifuged at 10^5g for 30 min at 4° C in a Sorvall AT2-120 rotor to separate cytosol (10^5g supernatant) and membrane (10^5g pellet) fractions. The membrane pellet was resuspended in 0.4 ml SDS-lysis buffer (10 mM Tris-HCl at pH 6.8, 1% (w/v) SDS, 100 mM NaCl, 1 mM sodium EDTA, and 1 mM sodium EGTA) supplemented with the same protease and phosphatase inhibitor cocktails as above. To prepare the nuclear extract fraction, the 1000g pellet was resuspended in nuclear extract buffer containing 20 mM HEPES-NaOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, and 1 mM sodium EGTA supplemented with protease and phosphatase inhibitor cocktails. Aliquots from each of the above fractions were measured for protein concentration by the BCA kit (Pierce). Protein samples were diluted to the same concentration after addition of 4X SDS loading buffer (0.15 mM Tris-HCl at pH 6.8, 12% (w/v) SDS, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol, and 6% (v/v) β-mercaptoethanol). The samples were then boiled at 95° C for 5 min and subjected to SDS-PAGE (8% for membrane and nuclear fractions; 12% for cytosol). Immunoblot analysis for SREBP-1c and protein kinases were done as described by Shimomura, *et al.* (Shimomura et al., 1999b) and Li, *et al.* (Li et al., 2010), respectively. The following antibodies were used: 5 μg/ml rabbit anti-rat SREBP-1 (Shimomura et al., 1999b); anti-HA (1:4000 dilution; Bethyl Laboratories, No. A190-108A); Akt (1:2000; Cell Signaling, No. 9272); phospho-Ser473-Akt (1:2000; Cell Signaling, No. 4060); ribosomal protein S6 (1:2000; Cell Signaling. No. 2217); and phospho-Ser235/236-ribosomal protein S6

(1:2000; Cell Signaling, No. 2211). Gels for immunoblots were scanned and quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Quantitative real-time PCR

Total RNA was prepared from tissues or hepatocytes and subjected to real-time PCR analysis with cyclophilin as the invariant control as previously described (Li et al., 2010).

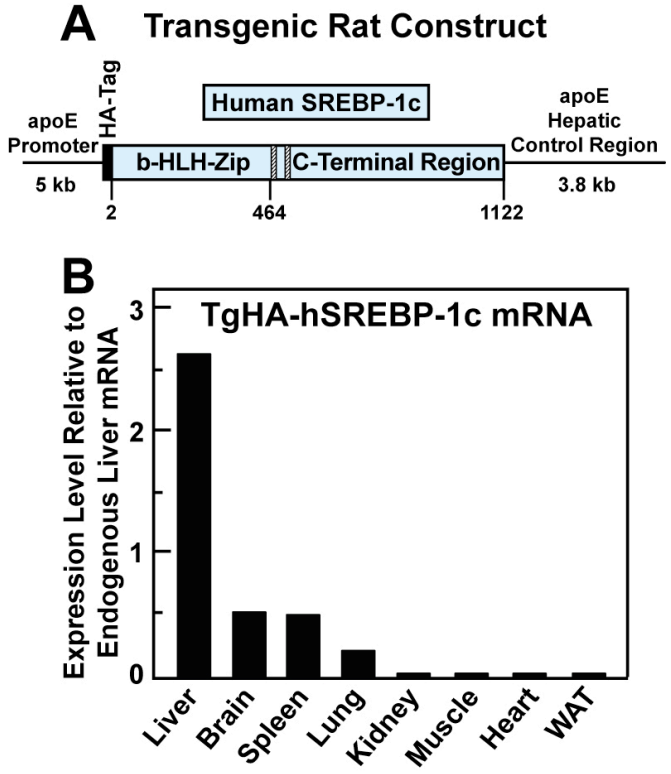


Figure 2-1. Generation of transgenic rats expressing HA-tagged full-length human SREBP-1c in liver. (A) The transgene construct contains a cDNA fragment encoding HA-tagged full-length human SREBP-1c (amino acids 2-1122) under control of the human *apoE* promoter and its hepatic control region. (B) Tissue distribution of human *HA-hSREBP-1c* transgene. Male *TgHA-hSREBP-1c* rats (2-3 month-old) that were fed a chow diet *ad libitum* were euthanized 6 h into the dark cycle. Equal amounts of total RNA from the indicated tissues of 4 transgenic rats were pooled and subjected to real-time PCR. Each value represents the amount of transgenic human SREBP-1c mRNA in the indicated tissue relative to that of endogenous rat SREBP-1c mRNA in the liver, which is arbitrarily defined as 1. The C_T values for endogenous and transgenic SREBP-1c in the liver were 24.4 and 23.0, respectively.

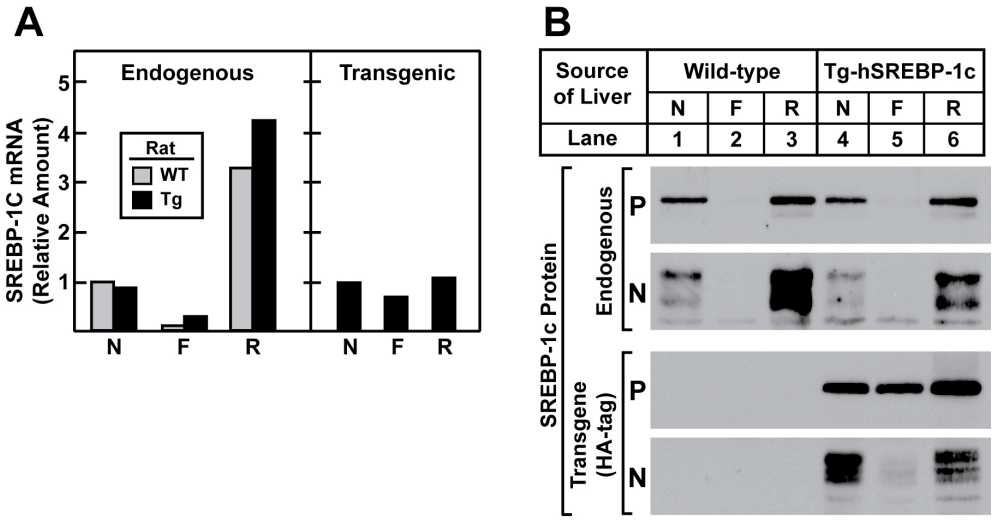


Figure 2-2. Effect of fasting and refeeding on the hepatic levels of endogenous and transgenic SREBP-1c mRNAs and proteins in WT and *TgHA-hSREBP-1c* rats. Male WT and transgenic *TgHA-hSREBP-1c* (Tg) littermates (2-3 month-old, 4 rats/group) were subjected to fasting and refeeding as described in Materials and Methods. The nonfasted group (N) was fed a chow diet *ad libitum*, the fasted group (F) was fasted for 48 h, and the refed group (R) was fasted for 48 h and refed a high-carbohydrate diet for 6 h prior to study. (A) Equal amounts of total RNA from livers of 4 rats were pooled and subjected to real-time PCR. For the endogenous rat SREBP-1c mRNA, each value represents the amount relative to that in the nonfasted WT rats, which was arbitrarily defined as 1 (C_T value, 24.2). For the transgenic human SREBP-1c mRNA, the level in the nonfasted transgenic rats was arbitrarily defined as 1 (C_T value, 23.2). (B) Liver membrane and nuclear extract fractions were prepared individually and pooled from 4 rats per group. Aliquots of membrane (30 μg) and nuclear extract (30 μg) fractions were subjected to 8% SDS-PAGE and immunoblot analysis with both anti-rat SREBP-1 and anti-HA antibodies to detect membrane-bound precursor (P) and cleaved nuclear (N) forms of endogenous rat SREBP-1 and HA-tagged transgenic human SREBP-1c, respectively. Gels were exposed to film for 60 s (endogenous N panel) or 1-10 s (other 3 panels).

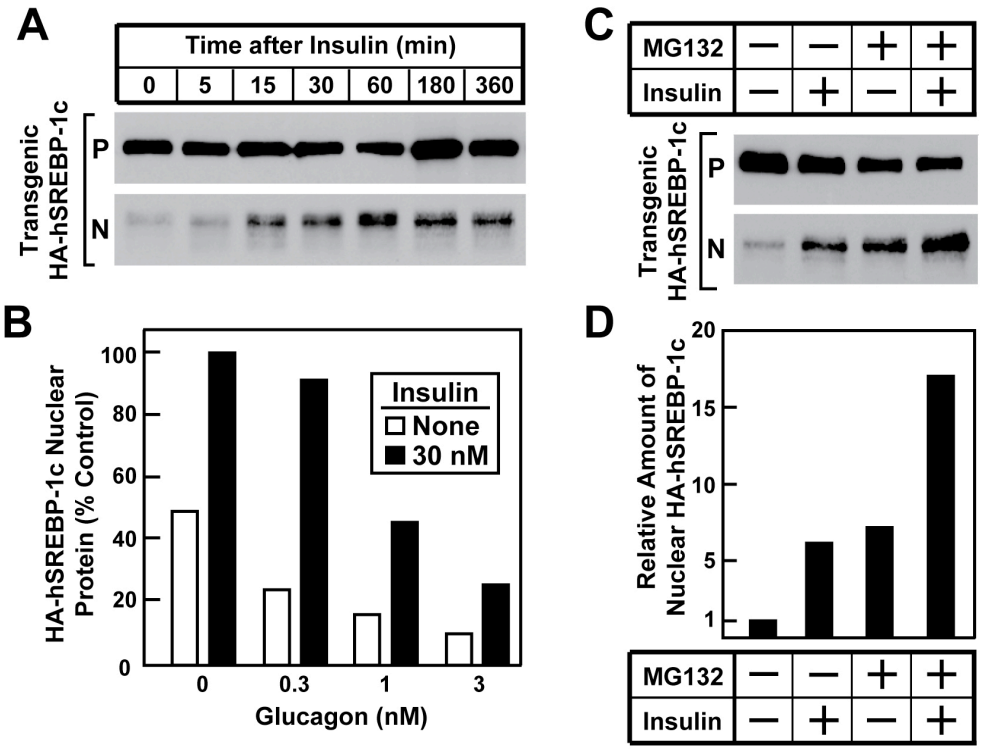


Figure 2-3. Insulin-mediated stimulation of proteolytic processing of transgenic SREBP-1c in primary rat hepatocytes. Hepatocytes from nonfasted *TgHA-hSREBP-1c* rats were prepared and plated on day 0 as described in Materials and Methods. (A) Time course of insulin effect. On day 1, the cells were treated with or without 100 nM insulin for the indicated time, harvested, and then pooled (3 dishes of cells/sample) for immunoblot analysis of precursor (P) and nuclear (N) forms of transgenic HA-hSREBP-1c protein. The gels were exposed to film for 5 s. (B) Effect of glucagon. On day 1, cells were pretreated for 2 h with or without the indicated concentration of glucagon, after which the cells were treated with or without 30 nM insulin for 1 h prior to harvest as above. Immunoblot analysis of nuclear transgenic HA-hSREBP-1c was carried out and then scanned and quantified by densitometry as described in Materials and Methods. The amount of the cleaved nuclear HA-hSREBP-1c protein in the cells without any treatment was arbitrarily set at 1. (C,D) Effect of proteasomal inhibitor. On day 1, hepatocytes were treated with or without 10 nM insulin for 1 h in the absence or presence of 10 μM MG132 (proteasomal inhibitor) and then harvested for immunoblot analysis as in A. The gels were exposed to film for 5-10 s (C). The gel of nuclear extract fractions (N) in C was scanned and quantified by densitometry as in B, with the amount of nuclear HA-hSREBP-1c protein in cells without any treatment arbitrarily set at 1 (D).

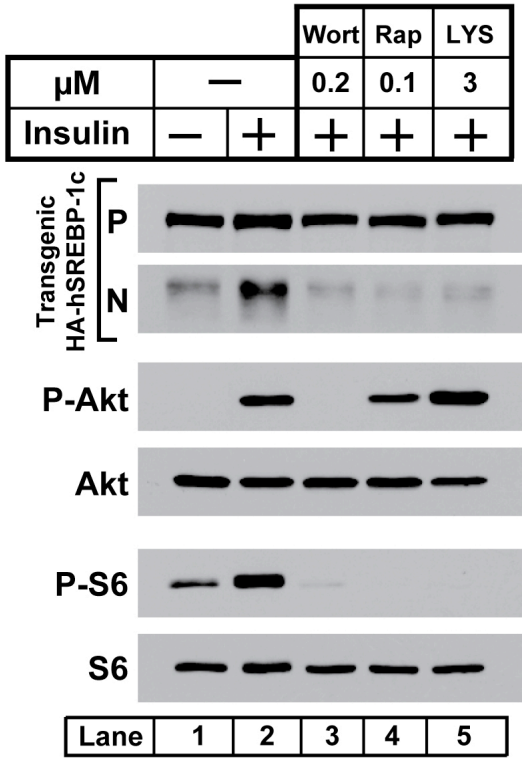


Figure 2-4. Effects of protein kinase inhibitors on insulin-stimulated proteolytic processing of transgenic SREBP-1c in rat hepatocytes. Hepatocytes from nonfasted *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 3 h with or without the indicated protein kinase inhibitor: 0.2 μM wortmannin (lane 3), 0.1 μM rapamycin (lane 4), or 3 μM LYS6K2 (lane 5). After this preincubation, the cells were treated with or without 10 nM insulin for 30 min, harvested, and then pooled (3 dishes of cells/sample). Each sample was subjected to immunoblot analyses of the following proteins: precursor (P) and nuclear (N) forms of transgenic HA-hSREBP-1c, phosphorylated Akt (P-Akt), total Akt, phosphorylated ribosomal protein S6 (P-S6), and total S6. Gels were exposed to film for 3-25 s.

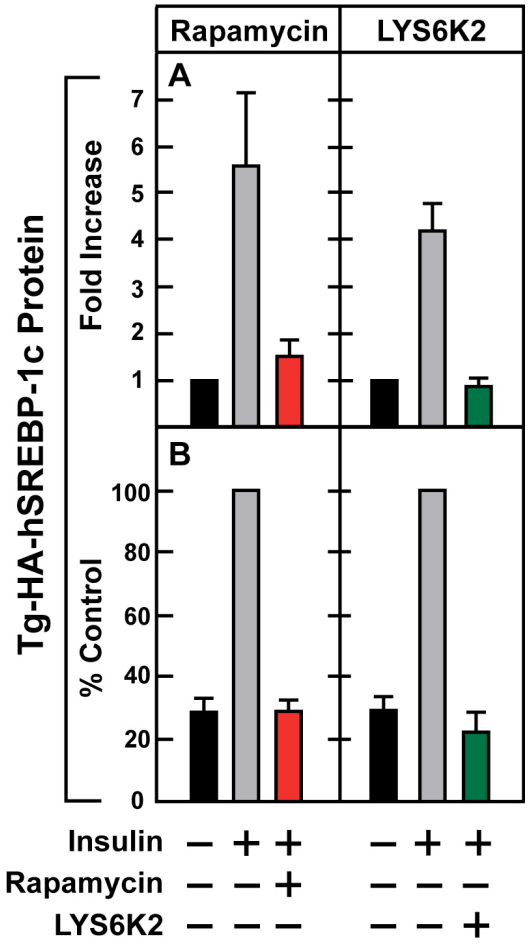


Figure 2-5. Compilation of multiple experiments demonstrating that mTORC1 and S6K are required for insulin to stimulate SREBP-1c proteolytic processing in rat hepatocytes. Ten (rapamycin) or 8 (LYS6K2) independent primary hepatocyte experiments from *TgHA-hSREBP-1c* rats were performed. Hepatocytes from nonfasted *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 2 h with or without 0.1 μM rapamycin or 3 μM LYS6K2, after which the cells were treated with or without 30 nM insulin for 1 h, harvested, and then pooled (3 dishes of cells/sample) for immunoblot analysis as in Figure 2-4. Nuclear HA-hSREBP-1c protein gels were scanned and quantified by densitometry. (A) The amount of nuclear HA-hSREBP-1c protein from cells treated without insulin (black bars) was arbitrarily set at 1. (B) The amount of nuclear HA-hSREBP-1c protein from cells treated with insulin alone (grey bars) was arbitrarily set at 100 percent. Each error bar in A and B represents the mean ± SEM value of measurements obtained from 10 (rapamycin) or 8 (LYS6K2) independent experiments.

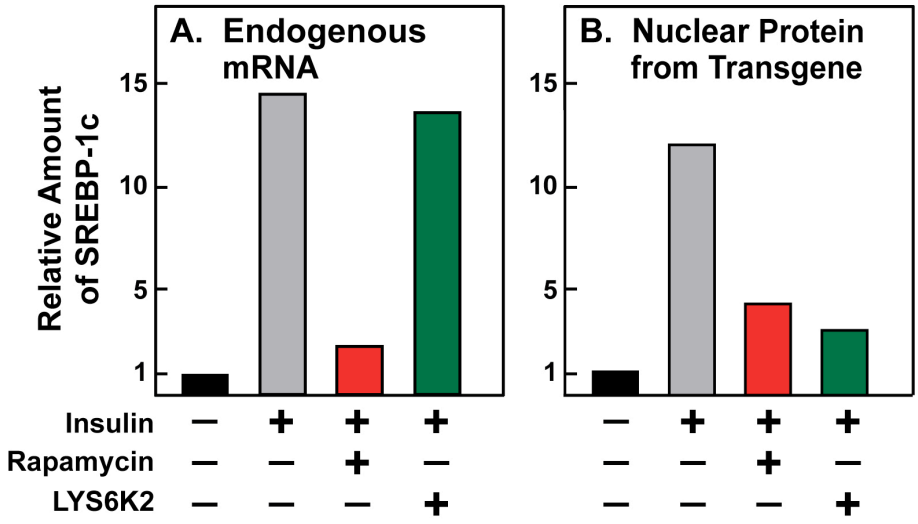


Figure 2-6. Differential effects of mTORC1 and S6K inhibition on insulin-stimulated transcription (A) and proteolytic processing (B) of SREBP-1c in rat hepatocytes. Hepatocytes from nonfasted *TgHA-hSREBP-1c* rats were prepared and plated on day 0. (A) Endogenous rat SREBP-1c mRNA. On day 1, cells were pretreated with or without 0.1 μM rapamycin or 3 μM LYS6K2 for 30 min, after which the cells were treated with or without 30 nM insulin for 6 h prior to study. Endogenous rat SREBP-1c mRNA levels were determined by real-time PCR from equal amounts of total RNA pooled from 3 dishes. Each value represents the amount of rat SREBP-1c mRNA relative to that in cells without any treatment, which was arbitrarily defined as 1. The C_T value for endogenous SREBP-1c mRNA in cells without any treatment was 26.4. (B) Transgene-encoded nuclear SREBP-1c protein. On day 1, cells were pretreated with or without 0.1 μM rapamycin or 3 μM LYS6K2 for 2 h, after which the cells were treated with or without 30 nM insulin for 6 h prior to study. The relative amounts of transgene-encoded nuclear HA-hSREBP-1c were determined by immunoblot analysis and quantified by densitometry. The amount of nuclear HA-hSREBP-1c protein in cells without any treatment (black bars) was arbitrarily set at 1.

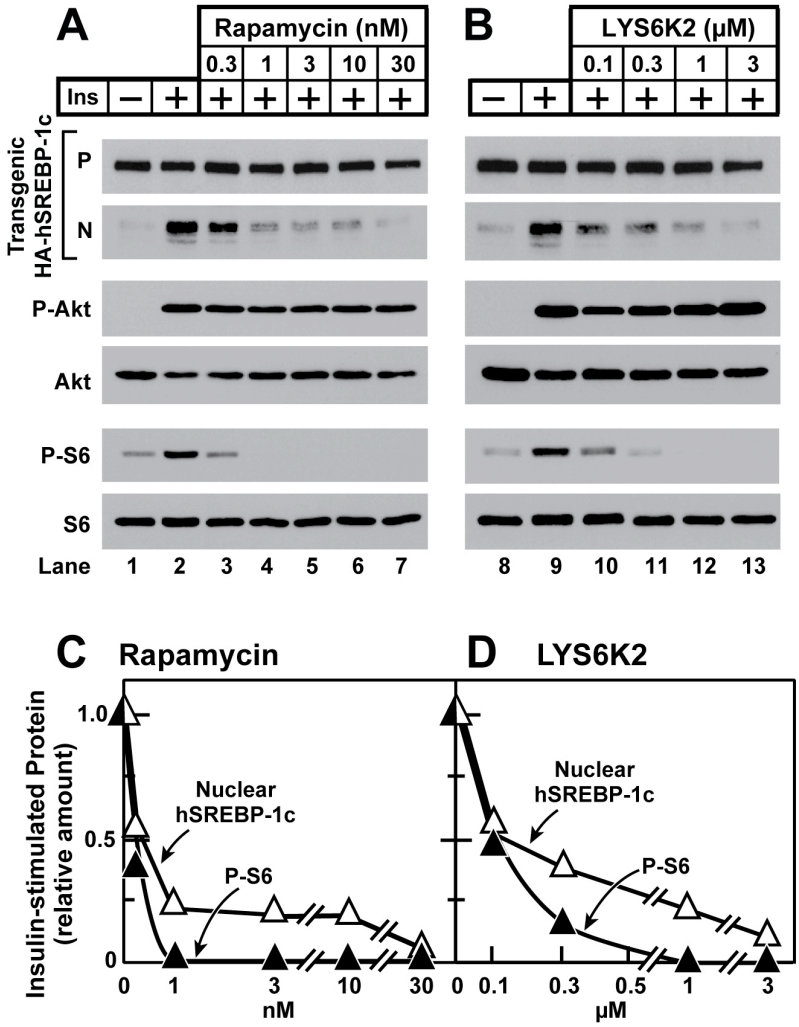


Figure 2-7. mTORC1 and S6K are required for insulin to stimulate proteolytic processing of transgenic SREBP-1c in rat hepatocytes. Hepatocytes from nonfasted *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 2 h with or without the indicated concentration of rapamycin or LYS6K2, after which the cells were treated with or without 30 nM insulin for 1 h, harvested, and then pooled (3 dishes of cells/sample) for immunoblot analysis. (A,B) Samples were subjected to immunoblot analysis as in Figure 2-4. (C,D) Quantification of immunoblots. The gels of nuclear extract fractions (N) of transgenic HA hSREBP-1c protein and cytosol fractions of phosphorylated ribosomal protein S6 (P-S6) in A and B were scanned and quantified by densitometry. The amounts of nuclear HA-hSREBP-1c or cytosol P-S6 protein in cells treated with insulin alone (lanes 2,9) were arbitrarily set at 1.

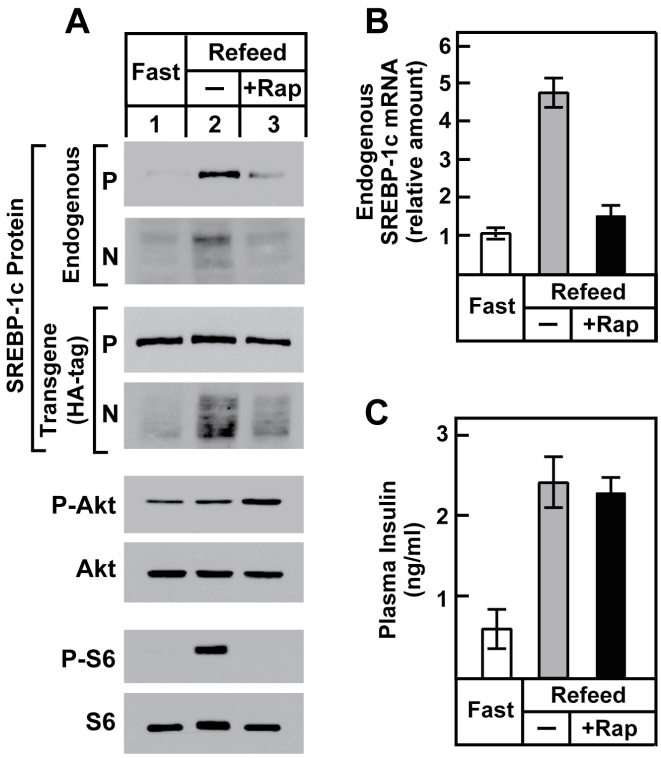
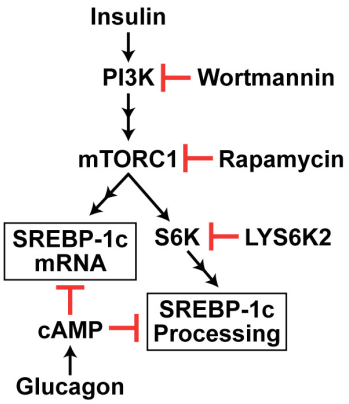


Figure 2-8. Effect of mTORC1 inhibition on SREBP-1c processing (A) and SREBP-1c transcription (B) in livers of *TgHA-hSREBP-1c* rats subjected to fasting and refeeding. Male transgenic rats (2-3 month-old, 4 rats/group) were fasted for 48 h, or fasted for 48 h and then refed a high-carbohydrate diet for 3 h prior to study. For the refed group, the animals were injected intraperitoneally with either vehicle or 20 mg/kg rapamycin (Rap) 1 h prior to refeeding. (A) Liver membrane, nuclear extract, and cytosol fractions were prepared individually and pooled (4 rats/group). Membrane and nuclear extract fractions were subjected to immunoblot analysis to detect precursor (P) and nuclear (N) forms of HA-tagged transgenic human SREBP-1c and endogenous rat SREBP-1 as in Figure 2-2. Immunoblot analysis of cytosolic phosphorylated ribosomal protein S6 (P-S6) and total S6 was carried out as described in Materials and Methods. Gels were exposed to film for 3-60 s. (B) Total liver RNA was prepared individually and subjected to real-time PCR. Each bar (mean ± SEM of data from 4 rats) represents the amount of endogenous SREBP-1c mRNA relative to that in the fasted group, which was arbitrarily defined as 1. (C) Plasma insulin levels. Insulin was measured in plasma obtained immediately after administration of anesthesia as described in Materials and Methods. Each bar represents mean ± SEM of data from 4 rats.



Kinase Inhibited	SREBP-1c	
	mRNA	Processing
PI3K	Blocked	Blocked
mTORC1	Blocked	Blocked
S6K	No Effect	Blocked

Figure 2-9. Pathways for insulin stimulation of SREBP-1c mRNA and proteolytic processing diverge after mTORC1. Both processes are inhibited by glucagon acting through cyclic AMP (cAMP).

CHAPTER THREE
Conclusions and Further Insights

The studies presented here establish a new model for the study of SREBP-1c processing in liver. Under the control of the non-insulin sensitive liver-specific *apoE* promoter/enhancer, epitope-tagged human SREBP-1c was expressed in a line of transgenic rats. Using freshly isolated primary hepatocytes or whole liver from these transgenic rats, the insulin-mediated signaling pathway required for the induction of SREBP-1c processing was studied without the confounding effects of insulin-stimulated increases in SREBP-1c mRNA.

Sterol-mediated Suppression of SREBP-1c Processing

Historically, the first characterized regulation of the SREBP pathway was by sterols (Brown and Goldstein, 1999; Goldstein et al., 2002). High intracellular levels of sterols blocked the processing of SREBP, thus preventing its maturation to an active transcription factor. This blockade of SREBP processing was seen for both SREBP-1 and SREBP-2 in cultured cells (Brown and Goldstein, 1997; Adams et al., 2004) and their regulation was initially thought to be coordinately regulated. However, early *in vivo* studies in hamster liver indicated that while both nSREBP-1 and nSREBP-2 were blocked by accumulation of sterols, nSREBP-1 was suppressed and nSREBP-2 was induced when sterols were depleted (Sheng et al., 1995; Shimomura et al., 1997a). It was later shown that insulin was able to selectively induce SREBP-1c (Shimomura et al., 1999b).

However, the question of sterol-mediated suppression of SREBP-1c processing can now be assessed using the *TgHA-hSREBP-1c* rat. In Figure 3-1, transgenic primary hepatocytes were isolated and treated with either cholesterol complexed with methyl-β-cyclodextrin or 25-hydroxycholesterol in ethanol. Both sterols completely suppressed proteolytic processing of HA-hSREBP-1c—even eliminating the basal level of nuclear HA-hSREBP-1c—but had no effect on the precursor form. Figure 3-2 demonstrates the potent effect of 25-hydroxycholesterol on the suppression of HA-hSREBP-1c processing. At the highest concentration, 25-hydroxycholesterol suppresses the basal level of nuclear HA-hSREBP-1c, and at lower concentrations only blocks the induction of nuclear HA-hSREBP-1c.

Insig Overexpression Blocks SREBP-1c Processing

The sterol-mediated suppression of SREBP processing requires the interplay between the polytopic ER membrane proteins Scap and Insigs. When intracellular levels of sterols are high, Scap adopts a conformation that allows it to bind to Insigs. Insigs will act as anchors, preventing the Scap/SREBP complex from exiting the ER (Yabe et al., 2002; Yang et al., 2002). Indeed, if Insigs are expressed at high enough levels, the Scap/SREBP complex becomes trapped in the ER, even when the sterol content of cells is low (Engelking et al., 2004).

The ability of overexpressed Insigs to block processing of SREBP-1c can also be assessed using the *TgHA-hSREBP-1c* rat. In Figure 3-3, transgenic primary hepatocytes were isolated and infected with adenovirus overexpressing LacZ (control), Insig-1, or Insig-2. Infection with LacZ adenovirus did not effect insulin-induced HA-hSREBP-1c

processing. However, infection with adenovirus overexpressing Insig-1 or Insig-2 blocked insulin's ability to stimulate SREBP-1c processing.

Sterol- and Insig-mediated suppressions of transgenic HA-hSREBP-1c processing were also useful as initial characterizations of the *TgHA-hSREBP-1c* rat. These results indicated that the transgenic HA-hSREBP-1c protein was integrating itself into and utilizing the endogenous rat machinery (e.g., Scap and Insigs) to be proteolytically cleaved to its nuclear form.

Glucagon and Forskolin Block SREBP-1c Processing

In vivo, the hormones insulin and glucagon act in opposition to one another: insulin levels are highest during nutrient abundance, activating nutrient uptake and storage pathways, while glucagon levels are highest during nutrient deficits, activating nutrient mobilization and utilization. Since SREBP-1c activates all of the genes necessary for FA and TG synthesis, it should be activated by insulin and repressed by glucagon. This has been shown previously at the transcriptional level (Foretz et al., 1999a, 1999b; Shimomura et al., 2000), and in Figure 2-3 at the level of processing.

When glucagon binds to its cell surface receptor, it activates signaling pathways that increase intracellular levels of cAMP, which acts as a second messenger to further propagate the signaling cascade. Therefore, if hepatocytes are treated with another chemical that raises intracellular cAMP levels in a fashion similar to glucagon, this should also block SREBP-1c processing. This experiment was performed in Figure 3-4. Panel 3-4A shows the effect of increasing concentrations of glucagon on the processing of SREBP-1c (it is also the immunoblot that was scanned and quantified by densitometry

in Figure 2-3B). It should be noted that the concentration of glucagon (0.3 nM) that has no effect on SREBP-1c processing is 100-fold less than the insulin (30 nM) used to stimulate SREBP-1c processing, a ratio that was also observed for the induction of SREBP-1c mRNA (Shimomura et al., 2000). Panel 3-4B shows the effect of forskolin on SREBP-1c processing; forskolin raises intracellular levels of cAMP. At higher concentrations of forskolin (10 μM), insulin-induced SREBP-1c processing is blocked as it was with glucagon. This provides some initial clues as to what signaling events are required for the glucagon-mediated suppression of SREBP-1c processing.

Scap Conformation and ER Budding

From experiments with the *TgHA-hSREBP-1c* rat in Chapters 2 and 3, it is clear that insulin stimulates and glucagon represses SREBP-1c processing. However, the precise molecular event that either of these hormones induces or represses is not entirely clear. For instance, the Scap/SREBP complex must be released from the ER for processing to occur. There are at least 2 ways in which insulin signaling might enhance this: 1) a phosphorylation-induced Scap conformational change, causing Scap to dissociate from Insigs and escort SREBP-1c to the Golgi apparatus, and 2) increased budding of the Scap/SREBP complex from the ER in COPII-coated vesicles. The Brown and Goldstein lab has previously developed assays to study both of these processes in cultured cells (Nohturfft et al., 2000; Brown et al., 2002).

It would be interesting to adapt these methods for use in primary hepatocytes in order to assess how insulin or glucagon effects Scap conformation and/or Scap ER budding and what impact these have on the processing of SREBP-1c. Inhibitors of

mTORC1 and S6K might also then be used to assess whether their blockade of SREBP-1c processing is mediated by alteration of either of these two events.

Immunofluorescence in Transgenic Primary Rat Hepatocytes

The processing of SREBP-1c in freshly isolated transgenic primary hepatocytes was assessed by immunoblot using an antibody to the N-terminal HA-tag on the transgene. Another way of utilizing the epitope tag to visualize the processing of SREBP-1c would be to perform immunofluorescence on the transgenic primary hepatocytes. Cells would be treated with or without insulin and then fixed after a specified treatment time (which could also be assessed in a time course). Inhibitors of the insulin signaling pathway (especially rapamycin and LYS6K2) could also be used to visualize the blockade of SREBP-1c processing.

Depending on the robustness of the immunofluorescence assay, this could be a method for high-throughput screening of either chemical or RNA interference (RNAi) libraries for their effect on SREBP-1c processing. If a chemical inhibitor of SREBP-1c processing is found, it could be the starting point for a new drug to prevent SREBP-1c processing that is over-exuberant in T2DM and fatty liver disease. Its target molecule could either be a known SREBP pathway component or another as yet undetermined component that could be characterized for a fuller understanding of the SREBP-1c processing signaling pathway. Positive hits from the RNAi screen would certainly include known SREBP or insulin signaling pathway components (serving as positive controls), but it might also include novel molecules not yet known to play a role in the SREBP-1c processing pathway.

Phosphoproteomics to Determine the Downstream Target of S6K that Mediates SREBP-1c Processing

Figures 2-4 and 2-7 define S6K, a downstream target of mTORC1, as necessary for the processing of SREBP-1c. This begs the question, what is the target of S6K that mediates the insulin-induced processing of SREBP-1c? To date, S6K has been shown to phosphorylate several proteins (Fenton and Gout, 2011; Magnuson et al., 2012), but no phosphoproteomic studies have been performed looking specifically for targets of S6K, let alone such studies in primary hepatocytes. Phosphoproteomics has been performed for the mTORC1-regulated phosphoproteome (Hsu et al., 2011; Yu et al., 2011), but neither of these studies were performed in primary hepatocytes. It would be interesting to apply a triple-filtered, phosphoproteomics approach using primary hepatocytes: a putative candidate that affects SREBP-1c processing, when compared to the no treatment group, should exhibit increased phosphorylation in the insulin alone group, but not in the insulin plus either rapamycin or LYS6K2 groups. However, a putative candidate that effects SREBP-1c transcription should show increased phosphorylation in the insulin alone or insulin plus LYS6K2 groups, but not in the insulin plus rapamycin group because of the bifurcation of SREBP-1c transcription and processing signaling pathways downstream of mTORC1 (Figure 2-6).

SREBP Pathway Components as Potential S6K Targets

S6K is a member of the AGC family of serine/threonine kinases. It, along with other members of its kinase family, has a target phosphorylation sequence RXRXXS/T, where X is any amino acid (Fenton and Gout, 2011). If S6K phosphorylates one of the

SREBP pathway components, that protein should possess this amino acid sequence or a very similar one (R-3 residue is more important than the R-5) (Obata et al., 2000).

Two proteins in the SREBP pathway contain a perfect S6K consensus sequence: Scap and S1P. Unfortunately, there are complications with each of these sites. Scap has two consensus sites; the N-terminal consensus site on Scap is located in its first ER luminal loop. Recently, this loop has been shown as the binding site for cholesterol that allows Scap to exert its regulatory function on SREBP processing (Motamed et al., 2011). Since this loop resides in the ER lumen, S6K, a mostly cytosolic and potentially nuclear protein (Fenton and Gout, 2011; Magnuson et al., 2012), would require an ability to rapidly enter the ER lumen. The C-terminal consensus site in Scap resides in the C-terminal WD domain. This domain mediates the interaction of Scap with SREBP (Sakai et al., 1997). However, the perfect consensus sequence is only present in human Scap. Other mammals are missing the R-5 residue. While this is not ideal, it still might be interesting to pursue because of the importance of the Scap/SREBP interaction, mediated by the WD domain, in the regulation of SREBP-1c processing. S1P contains a single perfect S6K consensus site. However, the site is in the portion of the protein that resides in the lumen of the Golgi apparatus. S6K would therefore require the capability to rapidly enter the Golgi apparatus.

SREBP-1c as a Potential Phosphorylation Target

SREBP-1c and SREBP-2 both share the other SREBP pathway components (i.e., Scap, Insigs, S1P, and S2P), and yet insulin selectively activates the processing of SREBP-1c and not SREBP-2. There is no evidence that the different SREBPs interact

differently with the other protein components of the SREBP pathway. Since the primary mode of signal transduction in the insulin signaling pathway is via phosphorylation (Cohen, 2006), this begs the question whether SREBP-1c itself is being phosphorylated as it is the only component not shared with SREBP-2.

This question could be assessed using the epitope tag on the transgenic HA-hSREBP-1c. Using either fasting/refed rat whole liver, or insulin treated primary hepatocytes, precursor and/or nuclear SREBP-1c could be immunoprecipitated using an antibody to the HA-tag. Potential phosphorylation sites could then be mapped by mass spectrometry.

Using Transgenic Rats to Assess Processing in a Rat Model of Insulin Resistance

Zucker fatty rats have been used as a model to study the role of obesity and insulin resistance in the progression to T2DM (Unger, 1995). Zucker fatty rats harbor a recessive mutation that renders them obese and hyperinsulinemic, whereas their heterozygous or WT littermates are lean and normoinsulinemic (Zucker and Zucker, 1961).

Figure 3-5 is data from a pilot study performed in Zucker lean and fatty rats subjected to a fasting time course: either nonfasted, 18 h fasted, or 48 h fasted. Plasma insulin and liver SREBP-1c mRNA were measured at each time point. At all time points, Zucker fatty rats exhibited higher plasma insulin levels and higher hepatic levels of SREBP-1c mRNA than their lean littermates. Upon fasting, both Zucker lean and fatty rats showed decreased plasma insulin and hepatic SREBP-1c mRNA levels. When plotted, the relationship between plasma insulin and hepatic SREBP-1c mRNA was linear

and exhibited the same rate of change in both Zucker lean and fatty rats. This indicated that even though the fatty rats were obese and insulin resistant, the amount of SREBP-1c mRNA in their liver remained as sensitive to plasma insulin levels as in the lean, non-insulin resistant littermates.

This is an experimental demonstration of the selective insulin resistance present in the liver in the diabetic state. The next question then is to ask if the processing of SREBP-1c is similarly elevated with the hyperinsulinemia present in the Zucker fatty rats. If the *TgHA-hSREBP-1c* rat was crossed into the Zucker fatty rat line, the role of hyperinsulinemia and insulin resistance on elevated levels of processed nuclear SREBP-1c could be tested. Some of the questions that might be asked include: Is SREBP-1c processed at a higher basal level in Zucker fatty rats due to their hyperinsulinemia? Does the amount of nuclear SREBP-1c possess a linear relationship with plasma insulin levels like SREBP-1c mRNA (Figure 3-5)? Are inhibitors of mTORC1 and S6K still capable of blocking processing of SREBP-1c or has resistance arisen? Do hepatocytes taken from Zucker fatty rats have a "memory" and therefore exhibit a higher basal level of SREBP-1c processing in cell culture? Is SREBP-1c modified (e.g., phosphorylated) differently in states of insulin resistance/hyperinsulinemia? The *TgHA-hSREBP-1c* rat crossed into the Zucker line would represent a disease model that would complement the standard *TgHA-hSREBP-1c* rat in helping to fully characterize SREBP-1c processing in both normal and abnormal states, potentially providing further insights into the pathogenesis of insulin resistance, T2DM, and fatty liver disease.

Using Transgenic Rats to Study SREBP-1c Processing

The transgenic rat model presented here represents an entirely new model system for the study of SREBP-1c processing, both *in vivo* and in cell culture. Studies using kinase inhibitors to define the importance of mTORC1 and S6K in SREBP-1c processing are only the beginning of the discoveries that are possible with this model. For example, increased ER stress has been implicated in the activation of SREBP-1c (Kammoun et al., 2009). The transgenic rat model would allow for the study of ER stress pathway-induced SREBP-1c processing as well as its relative importance in insulin-induced activation of SREBP-1c processing. Future insights into the signaling pathway leading to SREBP-1c processing will help to clarify the role of insulin and SREBP-1c in disorders such as T2DM or fatty liver disease and may aid in the design of novel clinical treatments.

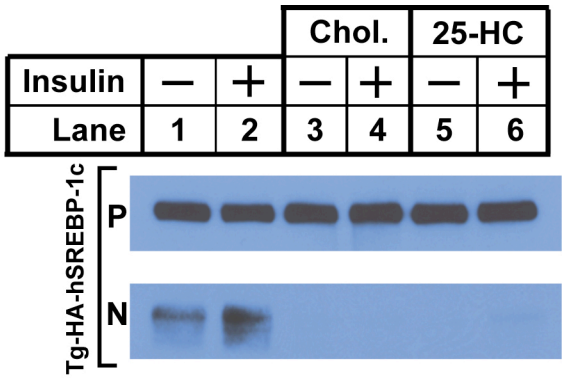


Figure 3-1. **Cholesterol and 25-hydroxycholesterol block insulin-stimulated proteolytic processing of transgenic SREBP-1c in rat hepatocytes.** Hepatocytes from *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 3 h with or without the indicated sterol: 30 μM cholesterol complexed with methyl-β-cyclodextrin (lanes 3-4) or 2.5 μM (1 μg/mL) 25-hydroxycholesterol in ethanol (lanes 5-6). After this preincubation, the cells were treated with or without 10 nM insulin for 30 min prior to harvest. Immunoblot analyses of precursor (P) and nuclear (N) forms of transgenic HA-hSREBP-1c were carried out as described in Materials and Methods in Chapter 2. Gels were exposed to film for 5-20 s.

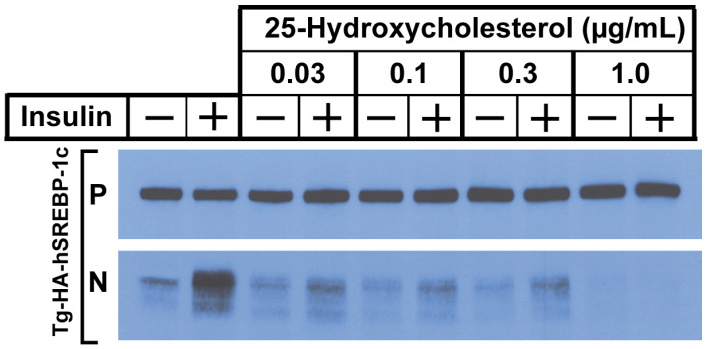


Figure 3-2. **25-Hydroxycholesterol blocks insulin-stimulated proteolytic processing of transgenic SREBP-1c in rat hepatocytes at low concentrations.** Hepatocytes from *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 3 h with or without the indicated concentration of 25-hydroxycholesterol (1 µg/mL equivalent to 2.5 µM), after which the cells were treated with or without 30 nM insulin for 30 min prior to harvest. Immunoblot analyses of precursor (P) and nuclear (N) forms of transgenic HA-hSREBP-1c were carried out as described in Materials and Methods in Chapter 2. Gels were exposed to film for 10-20 s.

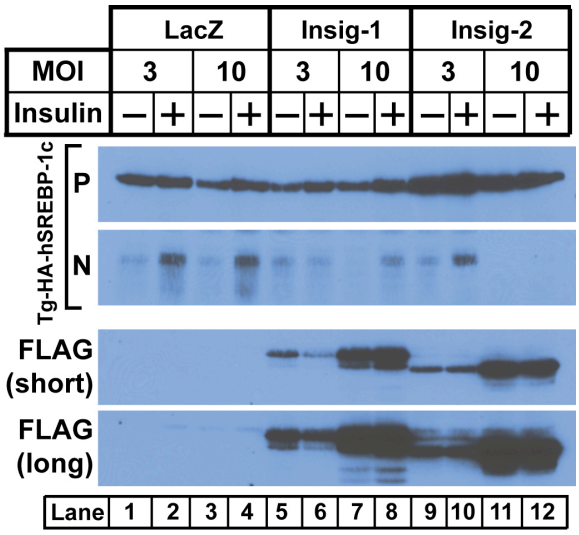


Figure 3-3. Overexpression of Insigs blocks insulin-stimulated proteolytic processing of transgenic SREBP-1c in rat hepatocytes. Hepatocytes from *TgHA-hSREBP-1c* rats were prepared and plated on day 0 and were incubated with the indicated concentration of the indicated FLAG-tagged adenovirus overnight (1 MOI was taken to be 30 particles/cell). On day 1, cells were treated with 30 nM insulin for 3 h prior to harvest. Immunoblot analyses of precursor (P) and nuclear (N) forms of transgenic HA-hSREBP-1c were carried out as described in Materials and Methods in Chapter 2. Immunoblot analysis of FLAG-tagged adenoviruses were performed on cytosol fractions using mouse monoclonal M2 anti-FLAG antibody (Sigma). Gels were exposed to film for 3-60 s.

Figure 3-4. **Glucagon and forskolin block insulin-stimulated proteolytic processing of transgenic SREBP-1c in rat hepatocytes.** Hepatocytes from *TgHA-hSREBP-1c* rats were prepared and plated on day 0. On day 1, cells were pretreated for 2 h with the indicated concentration of glucagon (A) or forskolin (B), after which the cells were treated with or without 30 nM insulin for 1 h prior to harvest. Immunoblot analysis of nuclear transgenic HA-hSREBP-1c was carried out as described in Materials and Methods in Chapter 2. Gels were exposed to film for 10 s.

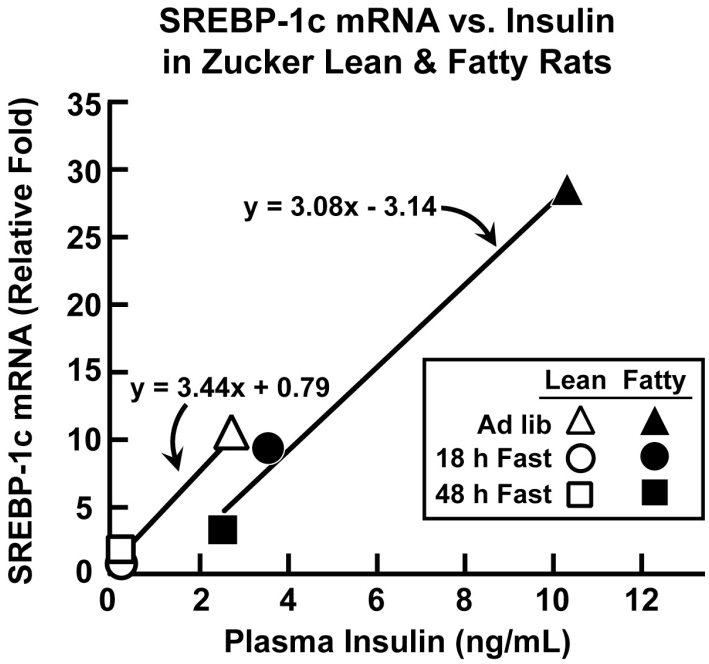


Figure 3-5. **Effect of fasting on SREBP-1c mRNA and plasma insulin in Zucker lean and fatty rats.** Zucker lean (open symbols) and fatty (closed symbols) rats (2 animals/group) were fed a chow diet *ad libitum* (triangles), fasted for 18 h (circles), or fasted for 48 h (squares). Treatments for each group were staggered such that all animals were sacrificed at the same time at the end of the dark cycle. Plasma was obtained from blood taken from the inferior vena cava immediately after anesthetization and frozen. Plasma insulin was measured using an insulin ELISA kit. Total liver RNA was isolated from each flash frozen rat liver. Quantitative real-time PCR for SREBP-1c mRNA was performed on pooled cDNA samples. The level of mRNA in the fed lean rats was arbitrarily set to 1.

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