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ATTENUATION OF mTORC1-DRIVEN SECRETION OF LIPOPROTEINS AND TRIACYLGLYCERIDES BY SHORT CHAIN FATTY ACIDS: MECHANISTIC INSIGHT INTO THE PATHOGENESIS OF HYPERTRIGLYCERIDEMIA

Joseph L. Roberts
University of Nebraska-Lincoln, joseph.roberts@huskers.unl.edu

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by

Joseph L. Roberts

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ATTENUATION OF mTORC1-DRIVEN SECRETION OF LIPOPROTEINS AND TRIACYLGLYCERIDES BY SHORT CHAIN FATTY ACIDS: MECHANISTIC INSIGHT INTO THE PATHOGENESIS OF HYPERTRIGLYCERIDEMIA

Joseph L. Roberts, M.S.

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Advisor: Regis Moreau

The mechanistic target of rapamycin complex 1 (mTORC1) is a serine/threonine kinase that drives several anabolic processes including lipid synthesis, protein synthesis, and adipogenesis. mTORC1 is highly active in the livers of obese rodents, in overnutrition, and is implicated in the development of obesity related metabolic disorders, including the overproduction of atherogenic lipoproteins. Direct inhibition of mTORC1 is not a viable treatment strategy because it prevents feedback inhibition of the insulin-signaling cascade, leading to increased lipid synthesis and secretion of lipoproteins. Thus, therapeutic approaches that drive catabolic pathways are considered promising mechanisms for overcoming mTORC1-driven anabolism. Human liver HepG2 cells were stably transduced with lentiviral shRNA targeting tuberous sclerosis complex 2 (TSC2, upstream inhibitor of mTORC1) to constitutively activate mTORC1 or lentiviral scramble shRNA as negative control. In TSC2 shRNA cells, TSC2 gene and protein expression were suppressed, and mTORC1 activity (phospho-p70 S6K/p70 S6K) was subsequently increased. Hyperactive mTORC1 upregulated lipogenic and lipid transport genes and significantly increased cellular triacylglycerol content when

compared to scramble shRNA cells. Furthermore, the constitutive activation of mTORC1 increased apolipoprotein B (apoB) and triacylglycerol secretion into culture media. To determine if dietary agents would reverse mTORC1-mediated secretion of lipoproteins and triacylglycerides, TSC2 cells were treated with one of two short chain fatty acids, alpha-lipoic acid (LA; 200 μM) or 4-phenylbutyric acid (PBA; 8 mM). LA and PBA significantly decreased secreted apoB and triacylglyceride despite their differential effects on mTORC1 activity; LA repressed mTORC1 signaling in TSC2 shRNA cells; whereas PBA did not. In addition to lowering secreted triacylglycerol LA decreased cellular triacylglycerol content but not PBA, which increased cellular triacylglycerol. We conclude that LA and PBA have the potential to lower the secretion of atherogenic lipoproteins associated with mTORC1 signaling hyperactivation.

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CHAPTER 1

LITERATURE REVIEW

Hypertriglyceridemia

Hypertriglyceridemia is a serious public health concern. Over 24% of the U.S.' population has hypertriglyceridemia, with a higher prevalence (42.8%) among people aged ≥50 years [1]. The prevalence of hypertriglyceridemia in the U.S. has slowly declined from 33.5% in 1999 to 24.3% in 2010; however, this trend is likely attributable to the increased use of lipid-lowering agents from 8% to 15.6% in 1999 and 2010, respectively [2]. Hypertriglyceridemia, defined as abnormally high fasting serum triacylglycerides (TG) levels (>150 mg/dL or 1.8 mM), often occurs in people who are overweight or obese, and those with type 2 diabetes [1]. Elevated TG is considered to be a significant risk factor for cardiovascular disease. For example, each 1 mM (89 mg/dL) increase in TG translated to a 12% and 37% increase in cardiovascular disease risk in men and women, respectively [3]. Hypertriglyceridemia is also a significant risk factor for pancreatitis, liver disease, type 2 diabetes, and metabolic syndrome [4].

The first-line therapies for treating hypertriglyceridemia are lifestyle changes such as dietary modification, exercise, and weight loss. In extreme and persistent cases (≥200 mg/dL or 2.26 mM), pharmacologic treatment options are utilized to lower TG including niacin, fish oil (omega-3 fatty acids), statins, fibrates, and thiazolidinediones (Table 1.1) [1]. The likelihood of controlling TG levels with lifestyle modifications at the population level is impractical, as obesity rates continue to climb despite countless public health initiatives [2]. At the individual level, drug therapies have demonstrated a notable ability to improve TG levels; however, negative side effects (e.g., flushing, gastrointestinal complaints, myopathy, worsening glycemia) remain a concern [1]. Thus, clinicians must

prescribe lipid-lowering drugs carefully, especially in patients with renal and hepatic impairments. This dilemma, along with the ongoing obesity and type 2 diabetes epidemics shed light on the importance of identifying safe and inexpensive therapeutic agents that would effectively reduce TG levels.

Table 1.1. Select therapies for treating dyslipidemia.

Therapy	TG	LDL-C	HDL-C	Potential adverse effects
Fibrates	↓40-60%	↑5-30%	↑15-25%	Myopathy, rhabdomyolysis, elevated liver enzymes
Statins	↓20-40%	↓18-55%	↑5-15%	Myopathy, rhabdomyolysis, elevated liver enzymes
Niacin	↓30-50%	↓5-25%	↑20-30%	Flushing, worsening glycemia, elevated liver enzymes
Fish oils	↓30-50%	↑5-10%	↑5-10%	Fishy aftertaste, gastrointestinal upset

Lipid Transport and Metabolism

Free fatty acids (FFA) are energy dense molecules (9 kcal/g) that are utilized by select tissues for energy (e.g., muscle), and as a source of energy during periods of starvation. FFAs originate from dietary sources (exogenous), and through *de novo* lipogenesis (endogenous) in the liver in response to excess energy. Excess FFA are stored within adipose tissue as TG, which are formed through a condensation reaction between three FFA and a glycerol molecule. This reaction yields three molecules of water and one TG. The fatty acid composition of TG can be considerably diverse, differing in acyl chain length and degree of saturation. All TG are hydrophobic, have detergent-like properties, and are generally insoluble in blood, thus they require specialized transport structures called lipoproteins (Figure 1.1).

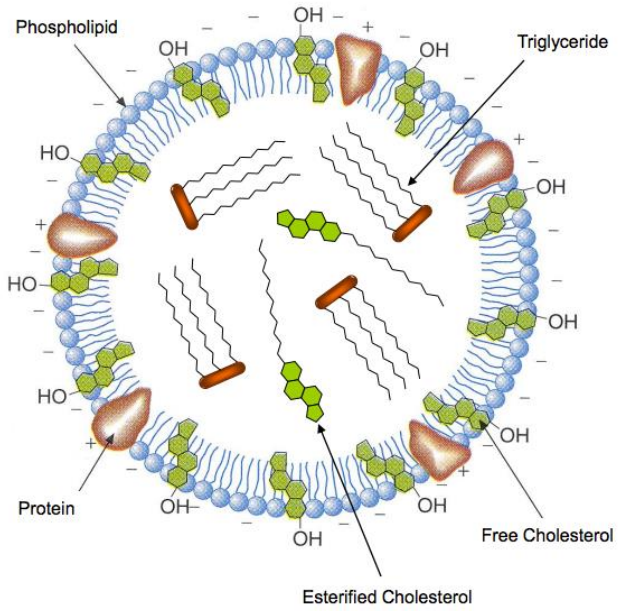

Figure 1.1. Generalized structure of a lipoprotein. Lipoproteins consist of a phospholipid monolayer with embedded proteins and free cholesterol. The neutral lipids (e.g., TG and cholesterol esters) that are nonpolar are found within the inner hydrophobic core of the lipoprotein.

Structurally, lipoproteins are comprised of a phospholipid monolayer shell that also contains free cholesterol and proteins. The phospholipids are positioned such that the polar head groups interact with aqueous environment of the blood, while the nonpolar acyl chains form a hydrophobic core that is compatible with the nonpolar nature of TG and cholesterol esters. This arrangement allows for the dispersion and transport of lipids within the aqueous environment of the blood. Lipoproteins also contain a variety of surface proteins known as apolipoproteins that serve as structural components, as well as cofactors and ligands for lipid metabolism.

Lipoproteins are classified according to their apolipoproteins, lipid composition, and density (lipid:protein ratio) (Table 1.2). There are four classes of lipoproteins that

include chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Chylomicrons are TG-rich lipoproteins that are synthesized and secreted by intestinal enterocytes after consumption of a lipid containing meal. The TG core is comprised of dietary lipid that had been hydrolyzed by digestive lipases (e.g., gastric lipase, pancreatic lipase) into FFA, transported inside of the cell, and then reassembled into TG. The resynthesized TG, cholesterol, phospholipids, and a truncated form of apolipoprotein B (apoB), apoB48, are assembled into chylomicrons and secreted into the lymphatic system. Chylomicrons enter systemic circulation through the thoracic duct, and subsequently deliver dietary TG to target tissues (e.g., adipose) via the action of lipoprotein lipase (LPL). LPL is expressed in adipose, muscle, mammary gland, brain, and luminal surface of capillaries and arteries. LPL catalyzes the hydrolysis of esterified fatty acids, yielding glycerol and free fatty acids that then enter the peripheral tissue for usage or storage. This reaction generates smaller, TG-poor chylomicron remnants that bind with hepatic lipoprotein receptor-related protein 1 (LRP1) and apoB/E receptors to initiate receptor mediated endocytosis and clearance.

Table 1.2. Defining features of the four classes of lipoproteins.

Lipoprotein	Chylomicrons	VLDL	LDL	HDL
Density (g/mL)	< 0.95	0.95-1.006	1.019-1.063	1.063-1.225
Diameter (Å)	1,000-10,000	300-800	200-250	50-120
Defining Apolipoprotein	apoB48	apoB100	apoB100	apoAI

The remaining classes of lipoproteins (VLDL, LDL, HDL) are responsible for transporting endogenous lipids, or those that arose from sources other than diet. TG-rich VLDL are synthesized and secreted from the liver and are characterized by the presence

of one full-length apoB100. In a process analogous to chylomicron metabolism, the TG within VLDL is catabolized into FFA and glycerol through the action of LPL. This reaction produces TG-poor IDL, then LDL through further processing. ApoB100 and ApoE found on the surface of LDL can then interact with hepatic LDL receptor (LDLR) to initiate clearance. Aberrant production and secretion of VLDL from the liver can lead to a host of conditions including hypertriglyceridemia, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, and type 2 diabetes. However, the molecular mechanisms involved in abnormal lipoprotein metabolism remain unclear.

Assembly and Secretion of Very-Low Density Lipoprotein (VLDL)

VLDL particles are triacylglyceride-rich lipoproteins that are synthesized and secreted from the liver. VLDL transports TG that arise from *de novo* lipogenesis (fed state), and TG formed from FFA released from adipocytes (fasted state) to peripheral tissues including heart, adipose, and skeletal muscle. VLDL plays an essential role in lipid homeostasis; thus, its biogenesis is tightly controlled, primarily through regulation of apolipoprotein B (apoB) [5]. ApoB is an abundant protein that occurs in plasma as two distinct isoforms. The *APOB* gene is located on chromosome 2, and is expressed exclusively in the liver and small intestine. Though the entire gene is expressed in both tissues, mRNA editing in the intestine introduces a stop codon at residue 2153 resulting in a truncated version of apoB [6]. As a result of mRNA editing the apoB molecule is 48% of full-length protein, designated apoB48, which is incorporated and secreted as the key identifier of chylomicrons [7]. Conversely, hepatocytes produce the full-length apoB protein that is referred to as apoB100 [7]. ApoB100 is a large protein that contains 4,536

amino acid residues and has a molecular weight of approximately 550 kDa [6]. Each VLDL contains a single, nontransferable apoB100 molecule that is subjected to a series of complex processing steps before it is secreted into circulation.

The amount of apoB secreted is primarily determined by the amount of protein that evades co- and post-translational degradation. For instance, in human hepatocellular carcinoma (HepG2) cells approximately 30-75% of newly synthesized apoB is degraded within a 2-3 hr period before the remaining protein is secreted [8–11]. ApoB mRNA is constitutively expressed and can be controlled by a process that involves the trafficking of apoB mRNA into P-bodies, aggregates of translationally repressed mRNAs [12,13]. ApoB mRNA that escapes this initial degradation is then translated, and subsequently lipidated by microsomal triacylglyceride transfer protein (MTTP) that prevents proteosomal degradation [14]. The addition of TG to nascent apoB by MTTP occurs during its contranslational translocation to the endoplasmic reticulum (ER) and gives rise to a dense, lipid-poor pre-VLDL particle [14]. Once inside of the ER lumen apoB can be regulated by ER60, a chaperone and protease that binds to and degrades overexpressed apoB [15]. Remaining apoB is then trafficked to the Golgi apparatus where it undergoes further lipidation in an MTTP-independent manner that involves apoCIII, an apolipoprotein secreted by the liver [16,17]. The TG that are used to lipidate apoB in the ER and Golgi are derived from FFA released from adipocytes, uptake of chylomicron and VLDL remnants, and *de novo* lipogenesis [5]. Secretion of the fully lipidated apoB particle can be inhibited by lysosomal and autophagic degradation [5]. Mature VLDL that escapes degradation and inhibitory mechanisms is then secreted from the hepatocyte

where it is either subjected to reuptake mediated by LDLR or enters circulation to deliver its cargo to peripheral tissues (Figure 1.2) [5].

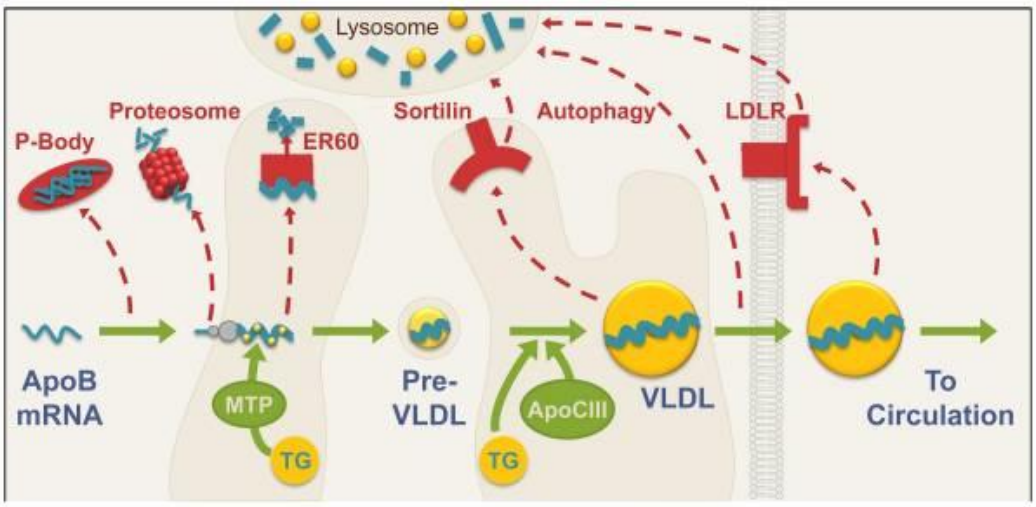

Figure 1.2. Molecular control mechanisms in the synthesis and secretion of very low-density lipoprotein (VLDL). ApoB is lipidated with TG by MTTP in the ER to form a lipid-poor pre-VLDL particle. The pre-VLDL particle is then trafficked to the Golgi compartment where it is further lipidated in a MTTP independent manner that is promoted by apoCIII. ApoB is controlled by a variety of mechanisms, including proteasomal, autophagic, and lysosomal degradation. Green arrows indicate apoB-VLDL synthesis pathways; red dashed arrows indicate apoB degradation pathways. [Figure source: Haas et al., 2013] [5].

Mechanistic Target of Rapamycin Complex 1 (mTORC1)

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved threonine/serine kinase that exists within two distinct protein complexes, mechanistic target of rapamycin complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is comprised of five protein components: mechanistic target of rapamycin (mTOR), the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8); proline-rich Akt substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) [18]. Raptor is essential for the

assembly of mTORC1, and recruitment and binding of mTOR substrates (p70 S6K1 and 4E-BP1) [19,20]. mLST8 helps stabilize the mTOR-Raptor association by binding to interacting with the kinase domain of mTOR and plays a positive role in mTOR activation [21]. Conversely, PRAS40 and Deptor interact with and inhibit mTORC1 activity and are degraded upon mTORC1 activation [18,22].

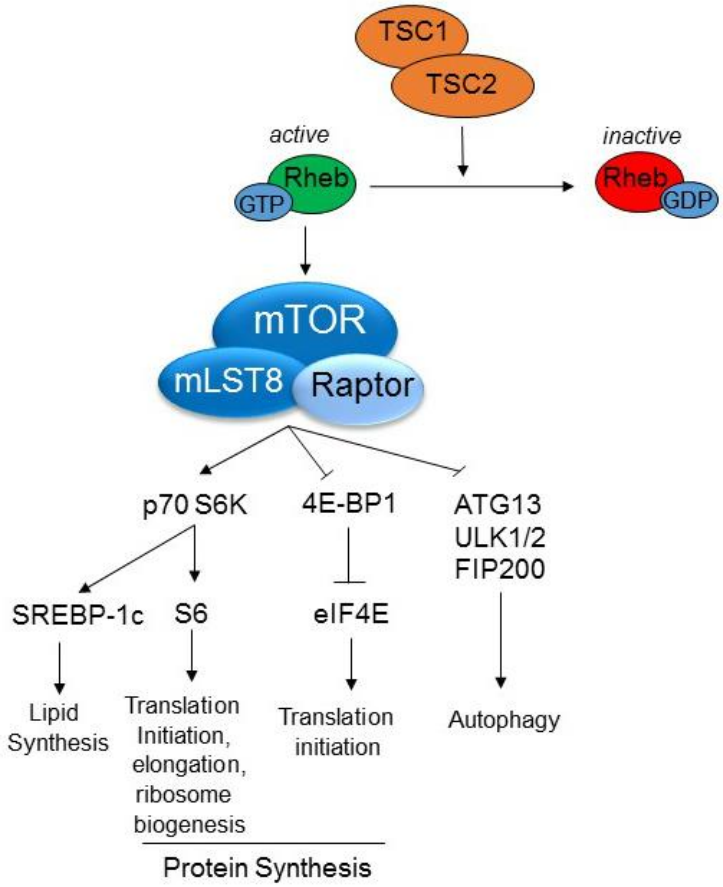

Figure 1.3. **Induction of mTORC1 signaling leads to activation of anabolic metabolism**. Inactivation of the TSC1/2 complex allows GTP-bound Rheb to bind and activate mTORC1 kinase activity. mTORC1 phosphorylates p70 S6K which phosphorylates S6 and activates SREBP1; conversely, mTORC1inhibits the action of 4E-BP1, ATG13 (autophagy-related protein 13), and ULK1/2 (UNC-51 like autophagy activating kinase) through phosphorylation. These reactions drive the anabolic processes of lipid synthesis and protein synthesis, while inhibiting the catabolic process of autophagy.

mTORC1 senses and integrates many intracellular and extracellular signals including, nutrients (e.g., amino acids, glucose), hormones (e.g., insulin), growth factors, oxygen, ATP levels, and cytokines [23]. These signals potentiate a cascade that involves inhibition of the GTPase-activating protein (GAP) complex comprised of TSC1 (hamartin) and TSC2 (tuberin) (TSC1/2) [23–25]. When the TSC1/2 complex is inhibited, it allows the activation of the small Ras-related small G protein Rheb (Ras homolog enriched in brain) via GTP-loading [26]. The GTP-bound Rheb acts as up-stream activator of mTORC1, and is required for mTORC1 activity [22,23]. In instances of nutrient or energy depletion, the TSC1/2 complex hydrolyzes GTP-Rheb (active) to GDP-Rheb (inactive), thus preventing the activation of mTORC1 [24–26]. Upon activation, mTORC1 directly phosphorylates p70 S6 Kinase 1 (p70 S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) [27]. Phosphorylation of mTORC1's downstream effectors potentiates the induction of anabolic cell growth (e.g., biosynthesis of proteins, lipids, organelles) and inhibition of catabolic processes (e.g., autophagy, ketogenesis) (Figure 1.3) [26,27].

The role of mTORC1 in lipogenesis

mTORC1 is often regarded as a master regulator of cellular growth and metabolism through its induction of protein synthesis, lipogenesis, adipogenesis, and organelle biogenesis. mTORC1 has emerged as a critical regulator of lipid homeostasis in physiological and pathological states [26]. In a postprandial state mTORC1 is transiently activated by insulin and nutrient sufficiency in metabolically active tissues (e.g., liver, adipose, muscle) promoting lipogenesis and blocking β-oxidation. In a normal

physiological state, insulin binds with its tyrosine kinase receptor induces autophosphorylation of the receptor, and recruitment and phosphorylation of insulin receptor substrates 1 and 2 (IRS1 and IRS2) at multiple tyrosine residues [28]. The activated IRS then complex with PI3K and promotes the production of phosphatidylinositol (3,4, 5)-triphosphate that increases the recruitment and activation of protein kinase B (Akt) [26]. Akt phosphorylates and inactivates TSC2 and PRAS40 allowing mTORC1 to become active and phosphorylate p70 S6K, which in turn phosphorylates several targets including ribosomal protein S6 and IRS1 (Figure 1.4) [26]. p70 S6K is required for mTORC1-mediated lipogenesis and is purported to play a role in the processing of sterol regulatory element-binding proteins (SREBP) in hepatocytes [29].

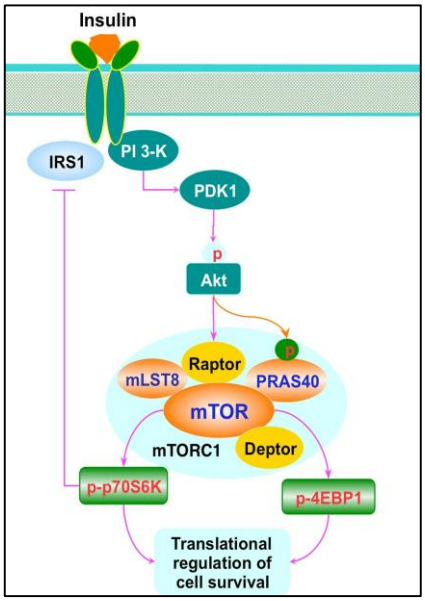

Figure 1.4. Activation of mTORC1 through insulin signaling. Insulin binds to its tryrosine kinase receptor and induces the recruitment of IRS1 and PI3-K leading to the activation of Akt. PRAS40 is inhibited by Akt, allowing mTORC1 to become active and phosphorylate its downstream effectors. p-p70 S6K inhibits IRS1 via a negative feedback loop, thus suppressing insulin signaling. [Figure adapted from: Chong and Maise, 2012] [30].

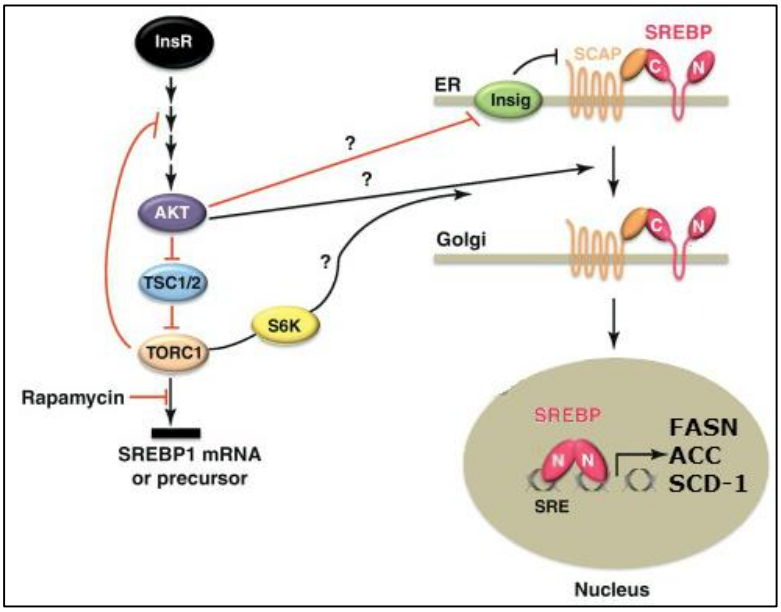

Figure 1.5. Proposed activation mechanism of SREBP through insulin and mTORC1 signaling. Red and black arrows indicate inhibitory and stimulatory actions, respectively. The question marks indicate unknowns associated with the mechanism. [Figure adapted from: Jeon and Osborne, 2012] [31].

SREBP-1 is a helix-loop-helix transcription factor that induces the expression of genes involved in cholesterol, fatty acid, triacylglyceride, and phospholipid synthesis [26]. SREBP-1 is synthesized in the ER as a precursor protein (125 kDa) that is bound to sterol-sensing binding partner (SCAP) and is retained in the ER by INSIG proteins (Figure 1.5) [26]. Upon sterol depletion, the SREBP1-SCAP is released from Insig2a and transported to the Golgi apparatus where they undergo proteolytic cleavage into a mature SREBP-1c (68 kDa) [26]. The mature SREBP-1c can then translocate into the nucleus, bind to the sterol response element (SRE), and induce transcription of lipogenic genes (e.g., acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD-1)) [26,32–34]. In primary rodent hepatocytes and intact liver, insulin or feeding increases SREBP-1c and its targets and promotes *de novo* lipogenesis in a

manner that is sensitive to the mTORC1-specific inhibitor rapamycin [32,35,36]. Additionally, expression of constitutively active Akt or loss of either TSC1 or TSC2 results in insulin-independent activation of mTORC1 signaling, expression of SREBP-1c targets, and lipogenesis [29,37].

The activation of lipogenesis is dependent on insulin signaling [26]; however, obese rodents with severe hepatic insulin resistance have hyperactive mTORC1 and lipogenic activity, suggesting that insulin resistance does not affect the mTORC1 signaling cascade [26,38]. This hyperactivation of mTORC1 by overnutrition may promote lipogenesis by inducing SREBP-1c processing and explain why hepatic SREBP-1c remains active during liver insulin resistance [26]. Although mTORC1 activation *in vivo* is essential, it is not sufficient to stimulate hepatic SREBP-1c and targets in response to feeding [35,39]. For instance, mice with liver-specific Raptor knockout (constitutive mTORC1 inhibition) fail to induce SREBP-1c and lipogenesis [39,40]; however, mice with liver-specific TSC1 knockout (constitutive mTORC1 activation) revealed that mTORC1 is not capable of activating SREBP-1c and hepatic lipogenesis on its own [35]. This paradoxical scenario is likely a result of a strong negative feedback mechanism in chronic mTORC1 activation that attenuates Akt signaling. Consistent with this idea, restoration of Akt activity in liver-specific TSC1 knockout mice leads to SREBP-1c activation and lipogenesis [35].

The role of mTORC1 in lipid transport

There is mounting evidence suggesting that mTORC1 signaling is involved in lipid mobilization and transport. Conditions of overnutrition and obesity can lead to

chronically elevated mTORC1 signaling in liver and adipose, further contributing to the development of hypertriglyceridemia [41–43]. Evidence suggests that this phenomenon is a result of hyperglycemia and hyperinsulinemia associated with obesity [5]. In type 2 diabetes and insulin resistance, mTORC1 remains active likely due to persistent hyperinsulinemia leading to increased *de novo* lipogenesis [42]. Concomitantly, insulin resistance in adipose promotes slow, yet constant increase in lipolysis that causes an increase in FFA that are delivered to the liver [5]. The FFAs are then incorporated into TG and used to lipidate apoB100, allowing the apoB protein to evade degradation and be secreted from the liver as VLDL. Recent reports have also suggested that ER stress, potentiated through chronic mTORC1 activation, may play a role in the secretion of apoB from hepatocytes [44,45]. The ER is a dynamic organelle that plays an important role in protein and lipid metabolism. When the ER is under stress (e.g, accumulation of misfolded or unfolded proteins), the unfolded protein response (UPR) is initiated through three proteins: inositol-requiring enzyme 1, activating transcription factor-6 (ATF6), and protein kinase-like ER kinase (PERK) [45]. Activation of the UPR leads to translation arrest, degradation of misfolded proteins, expression of ER chaperones, and ER membrane expansion to increase the protein-folding capacity. Overnutrition appears to induce the ER stress response, activate SREBP-1, and increase intracellular lipid levels in an mTORC1-dependent manner [45]. Induction of ER stress and mTORC1 activity has also been shown to suppress sortilin-1, a protein that may be involved in post-Golgi regulation of apoB by trafficking the protein towards degradation pathways [44]. The

molecular mechanisms involved in mTORC1 regulation of ER stress, apolipoprotein expression, and metabolism remains to be elucidated.

The central role of mTORC1 in regulating lipid homeostasis and its persistent activation in obesity and type 2 diabetes suggests mTORC1 inhibitors would offer therapeutic benefits in metabolic diseases. However, patients treated with mTORC1 inhibitors experience increased insulin resistance, hypertriglyceridemia, hypercholesterolemia, and elevated plasma FFA [46]. Accordingly, plasma levels of both apoB100 and apoCIII were increased in rapamycin-treated patients [47], and rapamycin-treated guinea pigs experienced elevated circulating VLDL [48]. This phenomenon is thought to arise from a loss of the negative feedback on insulin signaling, allowing Akt to remain active and promote lipogenesis through SREBP-1c by suppressing Insig2a [35,49]. The negative side effects associated with direct inhibition of mTORC1 provide support for the identification of potential bioactive and therapeutic compounds capable of activating catabolism.

(R)-α-Lipoic acid

(R)-α-Lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid or thioctic acid, is a naturally occurring dithiol compound that is synthesized enzymatically from octanoic acid in most prokaryotic and eukaryotic microorganisms as well as in plant and animal mitochondria [50]. In the mitochondrion, LA serves as an essential cofactor for the lipoamide containing enzyme of aerobic metabolism [51]. Though *de novo* synthesis provides all the LA necessary for its function in the mitochondria, LA can also be absorbed from dietary sources such as muscle and organ meats (e.g., heart, liver), and to a

lesser degree dark green, leafy vegetables (e.g., spinach). In food, LA is present in a lipollysine form and ranges from 0.55 to 2.36 μg/g dry weight in meats [52] and 0.16 to 3.15 μg/g dry weight in vegetables [53]; however, a typical adult consuming a Western type diet, no LA can be detected in circulation [54]. Conversely, commercially available LA dietary supplements (ranging from 50 to 600 mg) are able to markedly increase plasma concentrations of LA to 1-225 μM, and are generally responsible for the positive health outcomes reported in the literature derived from LA supplements [54–58].

Growing evidence suggests that orally supplied LA promotes several biochemical activities with potential therapeutic value against a host of pathophysiological conditions, including diabetic polyneuropathies, inflammation, hepatic diseases [51]. LA has also been described as a potent biological antioxidant and a detoxification agent that is capable of scavenging a variety of reactive oxygen species, chelating transition metals, regenerating vitamins C and E, and increasing intracellular ubiquinone and reduced glutathione levels [51]. This array of cellular and molecular functions has stimulated considerable interest among the lay public and the research community for the use of LA both as a nutraceutical and as a pharmacotherapy. Recently, the TG-lowering properties of dietary LA have been recognized both in laboratory animals [59,60] and humans [61,62]. Despite these reports, the molecular mechanisms by which LA regulates blood TG remain ill defined.

4-Phenylbutyric acid

4-Phenylbutyric acid (PBA) is an aromatic short chain fatty acid (SCFA) and chemical chaperone commonly used to alleviate ER stress and facilitate proteostasis [63].

PBA is a chemical derivative of butyric acid, a SCFA produced via microbial fermentation in the gut. Like butyric acid, PBA has demonstrated considerable ability to regulate gene expression by inhibiting histone deacetylase activity (HDAC) [63]. Currently, sodium PBA was approved in 2013 by the US Food and Drug Administration for use as an ammonia scavenger in patients with urea cycle disorders [64].

There are an increasing number of studies that suggest that PBA, acting as a chaperone or HDAC inhibitor, can impart beneficial effects on several pathologies including cancer, metabolic syndrome, diabetes, and urea cycle disorders. There is also mounting evidence that PBA has anti-obesity and lipid-lowering properties [44,65–67]. For instance, PBA supplementation induced the expression of peroxisome proliferator-activated receptor alpha (PPARα) and genes involved in peroxisomal β-oxidation in livers of mice [65], while reducing lipogenic protein (e.g., ACC, FAS, SREBP-1, SCD-1) levels in livers of rats [66]. PBA also reduced plasma triacylglycerols in mice [65], hepatic steatosis, and body weights in rats [66,67].

REFERENCES

1. Pejic RN, Lee DT. Hypertriglyceridemia. J Am Board Fam Med. 2006;19(3):310–6.

2. Beltrán-Sánchez H, Harhay MO, Harhay MM, McElligott S. Prevalence and trends of Metabolic Syndrome in the adult US population, 1999–2010. J Am Coll Cardiol. 2013 Aug 20;62(8):697–703.

3. Abdel-Maksoud MF, Hokanson JE. The complex role of triacylglycerides in cardiovascular disease. Semin Vasc Med. 2002;2(3):325–33.

4. Ford ES, Giles WH, Dietz WH. Prevalence of the Metabolic Syndrome Among US Adults. Jama. 2002 Jan 16;287(3):356.

5. Haas ME, Attie AD, Biddinger SB. The regulation of ApoB metabolism by insulin. Trends in Endocrinology and Metabolism. 2013. p. 391–7.

6. Davidson NO, Shelness GS. APOLIPOPROTEIN B: mRNA editing, lipoprotein assembly, and presecretory degradation. Annu Rev Nutr. 2000;20:169–93.

7. Blasiole DA, Davis RA, Attie AD. The physiological and molecular regulation of lipoprotein assembly and secretion. Mol Biosyst. 2007;3(9):608–19.

8. Ginsberg HN. Synthesis and secretion of apolipoprotein B from cultured liver cells. Curr Opin Lipidol. 1995;6(5):275–80.

9. Yao Z, Tran K, McLeod RS. Intracellular degradation of newly synthesized apolipoprotein B. J Lipid Res. 1997;38(10):1937–53.

10. Jim Yeung S, Chen SH, Chan L. Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein B. Biochemistry. 1996;35(43):13843–8.

11. Liao W, Kobayashi K, Chan L. Adenovirus-mediated overexpression of microsomal triacylglyceride transfer protein (MTP): mechanistic studies on the role of MTP in apolipoprotein B-100 biogenesis. Biochemistry. 1999;38(23):7532–44.

12. Karimian Pour N, Adeli K. Insulin silences apolipoprotein B mRNA translation by inducing intracellular traffic into cytoplasmic RNA granules. Biochemistry. 2011;50(32):6942–50.

13. Parker R, Sheth U. P Bodies and the Control of mRNA Translation and Degradation. Molecular Cell. 2007. p. 635–46.

14. Fisher EA, Ginsberg HN. Complexity in the secretory pathway: The assembly and secretion of apolipoprotein B-containing lipoproteins. Journal of Biological Chemistry. 2002. p. 17377–80.

15. Qiu W, Kohen-Avramoglu R, Rashid-Kolvear F, Au CS, Chong TM, Lewis GF, et al. Overexpression of the Endoplasmic Reticulum 60 Protein ER-60 Downregulates ApoB100 Secretion by Inducing Its Intracellular Degradation via a Nonproteasomal Pathway: Evidence for an ER-60-Mediated and pCMB-Sensitive Intracellular Degradative Pathway. Biochemistry. 2004;43(16):4819–31.

16. Choi SH, Ginsberg HN. Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance. Trends in Endocrinology and Metabolism. 2011. p. 353–63.

17. Pan M, Liang JS, Fisher EA, Ginsberg HN. The late addition of core lipids to nascent apolipoprotein B100, resulting in the assembly and secretion of triacylglyceride-rich lipoproteins, is independent of both microsomal triacylglyceride transfer protein activity and new triacylglyceride synthesis. J Biol Chem. 2002;277(6):4413–21.

18. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. Cell. 2009;137(5):873–86.

19. Hara K, Maruki Y, Long X, Yoshino K ichi, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110(2):177–89.

20. Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino KI, et al. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J Biol Chem. 2003;278(18):15461–4.

21. Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KVP, Erdjument-Bromage H, et al. G??L, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell. 2003;11(4):895–904.

22. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, et al. PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. Mol Cell. 2007;25(6):903–15.

23. Laplante M, Sabatini DM. mTOR at a glance. J Cell Sci. 2009;

24. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous Sclerosis Complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003;13(15):1259–68.

25. Inoki K, Zhu T, Guan K-L. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577–90.

26. Laplante M, Sabatini D. An emerging role of mTOR in lipid biosynthesis. Curr Biol. 2009 Jul;19(22):R1046–52.

27. Laplante M, Sabatini DM. MTOR signaling in growth control and disease. Cell. 2012. p. 274–93.

28. White MF. The IRS-signalling system: A network of docking proteins that mediate insulin action. Mol Cell Biochem. 1998;182(1-2):3–11.

29. Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 2010;39(2):171–83.

30. Chong ZZ, Maiese K. Mammalian target of rapamycin signaling in diabetic cardiovascular disease. Cardiovasc Diabetol. 2012;11.

31. Jeon T-I, Osborne TF. SREBPs: metabolic integrators in physiology and metabolism. Trends Endocrinol Metab. 2012 Feb;23(2):65–72.

32. Brown NF, Stefanovic-Racic M, Sipula IJ, Perdomo G. The mammalian target of rapamycin regulates lipid metabolism in primary cultures of rat hepatocytes. Metabolism. 2007;56(11):1500–7.

33. Peng T, Golub TR, Sabatini DM. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol Cell Biol. 2002;22(15):5575–84.

34. Mauvoisin D, Rocque G, Arfa O, Radenne A, Boissier P, Mounier C. Role of the PI3-kinase/mTor pathway in the regulation of the stearoyl CoA desaturase (SCD1) gene expression by insulin in liver. J Cell Commun Signal. 2007;1(2):113–25.

35. Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, et al. Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. Cell Metab. 2011;14(1):21–32.

36. Li S, Brown MS, Goldstein JL. Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc Natl Acad Sci U S A. 2010;107(8):3441–6.

37. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. Cell Metab. 2008;8(3):224–36.

38. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146(3):1328–37.

39. Wan M, Leavens KF, Saleh D, Easton RM, Guertin DA, Peterson TR, et al. Postprandial hepatic lipid metabolism requires signaling through Akt2 independent

of the transcription factors FoxA2, FoxO1, and SREBP1c. Cell Metab. 2011;14(4):516–27.

40. Peterson TR, Sengupta SS, Harris TE, Carmack AE, Kang SA, Balderas E, et al. MTOR complex 1 regulates lipin 1 localization to control the srebp pathway. Cell. 2011;146(3):408–20.

41. Haas JT, Miao J, Chanda D, Wang Y, Zhao E, Haas ME, et al. Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression. Cell Metab. 2012;15(6):873–84.

42. Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. Endocrinology. 2005;146(3):1473–81.

43. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431(7005):200–5.

44. Ai D, Baez JM, Jiang H, Conlon DM, Hernandez-Ono A, Frank-Kamenetsky M, et al. Activation of ER stress and mTORC1 suppresses hepatic sortilin-1 levels in obese mice. J Clin Invest. 2012;122(5):1677–87.

45. Li H, Min Q, Ouyang C, Lee J, He C, Zou MH, et al. AMPK activation prevents excess nutrient-induced hepatic lipid accumulation by inhibiting mTORC1 signaling and endoplasmic reticulum stress response. Biochim Biophys Acta - Mol Basis Dis. 2014;1842(9):1844–54.

46. Kasiske BL, de Mattos A, Flechner SM, Gallon L, Meier-Kriesche H-U, Weir MR, et al. Mammalian target of rapamycin inhibitor dyslipidemia in kidney transplant recipients. Am J Transplant. 2008;8(7):1384–92.

47. Morrisett JD, Abdel-Fattah G, Hoogeveen R, Mitchell E, Ballantyne CM, Pownall HJ, et al. Effects of sirolimus on plasma lipids, lipoprotein levels, and fatty acid metabolism in renal transplant patients. J Lipid Res. 2002;43(8):1170–80.

48. Aggarwal D, Fernandez ML, Soliman GA. Rapamycin, an mTOR inhibitor, disrupts triacylglyceride metabolism in guinea pigs. Metabolism. 2006;55(6):794–802.

49. Ricoult SJH, Manning BD. The multifaceted role of mTORC1 in the control of lipid metabolism. EMBO Rep. 2013;14(3):242–51.

50. Herbert AA, Guest JR. Lipoic acid content of Escherichia coli and other microorganisms. Arch Microbiol. 1975 Dec 31;106(3):259–66.

51. Shay KP, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. Biochim Biophys Acta. Elsevier B.V.; 2009 Oct;1790(10):1149–60.

52. Kataoka H, Hirabayashi N, Makita M. Analysis of lipoic acid by gas chromatography with flame photometric detection. Methods Enzymol. 1997 Jan;279:166–76.

53. Lodge JK, Youn H-D, Handelman GJ, Konishi T, Matsugo S, Mathur V V, et al. Natural sources of lipoic acid: determination of lipoyllysine released from protease-digested tissues by high performance liquid chromatography incorporating electrochemical detection. J Appl Nutr. 1997;49:3–11.

54. Hermann R, Niebch G, Borbe HO, Fieger-Büschges H, Ruus P, Nowak H, et al. Enantioselective pharmacokinetics and bioavailability of different racemic α-lipoic acid formulations in healthy volunteers. Eur J Pharm Sci. 1996 May;4(3):167–74.

55. Breithaupt-Grögler K, Niebch G, Schneider E, Erb K, Hermann R, Blume HH, et al. Dose-proportionality of oral thioctic acid — coincidence of assessments via pooled plasma and individual data. Eur J Pharm Sci. 1999 Apr;8(1):57–65.

56. Teichert J, Tuemmers T, Achenbach H, Preiss C, Hermann R, Ruus P, et al. Pharmacokinetics of alpha-lipoic acid in subjects with severe kidney damage and end-stage renal disease. J Clin Pharmacol. 2005 Mar;45(3):313–28.

57. Mignini F, Streccioni V, Tomassoni D, Traini E, Amenta F. Comparative crossover, randomized, open-label bioequivalence study on the bioequivalence of two formulations of thioctic acid in healthy volunteers. Clin Exp Hypertens. 2007 Nov;29(8):575–86.

58. Carlson DA, Smith AR, Fischer SJ, Young KL, Packer L. The plasma pharmacokinetics of R-(+)-lipoic acid administered as sodium R-(+)-lipoate to healthy human subjects. Altern Med Rev. 2007 Dec;12(4):343–51.

59. Butler JA, Hagen TM, Moreau R. Lipoic acid improves hypertriglyceridemia by stimulating triacylglycerol clearance and downregulating liver triacylglycerol secretion. Arch Biochem Biophys. 2009;485(1):63–71.

60. Kim M-S, Park J-Y, Namkoong C, Jang P-G, Ryu J-W, Song H-S, et al. Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat Med. 2004;10(7):727–33.

61. Zhang Y, Han P, Wu N, He B, Lu Y, Li S, et al. Amelioration of lipid abnormalities by α-lipoic acid through antioxidative and anti-inflammatory effects. Obesity (Silver Spring). 2011;19(8):1647–53.

62. Alken RG, Koegst D, Fries G. Treatment of lipid metabolic disorders using 5-(1,2-dithiolan-3-yl) valeric acid (alpha-lipoic acid) or its physiologically compatible salts. Google Patents; 2002.

63. Cuadrado-Tejedor M, Ricobaraza AL, Torrijo R, Franco R, Garcia-Osta A. Phenylbutyrate is a multifaceted drug that exerts neuroprotective effects and reverses the Alzheimer´s disease-like phenotype of a commonly used mouse model. Curr Pharm Des. 2013;19(28):5076–84.

64. Lichter-Konecki U, Diaz GA, Merritt JL, Feigenbaum A, Jomphe C, Marier JF, et al. Ammonia control in children with urea cycle disorders (UCDs); Phase 2 comparison of sodium phenylbutyrate and glycerol phenylbutyrate. Mol Genet Metab. 2011;103(4):323–9.

65. Weng H, Endo K, Li J, Kito N, Iwai N. Induction of Peroxisomes by Butyrate-Producing Probiotics. Lobaccaro J-MA, editor. PLoS One. San Francisco, CA USA: Public Library of Science; 2015 Feb 6;10(2):e0117851.

66. Ren LP, Song GY, Hu ZJ, Zhang M, Peng L, Chen SC, et al. The chemical chaperon 4-phenylbutyric acid ameliorates hepatic steatosis through inhibition of de novo lipogenesis in high-fructose-fed rats. Int J Mol Med. 2013;32(5):1029–36.

67. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006;313(5790):1137–40.

CHAPTER 2

mTORC1 CONSTITUTIVE INHIBITION AND ACTIVATION: A MODEL AND ITS CHARACTERIZATION

INTRODUCTION

Hypertriglyceridemia is a serious public health issue that affects over 24% of Americans [1]. Defined as abnormally high fasting plasma triacylglycerols (TG; >150 mg/dL or 1.8 mM), hypertriglyceridemia is a comorbidity of obesity and type 2 diabetes and is a significant risk factor of cardiovascular disease [1,2]. Increased assembly and secretion of very low-density lipoproteins (VLDL) from the liver in obesity is thought to drive the development of hypertriglyceridemia. This process involves elevated lipidation of apolipoprotein-B100 (apoB; an essential protein of VLDL) from hepatic derived TG, which is then secreted into circulation. VLDL assembly and secretion pathways are well studied; however, the molecular mechanisms behind the pathogenesis of hypertriglyceridemia in obesity are ill defined.

The mechanistic target of rapamycin (mTORC1) is a critical regulator of anabolic metabolism and is highly active in the livers of obese rodents [3]. mTORC1 is a threonine/serine kinase that is structurally composed of mechanistic target of rapamycin (mTOR), regulatory-associated protein of mTOR (Raptor), and mammalian lethal with Sec13 protein 8 (mLST8), whereas inactive mTORC1 includes the aforementioned proteins plus DEP-domain-containing mTOR-interacting protein (Deptor) and proline-rich Akt substrate 40 kDa (PRAS40) [4,5]. mTORC1 is controlled by the upstream GTPase-activating protein tuberous sclerosis complex (TSC) (TSC1/2) comprised of TSC1 (hamartin) and TSC2 (tuberin) [5–7]. TSC1/2 negatively regulates mTORC1 activity by increasing the levels the GDP-bound Ras homolog enriched in brain (Rheb) [8]. Conversely, when TSC1/2 is inhibited, Rheb is GTP-bound and can bind to and

activate mTORC1 [8]. mTORC1 has emerged as a critical coordinator between nutritional status and cellular growth machinery. In overnutrition mTORC1 is constitutively active leading to increased activity of its downstream effectors, including p70 S6 kinase 1 (p70 S6K1) [5,8]. Activation of mTORC1 also induces the expression of hepatic SREBP-1c and lipogenesis [9,10]. Additionally, hyperinsulinemic obese mice experience hyperactive hepatic mTORC1 signaling and increased levels of circulating VLDL and TG [11–13]. The exact mechanism in which mTORC1 potentiates the development of hypertriglyceridemia are unknown. These observations led us to investigate the effects of constitutive activation or inhibition or mTORC1 activity in the secretion of apoB-containing lipoproteins and TG from hepatocytes.

MATERIALS AND METHODS

Cell Culture

HepG2 cells were purchased from American Tissue Culture Collection (Manasssa, VA). Lenti-X 293T cells were purchased from Clontech Laboratories (Mountain View, CA). Huh7 cells were kindly provided by Dr. Qiaozhu Su (University of Nebraska-Lincoln, NE, USA). HepG2 cells were maintained in low glucose (5.5 mM) Dulbecco's Modified Eagle's Medium (#11885-076; Life Technologies) supplemented with 10% fetal bovine serum (FBS) (#30-2020; ATCC) and antibiotics/antimycotics (#A5955; Sigma). Lenti-X 293T cells were maintained in high glucose (25 mM) Dulbecco's Modified Eagle's Medium (#11995-040; Life Technologies) supplemented with 10% FBS and antibiotics/antimycotics. Huh7 cells were maintained in high glucose

(25 mM) Dulbecco's Modified Eagle's Medium (#11995-040; Life Technologies) supplemented with 10% FBS, antibiotics/antimycotics, and Hyclone MEM-nonessential amino acids (Thermo Scientific). All cells were maintained at 37^{o}C in 5% CO_2 with medium renewal ever 3-4 days. For experimental treatments, stably transduced HepG2 cells were washed twice with PBS, serum-starved overnight (~16 hrs), and treated as indicated in corresponding figure legends in serum-free DMEM (5.5 mM or 30 mM glucose).

Lentivirus-mediated Short Hairpin RNA Silencing

pLKO.1 plasmids containing shRNA expression cassettes were purchased from Addgene (Cambridge, MA). The oligonucleotides encoding the short hairpin RNA (shRNA) transgene targeting Raptor (#1858; Addgene) [14] and TSC2 (#15478; Addgene) [15] have been previously described. A scramble (#1864; Addgene) [14] shRNA sequence was used as a negative control. Lentivirus were produced by transfecting Lenti-X 293T cells grown in 6-well dishes with 1 µg of the pLKO.1 plasmid containing the appropriate shRNA and Lenti-vpack packaging kit (#TR30022; OriGene) for 18 hours. Two batches of virus containing supernatants were collected at 48 and 72 hours after transfection, combined, and concentrated using Lenti-X concentrator (#631231; Clontech). Viral titers were then calculated using Lenti-X qRT-PCR Titration Kit (#631235; Clontech). Target cells grown in 12-well dishes were infected using a multiplicity of infection of 10 in the presence of 8 µg/ml Polybrene (Sigma) for 24 hours then selected for with 2 µg/ml puromycin (Sigma) for 5 days. Cells were analyzed for knockdown efficiency 120 hours post-infection. Polyclonal stably transduced HepG2 and

Huh7 cells were generated by infecting cells with shRNA-containing lentivirus, and selection with puromycin (2 µg/ml) for 2 weeks.

Cell lysis and Western blotting

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and solubilized in modified RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na deoxycholate, 1% Igepal, 1 mM DTT) supplemented with Halt protease and phosphatase inhibitors (Thermo Scientific). The cells were then scraped, vortexed for 1 minute, and then clarified by centrifugation for 10 min at 14,000 x g at 4ºC. Total protein concentration of clarified supernatants was determined using the Pierce BCA Assay (Thermo Scientific) and Bio-Tek µQuant plate reader. Samples were then adjusted to appropriate concentrations using ice-cold PBS and SDS loading buffer supplemented with 100 mM DTT, and denatured for 5 min at 95ºC. Secreted proteins in conditioned media were prepared by adding Halt protease inhibitors (Thermo Scientific), vortexing, and centrifugation for 10 min at 14,000 x g at 4ºC. The clarified media was then transferred to a fresh tube, vortexed briefly, and equal volumes denatured with SDS loading buffer supplemented with 100 mM DTT denatured for 5 min at 95ºC, and subjected to Western blot analysis. Briefly, proteins were resolved by reducing SDS-PAGE, transferred onto a nitrocellulose membrane (LI-COR), blocked for 1 hour at room temperature in Odyssey Blocking Buffer (LI-COR) in TBS, and probed overnight at 4ºC with respective primary antibody. Antibodies against Raptor (#2280), TSC2 (#4308), p70 S6K (#2708), phospho-p70 S6K (Thr389; #9234), and FASN (#3180) were purchased from Cell Signaling (Danvers, MA); ApoB (#71307) and ApoE (#71707) from Midland

Bioproducts (Boone, IA); β-Actin (#A5441) from Sigma-Aldrich (St. Louis, MO). IRDye secondary antibodies were purchased from LI-COR (Lincoln, NE). HRP-conjugated secondary antibodies against rabbit, mouse, and goat were purchased from R&D Systems, Cell Signaling, and Santa Cruz Biotechnology, respectively. Blots were visualized using either the LI-COR Odyssey scanner or enhanced chemiluminescence (ECL) on the ProteinSimple imaging system. All band densitometry quantification was completed with the LI-COR Image Studio Lite software.

Measurement of intracellular and secreted TG content

Lipids were extracted from stably transduced HepG2 cells and conditioned media (2 mL) using the chloroform/methanol method previously described with modifications [16]. TG were then quantified enzymatically using Triacylglycerol determination reagents (Sigma) and Bio-Tek µQuant plate reader.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from cells using BioRad Aurum Total RNA Mini Kit. First strand cDNA was synthesized from 1 µg total RNA with oligo (dT) and random primers using BioRad iScript reverse transcription supermix according to manufacturer's instructions. All qRT-PCR were performed on a BioRad CFX96 Real-Time PCR Detection System using Sso Advanced SYBR Green supermix (BioRad). Amplicons were confirmed by melt curve analysis and agarose gel electrophoresis. PPIA was used as the internal reference gene to normalize expression levels of all targets. Primer sequences are given in Table 2.1.

Table 2.1. Oligonucleotide primer sequences used for qRT-PCR.

Gene	Sequence (5'→3')[a]	Product Size (bp)	GenBank[b]	Ref.
RPTOR	TGGAGTCCGAAATGCTGCAA ACTGACTGTCTTCATCCGATCC	176	NM_001163034.1	
TSC2	GGCAAGAGAGTAGAGAGGGACG AAGAAGGGGGAATGGTAGAGC	116	NM_000548.3	[25]
APOB	CAGCTGATTGAGGTGTCCAG CACTGGAGGATGTGAGTGGA	86	NM_001159323.1	
SREBF1	CAGCCCCACTTCATCAAGG ACTGTTGCCAAGATGGTTCCG	161	NM_004176.4	[26]
MTTP	TGTGGCCTTACTATGGAGGAA AAGGAGCGTAGGTCTTTGCAG	184	NM_000253.2	[27]
PPIA	TTCATCTGCACTGCCAAGAC TGTCCACAGTCAGCAATGGT	152	NM_001300981.1	
FASN	ACAGCGGGGAATGGGTACT GACTGGTACAACGAGCGGAT	188	NM_004104.4	
DGAT1	GTTATTGCGGCCAATGTCTT AACCAGTAAGACCACAGCCG	150	NM_012079.5	
DGAT2	CAGGTCATCTCAGTGCTCCA TCCAGTCAAACACCAGCCAA	139	NM_032564.4	

[a] Shown as sense primer followed by antisense primer
[b] GenBank accession number

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance was determined as denoted in figure legends using an unpaired two-tailed Student's *t* test, One-Way ANOVA Tukey's Multiple Comparisons Test, or Two-Way ANOVA with Bonferroni Post-Test. All statistical tests were performed at the 5% significance level.

RESULTS

Lentiviral-mediated shRNA transduction transiently decreased Raptor and TSC2 expression in HepG2 and Huh7 cells.

HepG2 and Huh7 were transiently infected using lentiviral-mediated gene delivery with an MOI of 10 to determine if the shRNAs targeting Raptor (shRaptor) and TSC2 (shTSC2) would effectively reduce the expression of the respective mRNAs and proteins. shRaptor significantly decreased mRNA levels by 78% and 61% in HepG2 and Huh7 cells, respectively when compared to shScramble control. Raptor protein levels were also markedly lower in both HepG2 and Huh7 cells (Fig. 2.1, A). shTSC2 significantly decreased mRNA levels of TSC2 by 74% and 73%, respectively when compared to scramble controls, and equated to lower TSC2 protein in HepG2 and Huh7 cells (Fig. 2.2, B).

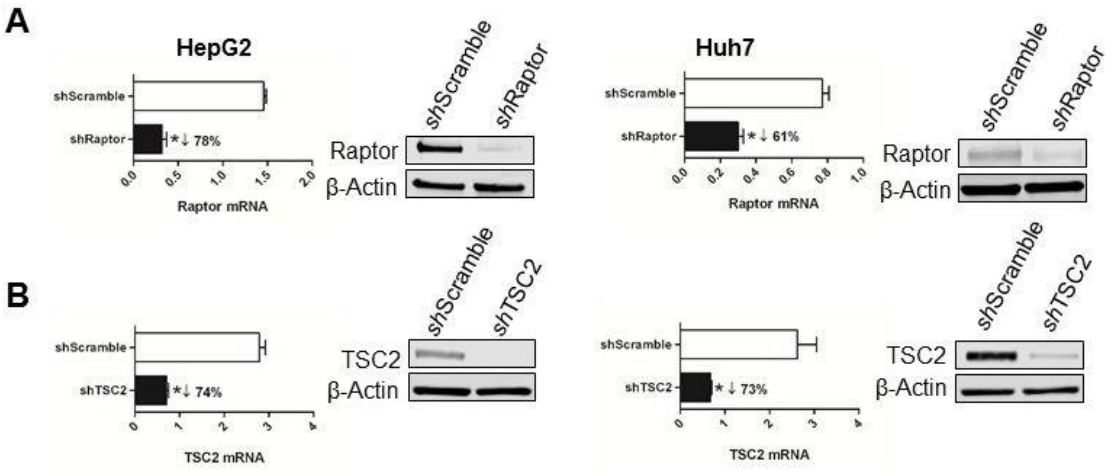

Fig. 2.1. Validation of lentiviral-mediated knockdown of TSC2 and Raptor at the mRNA and protein level in HepG2 and Huh7 cells. Raptor and TSC2 mRNA levels were normalized to the housekeeping gene *PPIA*; whereas, Raptor and TSC2 protein levels were normalized to β-Actin. Data represent mean ± SEM, n = 4. (**A**) The Raptor shRNA significantly reduced Raptor mRNA levels in both HepG2 and Huh7 cell lines by 78% and 61%, respectively. Raptor protein levels were also reduced in both cell lines. (**B**) The TSC2 shRNA significantly reduced TSC2 mRNA levels in both HepG2 and Huh7 cell lines by 74% and 73%, respectively. TSC2 protein levels were also reduced in both cell lines. Statistical significance was determined using an unpaired Student's *t*-test. **P* < 0.05 versus scramble control.

Knockdown of Raptor and TSC2 was maintained in stably transduced HepG2 cells, and leads to modulation of mTORC1 activity

Stably transduced HepG2 cell lines containing shScramble, shRaptor, or shTSC2 were created to increase reproducibility of downstream experiments and analyses. The shRaptor cells had a 69% and 90% knockdown of Raptor mRNA and protein levels, respectively (Fig. 2.2, A); whereas the shTSC2 cells had a 71% and 72% knockdown of TSC2 mRNA and protein levels, respectively (Fig. 2.2, B). Next, we examined the phosphorylation state of p70 S6K, an mTORC1 downstream effector, to determine if the suppression of Raptor and TSC2 affects mTORC1 activity. Knockdown of Raptor significantly decreased the kinase activity of mTORC1 as denoted by 29% lower phospho-p70 S6K (Fig. 2.2, A). Conversely, knockdown of TSC2 significantly increased the phosphorylation of p70 S6K by 82% suggesting heightened mTORC1 kinase activity (Fig. 2.2, B).

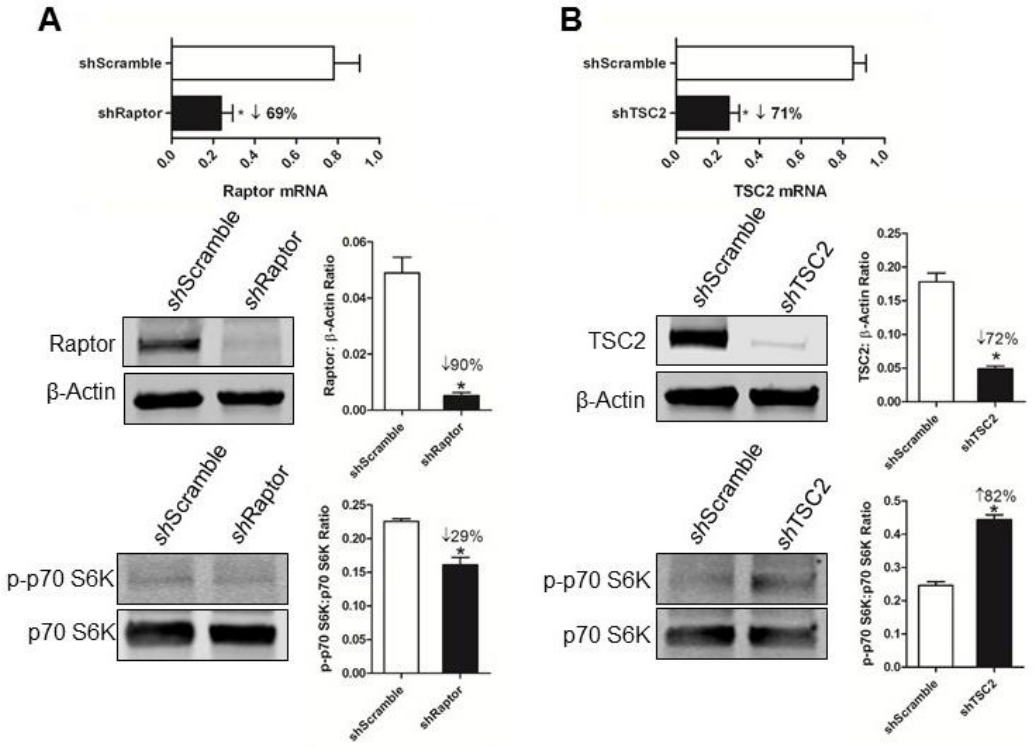

Fig 2.2. Knockdown of Raptor and TSC2 is maintained in stably transduced HepG2 cells, leading to modulation of mTORC1 activity. Raptor and TSC2 mRNA levels were normalized to housekeeping gene *PPIA*. Raptor and TSC2 protein were normalized to β-Actin, whereas phospho-p70 S6K was normalized to total p70 S6K. Data represent mean ± SEM, n = 4. (**A**) Raptor cells have a 69% decrease in Raptor mRNA levels, and a 90% reduction of Raptor protein. The knockdown of Raptor lead to a 29% reduction in phosho-p70 S6K (Thr389). (**B**) TSC2 protein levels were reduced by 72% in stable TSC2 cells. TSC2 cells have a 71% decrease in TSC2 mRNA levels. The knockdown of TSC2 lead to an 82% increase in phosho-p70 S6K (Thr389). Statistical significance was determined using an unpaired Student's *t*-test. $*P < 0.05$ versus scramble control.

Serum withdrawal does not alter cellular morphology or differences in mTORC1 activity

Fetal bovine serum (FBS), a common additive to cell culture mediums, contains a wide variety of growth factors needed for survival and propagation of mammalian cells. While FBS is essential to maintaining healthy cultures, it can contain a variety of factors

(e.g., fatty acids, cytokines) that can impact the outcome of cellular experiments. In order to control for this potential confounding factor, we conducted our experiments in medium devoid of FBS. Culturing stably transduced HepG2 cells in serum-free medium did not negatively impact cellular morphology (Fig. 2.3). Additionally, the percent knockdown of Raptor and TSC2 protein levels was not affected by serum withdrawal (Fig. 2.4), and the percent decrease in phosphorylation of p70 S6K in shRaptor cells was not significantly different between serum and serum-free conditions (Fig. 2.4). The phosphorylation of p70 S6K remained elevated in shTSC2 cells under both serum conditions, however, shTSC2 cells cultured without FBS for 48 hrs exhibited a 25% increase in p70 S6K phosphorylation when compared to the p-p70 S6K increase in FBS treated cells (Fig. 2.4).

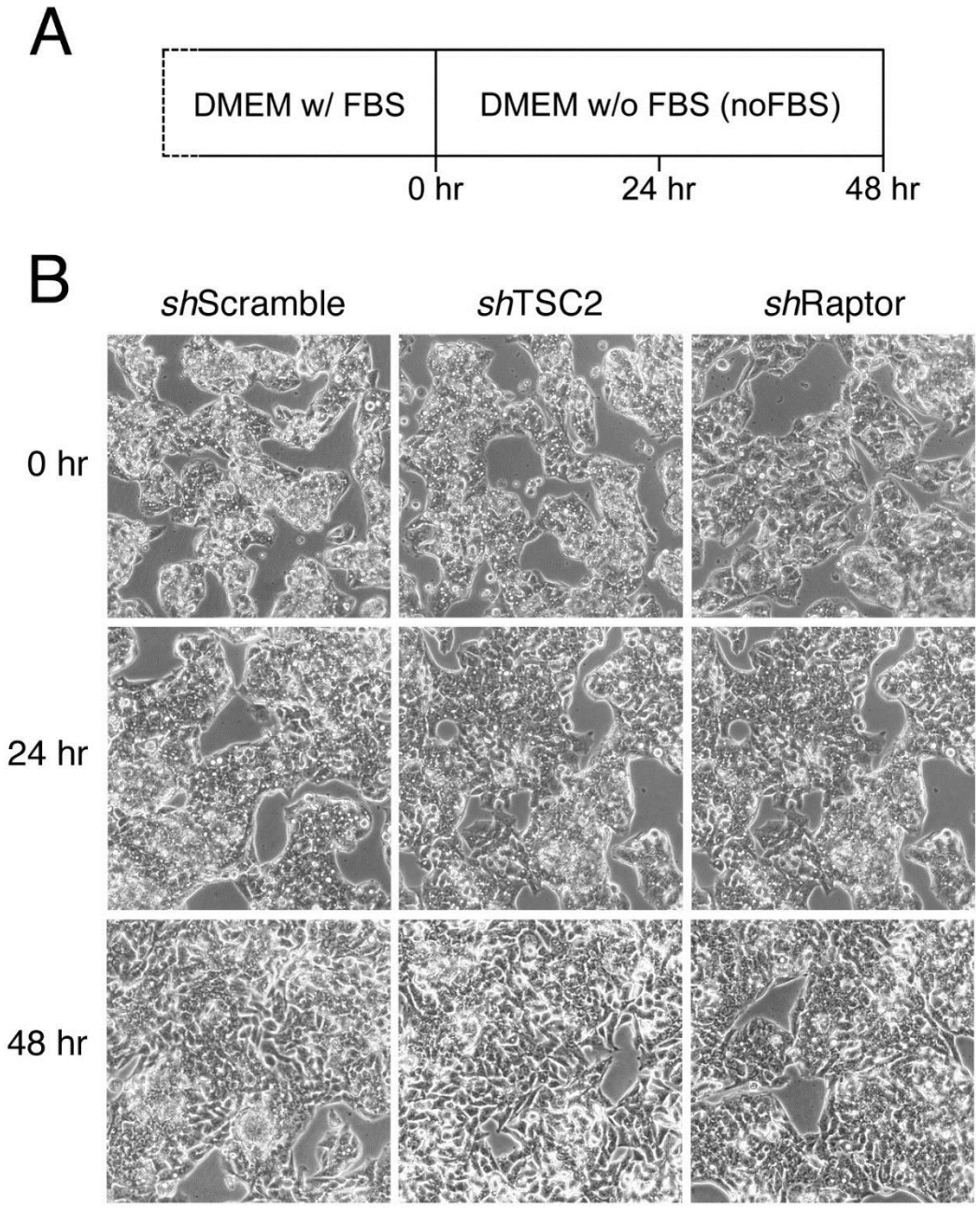

Fig. 2.3. Morphology of stably transduced HepG2 cells remains consistent during serum withdrawal. (**A**) Experimental timeline of serum withdrawal. (**B**) The morphology of stably transduced scramble, TSC2, and Raptor HepG2 cells remains consistent over a 48 hr serum withdrawal period.

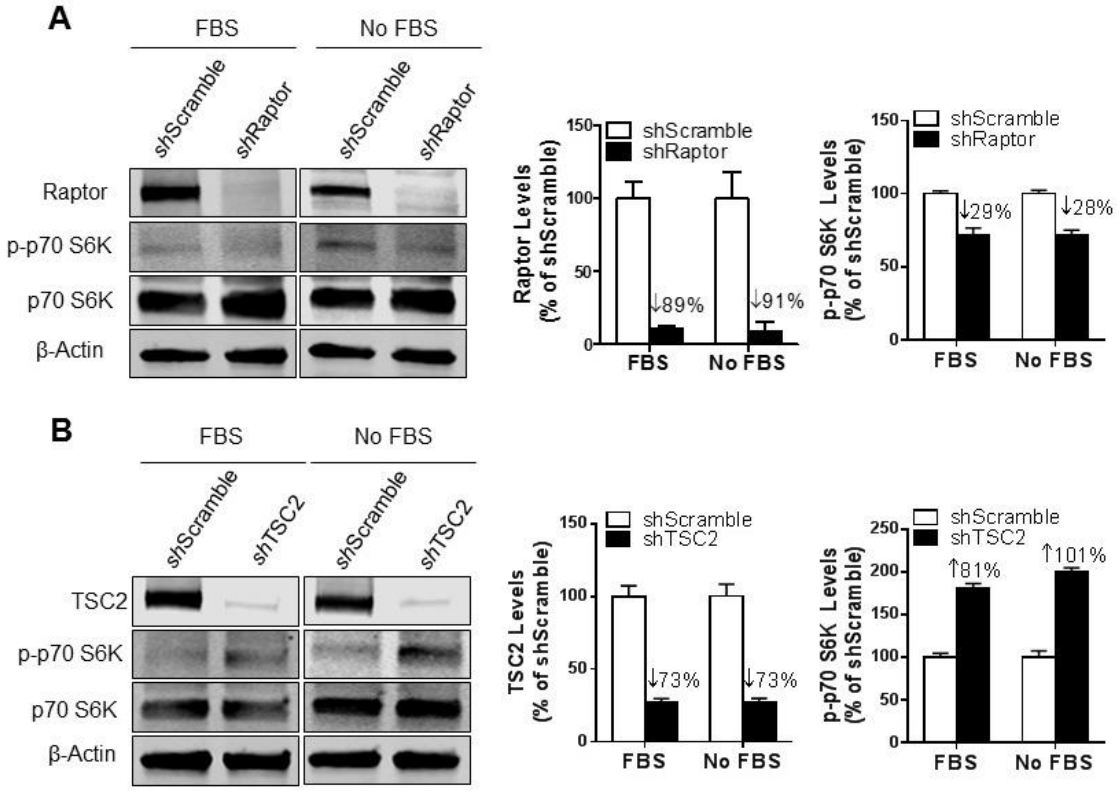

Fig. 2.4. Serum withdrawal does not alter changes in knockdown or mTORC1 activity. Cells were cultured in 5.5 mM glucose with or without FBS for 48 hrs, then harvested for total cellular proteins. Raptor and TSC2 protein levels were normalized to β-Actin, whereas p-p70 S6K was normalized to total p70 S6K. Data represent mean ± SEM, n = 4. (**A**) The removal of FBS from stably transduced shRaptor cells did not affect knockdown of Raptor ($F = 0.01$, $P = 0.9395$) or the phosphorylation of p70 S6K ($F = 0.0$, $P = 0.9719$). (**B**) Culturing shTSC2 cells did not affect knockdown of TSC2 ($F = 0.0$, $P = 1$) or the phosphorylation of p70 S6K ($F = 3.09$, $P = 0.1042$). Statistical significance was determined using a two-way ANOVA, with Bonferroni Post-Test.

Knockdown of TSC2 increases the expression of genes involved in lipogenesis and lipid transport

mTORC1 has emerged as a critical regulator in lipid homeostasis and its activation can induce *de novo* lipogenesis [17]. We examined the effects of genetic manipulation of mTORC1 activity on the expression of genes involved in lipid synthesis and mobilization. SREBP-1 (+51%) and fatty acid synthase (*FASN*; +55%) message

levels trended higher in shTSC2 cells, though the results did not reach significance (Fig. 2.5, A). Transcript levels of diacylglycerol O-acyltransferase protein 1 (*DGAT1*) and 2 (*DGAT2*) were significantly increased by 97% and 229% in shTSC2 cells, respectively when compared to shScramble (Fig. 2.5, A). The message levels of *SREBP1*, *FASN*, *DGAT1*, and *DGAT2* were not significantly affected by suppression of Raptor. Considering hepatic derived lipids are synthesized for storage and export, we examined the levels of two genes essential for the export of hepatic TG: microsomal triacylglyceride transfer protein (MTP) and apolipoprotein B (apoB). Knockdown of TSC2 led to a 130% and 182% significant increase in MTP and apoB message levels, respectively (Fig. 2.5, A).

We did not observe significant differences in FASN protein levels, a target gene of SREBP-1, (Fig. 2.5, B) which is consistent with the non-significant changes in *FASN* mRNA levels. There were significant differences in the cellular TG content between the three cell lines. Suppression of TSC2 significantly increased TG (+26.8%), whereas knockdown of Raptor significantly decreased cellular TG (-42.9%).

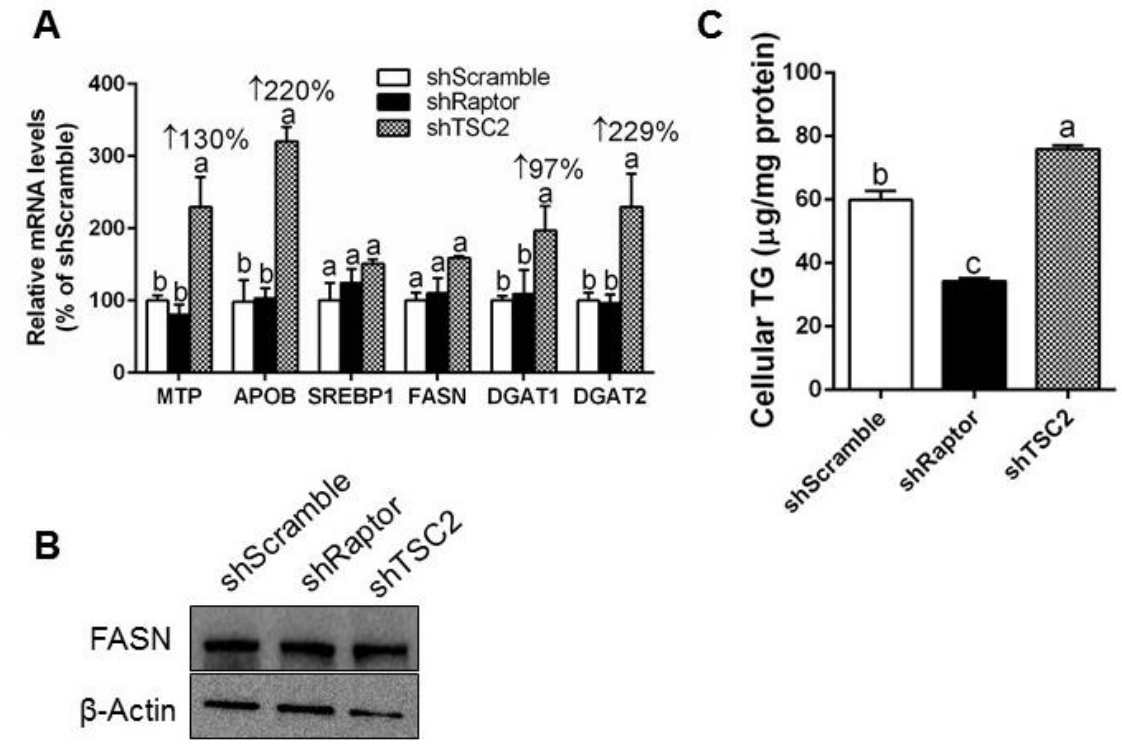

Fig. 2.5. mTORC1 activation increases the expression of genes involved in lipogenesis and lipid transport, and cellular triacylglycerides. Cells were treated with 30 mM glucose for 48 hrs and then harvested for total mRNA and protein. mRNA levels were normalized to the housekeeping gene *PPIA*. Protein levels were normalized to β-Actin. Data represent mean ± SEM, n = 3. (**A**) Gene expression of *MTP* (+130%), *APOB* (+220%), *DGAT1* (+97%), and *DGAT2* (+229%) were significantly higher in shTSC2 cells while *SREBP1* (+51%) and *FASN* (+55%) mRNA levels trended higher but did not reach significance. (**B**) FASN protein levels were not significantly different between the three stable lines. (**C**) The knockdown of TSC2 increased cellular TG content by 26.8%, while knockdown of Raptor decreased cellular TG by 42.9% when compared to shScramble (n = 4). Statistical significance was determined using a One-Way ANOVA with Tukey's Multiple Comparisons Test. Means without a common letter differ, $P < 0.05$.

mTORC1 is involved in lipogenesis and secretion of apoB-containing lipoproteins and TG

Previous reports have suggested that mTORC1 hyperactivation increases VLDL and TG secretion in rodents [11–13]. apoB is required for VLDL assembly and secretion,

and is used as a marker of VLDL levels in circulation; thus we examined secreted apoB and apoE protein levels in the media of cells treated with 5.5 mM and 30 mM glucose to determine if mTORC1 activity impacts secretion of VLDL. shRaptor cells secreted significantly less apoB under 5.5 mM (-60.3%) and 30 mM (-71%) glucose conditions, and secreted less apoE (-61.3%) under 30 mM glucose (Fig. 2.6, A). Hyperactivation of mTORC1 in shTSC2 cells promoted the secretion of significantly more apoB (+124.3%) under 30 mM glucose conditions. In euglycemic conditions (5.5 mM glucose), secreted apoB from shTSC2 was not significantly higher than the scramble control (Fig. 2.6, A). Secreted TG (Fig. 2.6, C) followed a similar trend to apoB, in that TG levels were lower (-39.7%) in the shRaptor cells and higher (+43.6%) in the shTSC2 cells.

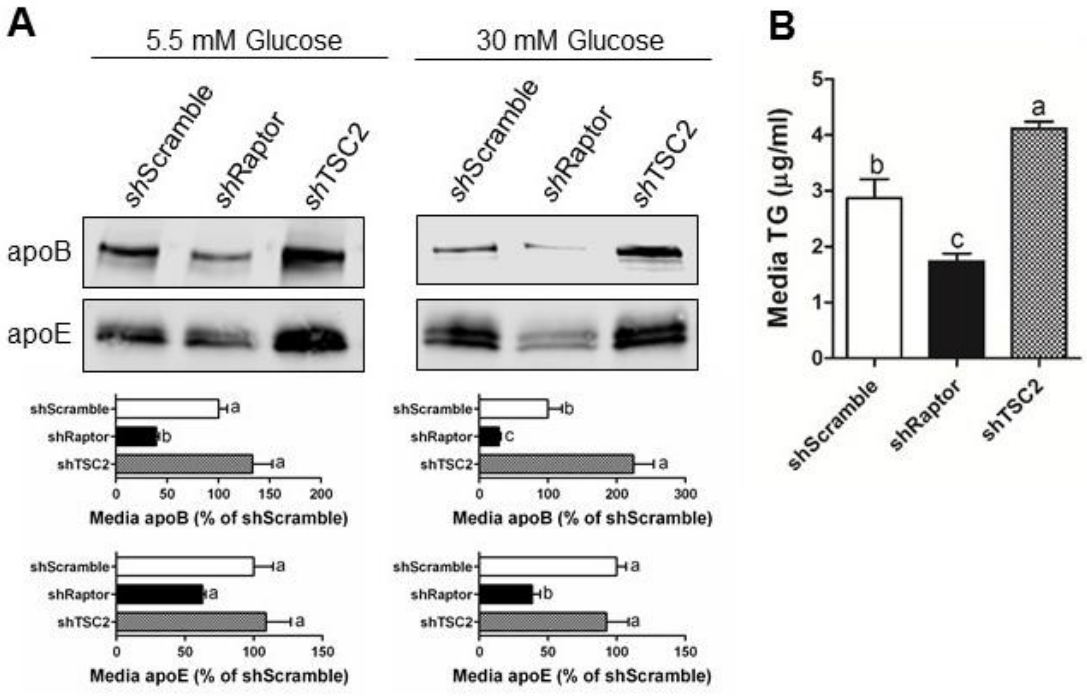

Fig. 2.6. Chronic activation of mTORC1 increases secretion of apoB-containing lipoproteins and triacylglycerides under high glucose conditions. Data represent mean ± SEM. (**A**) Activation of mTORC1 through TSC2 knockdown increased (+124.3%) secretion of apoB-containing lipoproteins under high glucose conditions; conversely, mTORC1 inhibition through Raptor knockdown decreased secretion of apoB-containing lipoproteins under low (-60.3%) and high (-71%) glucose conditions. Secreted apoE levels from TSC2 cells were not significantly different in either glucose condition; however, suppression of Raptor significantly reduced (-61.3%) apoE secretion under high glucose (n = 3). (**B**) The activation of mTORC1 through TSC2 knockdown increased secretion of TG by 43.6%, whereas knockdown of Raptor reduced the secretion of TG by 39.7% when compared to Scramble control (n = 6). Statistical Significance was determined using a One-Way ANOVA, with Tukey's Multiple Comparison Test. Means without a common letter differ, $P < 0.05$.

DMSO does not alter elevated secretion of apoB from shTSC2 cells or affect trends observed between shScramble, shRaptor, and shTSC2 cells

Dimethyl sulfoxide (DMSO) is an organosulfur compound commonly used as an organic solvent for polar and nonpolar compounds. DMSO will be used as a vehicle for bioactive compound delivery in future studies, thus it was important to determine if the DMSO would alter apoB secretion patterns observed in the stable cells. Secreted apoB and apoE levels from shTSC2 cells treated with 0.1% DMSO (24 hrs) did not change (Fig. 2.7, A). Further, the changes in apoB and apoE secretion seen between the three stable cell lines were also observed in the presence of 0.1% DMSO (Fig. 2.7, B).

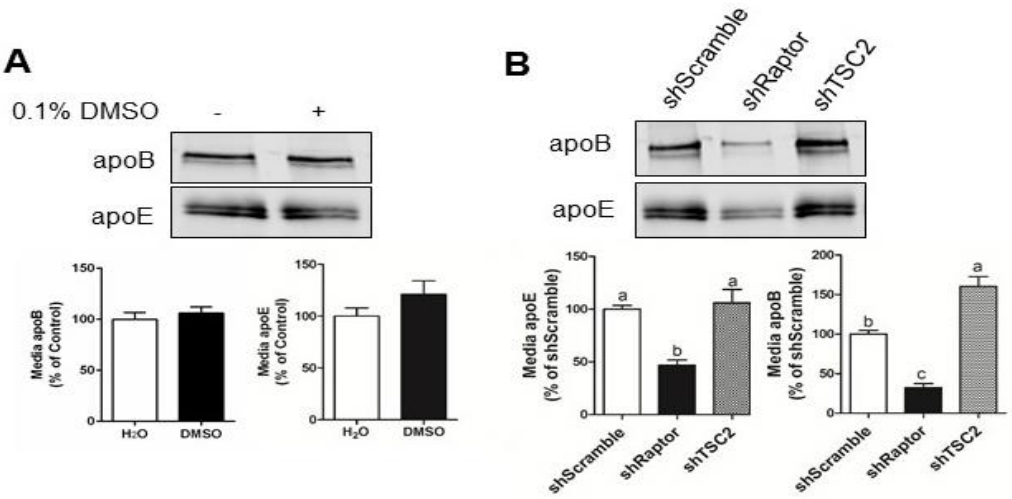

Fig. 2.7. mTORC1-driven secretion of apoB remains elevated when treated with DMSO under high glucose. Data represent mean ± SEM. (**A**) shTSC2 cells were treated with 0.1% DMSO or water control for 24 hrs in 30 mM glucose medium. The addition of DMSO did not significantly impact ($P > 0.05$) the secretion of apoB-containing lipoproteins from shTSC2 cells (n = 4). Statistical Significance was determined using an unpaired Student's *t*-test. (**B**) Stably transduced shScramble, shRaptor, and shTSC2 cells were treated with 0.1% DMSO in 30 mM glucose for 24 hrs. When compared to shScramble control shTSC2 cells secreted 60% more apoB-containing lipoproteins; conversely, shRaptor cells secreted 68% less apoB-containing lipoproteins (n = 3). Statistical Significance was determined using a One-Way ANOVA, with Tukey's Multiple Comparison Test. Means without a common letter differ, $P < 0.05$.

DISCUSSION

Overnutrition is strongly associated with obesity and is thought to promote the development of hypertriglyceridemia. However, mechanisms by which nutrient excess promote the development of hypertriglyceridemia are not well defined. Recently, a master regulator of anabolic metabolism, mTORC1, was shown to be hyperactive in the livers of obese rodents and to be activated in states of nutrient and energy excess [18,3,19]. This observation led us to develop a liver cell model in which to manipulate mTORC1 activity and evaluate the effects of dietary bioactive compounds on mTORC1 signaling and its consequences. Thus, this study was designed to accomplish two distinct aims: 1) create and characterize a model of mTORC1 hyperactivation and inhibition; and 2) validate experimental conditions for future studies.

Many studies of human apoB metabolism have utilized the HepG2 cell line, however these cells secrete relatively dense, lipid poor lipoproteins that resemble LDL. This is due to the cell's limited ability to fully lipidate apoB and secrete VLDL-sized particles [20]. An alternative human hepatoma cell line, Huh7, is being increasingly used to study apoB and VLDL metabolism [20–22]. Thus we validated the efficiency of shRNA-mediated knockdown of TSC2 and Raptor as models of mTORC1 hyperactivation and chronic inhibition, respectively in both cell lines. Transient transduction of the cell lines effectively decreased the expression of Raptor and TSC2 message and protein in HepG2 and Huh7 cells; however, the knockdown of each target was greater in HepG2 cells. The higher degree of gene silencing, along with the fact that Huh7 cells do not offer any advantage over HepG2 cells as a model of lipoprotein

metabolism [20] led us to create stably transduced HepG2 cell lines for shScramble, shRaptor, and shTSC2. The degree of target gene suppression remained high in stably transduced HepG2 cells, and resulted in significant modulations in mTORC1 kinase activity, as measured by phosphorylated p70 S6K levels. These results are consistent with previous reports that demonstrate activation of mTORC1 through disruption of the TSC1/2 complex, [23–25] and inhibition of mTORC1 through Raptor knockdown [26,27].

Genetic manipulation of mTORC1 activity has revealed a pivotal role of the kinase in lipid homeostasis [17,28]. mTORC1 activity is required for the activation of the transcription factor SREBP-1c, and constitutively active mTORC1 through loss of the TSC1/2 complex stimulates the expression of SREBP-1c targets and promotes lipogenesis [9,10,29]. Our model of constitutively active or inactive mTORC1 did not significantly affect *SREBP-1* or *FASN* transcript levels, nor did it affect protein levels of FASN under hyperglycemic conditions. However, constitutive activation of mTORC1 led to a marked increase in *DGAT1* and *DGAT2* mRNA levels. We observed significant differences in cellular TG content in our models, despite the absence of an effect on lipogenic gene expression. Recently, a report demonstrated that $TSC2^{-/-}$ MEFs experienced stimulation of *de novo* lipogenesis that was dependent on SREBP-1 [29]. We did not measure *de novo* lipid synthesis in the present study; however, our study suggests that our model of hepatic mTORC1 hyperactivation does not increase lipogenesis in a SREBP1/FASN mediated manner, rather it increases TG synthesis through DGAT1 and DGAT2. DGAT1 catalyzes the incorporation of exogenous FFAs into TG, whereas

DGAT2 incorporates glycerol-3-phosphate and endogenously derived FFAs into TG [30,31]. These two enzymes operate sequentially with DGAT2 functioning upstream to catalyze the initial synthesis of DAG that becomes the substrate for DGAT1 [31]. Glucose and high carbohydrate diets can upregulate the expression of DGAT1 and DGAT2, [32,33] however we did not observe an increase in *DGAT1* or *DGAT2* in our shScramble or shRaptor cells. This indicates that hyperactivation of mTORC1 induces the expression of *DGAT1* and *DGAT2* in a manner that is independent of glucose levels; however the exact mechanisms remain to be elucidated.

Constitutive activation of mTORC1 also led to a significant increase in two genes involved in lipid mobilization, *APOB* and *MTP*. MTP facilitates the lipidation of apoB from a cystosolic TG pool. This event is required for secretion of apoB-containing lipoproteins as it prevents proteasomal degradation of apoB. Our model of mTORC1 hyperactivation increased the secretion of apoB and TG, presumably due to increased availability of cellular TG to MTP for lipidation of apoB. Conversely, the chronic inhibition of mTORC1 attenuated the mobilization of apoB-containing lipoproteins and TG. This is possibly a result of decreased cytosolic TG available to lipidate apoB, and the ensuing degradation of underlipidated apoB.

mTORC1 activity is stimulated by growth factors through the canonical insulin and Ras signaling pathways [5,29]. FBS contains a variety of growth factors, and serum withdrawal did not impact mTORC1 activity in $TSC2^{-/-}$ MEFs [29]. Our results corroborate earlier reports that suggest removal of FBS does not affect knockdown or mTORC1 activity in cell models of mTORC1 chronic hyperactivation or inhibition. We

also examined whether DMSO would affect secretion of apoB from our cells. In agreement with data reported by Borradalie et al. [34], treating our cells with DMSO (0.1% v/v) did not affect apoB secretion in shTSC2 cells, nor did it alter the apoB secretion trends observed between the three cell lines. These results indicate that our experimental conditions are valid for future studies.

Our model of mTORC1 constitutive activation is unique, in that it mimics the pathophysiological conditions (e.g., insulin resistance, obesity) in which hypertriglyceridemia is present. In a healthy cell, mTORC1 is activated postprandially via insulin and other nutrients. mTORC1 then phosphorylates p70 S6K, which targets IRS1 to inhibit insulin-dependent activation of mTORC1. When a cell is insulin resistant and in a state of nutrient excess the negative feedback on IRS1 is maintained, however mTORC1 signaling remains active likely through the action of overnutrition. The inhibition of IRS1 and Akt turns off the insulin-mediated suppression of hepatic lipoprotein secretion, while maintaining mTORC1-dependent *de novo* lipogenesis. The newly formed lipids are then used to lipidate apoB and are secreted from the hepatocyte as VLDL. Suppressing TSC2 inhibits the GAP activity of the TSC1/2 complex, thus allowing the constitutive activation of mTORC1 signaling, independent of the insulin state of the cell. Our model demonstrates that chronic activation of mTORC1 leads to a significantly higher levels of lipogenic gene expression and TG synthesis, along with higher levels of secreted apoB and TG. Thus, we conclude that our model of constitutively active mTORC1 closely resembles the purported pathophysiology of

hypertriglyceridemia and is a useful tool to investigate the role of bioactive compounds in reversing aberrant mTORC1 signaling and its sequelae.

REFERENCES

1. Beltrán-Sánchez H, Harhay MO, Harhay MM, McElligott S. Prevalence and trends of Metabolic Syndrome in the adult US population, 1999–2010. J Am Coll Cardiol. 2013 Aug 20;62(8):697–703.

2. Abdel-Maksoud MF, Hokanson JE. The complex role of triacylglycerides in cardiovascular disease. Semin Vasc Med. 2002;2(3):325–33.

3. Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. Endocrinology. 2005;146(3):1473–81.

4. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. Cell. 2009;137(5):873–86.

5. Laplante M, Sabatini DM. mTOR at a glance. J Cell Sci. 2009;

6. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous Sclerosis Complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003;13(15):1259–68.

7. Inoki K, Zhu T, Guan K-L. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577–90.

8. Laplante M, Sabatini DM. MTOR signaling in growth control and disease. Cell. 2012. p. 274–93.

9. Peterson TR, Sengupta SS, Harris TE, Carmack AE, Kang SA, Balderas E, et al. MTOR complex 1 regulates lipin 1 localization to control the srebp pathway. Cell. 2011;146(3):408–20.

10. Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, et al. Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. Cell Metab. 2011;14(1):21–32.

11. Tremblay F, Brûlé S, Hee Um S, Li Y, Masuda K, Roden M, et al. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007;104(35):14056–61.

12. Bartels ED, Lauritsen M, Nielsen LB. Hepatic expression of microsomal triacylglyceride transfer protein and in vivo secretion of triacylglyceride-rich lipoproteins are increased in obese diabetic mice. Diabetes. 2002;51(4):1233–9.

13. Han S, Liang CP, Westerterp M, Senokuchi T, Welch CL, Wang Q, et al. Hepatic insulin signaling regulates VLDL secretion and atherogenesis in mice. J Clin Invest. 2009;119(4):1029–41.

14. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098–101.

15. Vander Haar E, Lee S-I, Bandhakavi S, Griffin TJ, Kim D-H. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol. 2007;9(3):316–23.

16. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37(8):911–7.

17. Laplante M, Sabatini D. An emerging role of mTOR in lipid biosynthesis. Curr Biol. 2009 Jul;19(22):R1046–52.

18. Haas JT, Miao J, Chanda D, Wang Y, Zhao E, Haas ME, et al. Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression. Cell Metab. 2012;15(6):873–84.

19. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431(7005):200–5.

20. Meex SJR, Andreo U, Sparks JD, Fisher EA. Huh-7 or HepG2 cells: which is the better model for studying human apolipoprotein-B100 assembly and secretion? J Lipid Res. The American Society for Biochemistry and Molecular Biology; 2011 Jan 31;52(1):152–8.

21. Ohsaki Y, Cheng J, Fujita A, Tokumoto T, Fujimoto T. Cytoplasmic Lipid Droplets Are Sites of Convergence of Proteasomal and Autophagic Degradation of Apolipoprotein B. Brodsky J, editor. Mol Biol Cell. The American Society for Cell Biology; 2006 Jun 21;17(6):2674–83.

22. Higashi Y, Itabe H, Fukase H, Mori M, Fujimoto Y, Sato R, et al. Distribution of microsomal triacylglyceride transfer protein within sub-endoplasmic reticulum regions in human hepatoma cells. Biochim Biophys Acta. 2002;1581(3):127–36.

23. Garami A, Zwartkruis FJT, Nobukuni T, Joaquin M, Roccio M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell. 2003;11(6):1457–66.

24. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol. 2003;5(6):578–81.

25. Ozcan U, Ozcan L, Yilmaz E, Düvel K, Sahin M, Manning BD, et al. Loss of the Tuberous Sclerosis Complex Tumor Suppressors Triggers the Unfolded Protein Response to Regulate Insulin Signaling and Apoptosis. Mol Cell. 2008;29(5):541–51.

26. Hara K, Maruki Y, Long X, Yoshino K ichi, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110(2):177–89.

27. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002;110(2):163–75.

28. Ricoult SJH, Manning BD. The multifaceted role of mTORC1 in the control of lipid metabolism. EMBO Rep. 2013;14(3):242–51.

29. Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 2010;39(2):171–83.

30. Qi J, Lang W, Geisler JG, Wang P, Petrounia I, Mai S, et al. The use of stable isotope-labeled glycerol and oleic acid to differentiate the hepatic functions of DGAT1 and -2. The Journal of Lipid Research. 2012. p. 1106–16.

31. Wurie HR, Buckett L, Zammit VA. Diacylglycerol acyltransferase 2 acts upstream of diacylglycerol acyltransferase 1 and utilizes nascent diglycerides and de novo synthesized fatty acids in HepG2 cells. FEBS J. 2012;279(17):3033–47.

32. Meegalla RL, Billheimer JT, Cheng D. Concerted elevation of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity through independent stimulation of mRNA expression of DGAT1 and DGAT2 by carbohydrate and insulin. Biochem Biophys Res Commun. 2002 Nov 1;298(3):317–23.

33. Zammit VA. Hepatic triacylglycerol synthesis and secretion: DGAT2 as the link between glycaemia and triglyceridaemia. Biochem J. 2013;451(1):1–12.

34. Borradaile NM, Carroll KK, Kurowska EM. Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. Lipids. 1999;34(6):591–8.

35. Xu Z, Wang M, Wang L, Wang Y, Zhao X, Rao Q, et al. Aberrant expression of TSC2 gene in the newly diagnosed acute leukemia. Leuk Res. 2009 Jul;33(7):891–7.

36. Ren S, Li X, Rodriguez-Agudo D, Gil G, Hylemon P, Pandak WM. Sulfated oxysterol, 25HC3S, is a potent regulator of lipid metabolism in human hepatocytes. Biochem Biophys Res Commun. 2007;360(4):802–8.

37. Dougan SK, Rava P, Hussain MM, Blumberg RS. MTP regulated by an alternate promoter is essential for NKT cell development. J Exp Med. The Rockefeller University Press; 2007 Mar 19;204(3):533–45.

CHAPTER 3

ATTENUATION OF mTORC1-DRIVEN SECRETION OF LIPOPROTEINS AND TRIACYLGLYCERIDES

INTRODUCTION

In Chapter 2, we report that constitutively active mTORC1 induces expression of genes involved in lipogenesis (e.g., *DGAT1*, *DGAT2*) and lipid transport (e.g., *APOB*, *MTP*) with a concomitant increase in secretion of apoB and TG from hepatocellular carcinoma (HepG2) cells. These results suggest that mTORC1 plays a critical role in the development of hypertriglyceridemia, and that targeting the complex therapeutically would be a useful tool in correcting elevated TG in humans. However, kidney transplant patients treated with mTORC1-specific inhibitors experience dyslipidemia consisting of hypertriglyceridemia, hypercholesterolemia, and elevated plasma FFA [1,2]. This seemingly paradoxical scenario arises from a loss of mTORC1 negative feedback on insulin-mediated signaling, allowing Akt to promote lipogenesis through induction of SREBP-1c. Elevated *de novo* lipid synthesis will facilitate lipidation of apoB, allowing the lipoprotein to escape proteasomal degradation and be secreted from the hepatocyte as a component of VLDL. Thus, direct inhibition of mTORC1 is not a viable treatment strategy. An alternative therapeutic method involves the activation of catabolic pathways to counter-act mTORC1-driven anabolism. Fibrates (e.g., Fenofibrate) are a class of amphipathic carboxylic acids that are used as the first line drug therapy to treat hypertriglyceridemia. Fibrates impart their lipid lowering properties by activating peroxisome proliferator-activator receptor alpha (PPARα), that in turn promotes catabolism and lipoprotein clearance [3]. Though effective, these drugs are associated with several side effects including stomach upset, myopathy, and increased risk of gallstones [3]. These negative effects led us to explore the lipid lowering properties of

two short chain fatty acids (SCFA) that have been suggested to promote catabolism through PPARα.

(R)-α-Lipoic acid (LA) is a naturally occurring organosulfur compound and SCFA. LA is enzymatically derived from octanoic acid and serves as an essential cofactor of lipoamide containing enzymes of aerobic metabolism. Recent reports have suggested that LA has TG-lowering properties and can increase whole body energy expenditure by activating catabolic pathways (e.g., β-oxidation) [4]. Specifically, dietary supplementation of LA induced the expression of PPARα target genes involved in medium- and long-chain fatty acyl metabolism in the liver of ZDF rats [5]. These observations led us to explore the therapeutic potential of LA in attenuating mTORC1-driven hypertriglyceridemia.

4-Phenylbutyric acid (PBA) is an aromatic SCFA that is a chemical derivative of butyric acid, produced in the colon by fermentative bacteria. PBA is a well-described chemical chaperone used to alleviate endoplasmic reticulum (ER) stress by rescuing conformational abnormalities of proteins. However, there is mounting evidence that the SCFA can activate liver catabolic processes. For instance, PBA supplementation induced the expression of PPARα and genes involved in peroxisomal β-oxidation in livers of mice [6], and decreased hepatic protein levels of ACC, FAS, SREBP-1, and SCD-1 [7]. PBA supplementation also lowered serum triacylglycerides [8], ameliorated hepatic steatosis [7,9], and decreased body weights in rodents [6]. PBA treated $TSC1^{-/-}$ and $TSC2^{-/-}$ MEFs also experienced a reduction in phospho-p70 S6K levels suggesting that the chaperone may attenuate mTORC1 signaling [10]. These observations led us to investigate the

therapeutic potential of PBA in reversing mTORC1-mediated hypertriglyceridemia through the induction of catabolism.

MATERIALS AND METHODS

Reagents

We obtained 4-Phenyl butyric acid (PBA) from Acros Organics (#130380250); (*R*)-α-Lipoic acid from MAK Wood (#RALA1100134); and Dimethyl Sulfoxide (DMSO) from ATCC. LA was dissolved in DMSO to a stock concentration of 200 mM and diluted to a final concentration of 200 μM. PBA was dissolved in DMEM medium to a final concentration of 8 mM.

Cell Culture

Previously validated stably transduced shScramble and shTSC2 HepG2 cells were maintained in low glucose (5.5 mM) Dulbecco's Modified Eagle's Medium (#11885-076; Life Technologies) supplemented with 10% fetal bovine serum (FBS) (#30-2020; ATCC) and antibiotics/antimycotics (#A5955; Sigma). Cells were maintained at 37ºC in 5% CO_2 with medium renewal every 3-4 days. For experimental treatments, cells were washed twice with PBS, serum-starved overnight (~16-24 hrs), and treated as indicated in corresponding figure legends in serum-free DMEM (5.5 mM or 30 mM glucose).

Cell lysis and Western blotting

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and solubilized in modified RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na deoxycholate, 1% Igepal, 1 mM DTT) supplemented with Halt

protease and phosphatase inhibitors (Thermo Scientific). The cells were then scraped, vortexed for 1 minute, and then clarified by centrifugation for 10 min at 14,000 x g at 4^{o}C. Total protein concentration of clarified supernatants was determined using the Pierce BCA Assay (Thermo Scientific) and Bio-Tek µQuant plate reader. Samples were then adjusted to appropriate concentrations using ice-cold PBS and SDS loading buffer supplemented with 100 mM DTT, and denatured for 5 min at 95^{o}C. Secreted proteins in conditioned media were prepared by adding Halt protease inhibitors (Thermo Scientific), vortexing, and centrifugation for 10 min at 14,000 x g at 4^{o}C. The clarified media was then transferred to a fresh tube, vortexed briefly, and equal volumes denatured with SDS loading buffer supplemented with 100 mM DTT denatured for 5 min at 95^{o}C, and subjected to Western blot analysis. Briefly, proteins were resolved by reducing SDS-PAGE, transferred onto a nitrocellulose membrane (LI-COR), blocked for 1 hour at room temperature in Odyssey Blocking Buffer (LI-COR) in TBS, and probed overnight at 4^{o}C with respective primary antibody. Antibodies against Raptor (#2280), TSC2 (#4308), p70 S6K (#2708), phospho-p70 S6K (Thr389; #9234), AMPKα (#2603), phospo-AMPKα (Thr172; #2535) were purchased from Cell Signaling (Danvers, MA); ApoB (#71307) and ApoE (#7107) from Midland Bioproducts (Boone, IA); β-Actin (#A5441); IRDye secondary antibodies were purchased from LI-COR (Lincoln, NE). HRP-conjugated secondary antibodies against rabbit, mouse, and goat were purchased from R&D Systems, Cell Signaling, and Santa Cruz Biotechnology, respectively. Blots were visualized using either the LI-COR Odyssey scanner or enhanced chemiluminescence

(ECL) on the ProteinSimple imaging system. All band densitometry quantification was completed with the LI-COR Image Studio Lite software.

Measurement of intracellular and secreted TG content

Lipids were extracted from stably transduced HepG2 cells and conditioned media (2 mL) using the chloroform/methanol method previously described with modifications [11]. TG were then quantified enzymatically using Triacylglyceride determination reagents (Sigma) and Bio-Tek μQuant plate reader.

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance was determined as denoted in figure legends using an unpaired two-tailed Student's *t* test. Statistical tests were performed at the 5% significance level.

RESULTS

LA decreases cellular TG and secretion of lipoproteins and TG by attenuating mTORC1 signaling in an AMPK independent manner

To determine if LA supplementation would overcome mTORC1-driven overproduction of lipoproteins and TG, we treated stably transduced shScramble and shTSC2 HepG2 cells with 200 μM LA for 24 and 36 hrs in the presence of 5.5 mM and 30 mM glucose (Fig. 3.1, A). LA (24 hrs) significantly decreased the phosphorylation of p70 S6K in shTSC2 cells, but had no effect on mTORC1 activity in shScramble cells (Fig. 3.1, B). Considering LA is purported to activate AMP-activated protein kinase (AMPK), which inhibits mTORC1 [12,13], we examined the phosphorylation of AMPKα

to determine if the suppression of mTORC1 activity stemmed from AMPK activation. We did not observe increased phospho-AMPKα in LA treated shTSC2 suggesting that the inhibitory effect of LA on hyperactive mTORC1 occurred in mechanism that is independent of AMPK (Fig. 3.1, C). We next sought to investigate the impact of LA (24 hrs) supplementation on the secretion of apoB and apoE from shScramble and shTSC2 cells under 5.5 mM and 30 mM glucose conditions. LA (24 hrs) significantly reduced secreted apoB (-26%) and apoE (-36%) from shTSC2 cells when cultured under 5.5 mM glucose (Fig. 3.1, E), but did not affect lipoprotein secretion from shScramble cells (Fig. 3.1, D). LA (36 hrs) significantly lowered cellular TG (Fig. 3.1, G) and secreted TG (Fig 3.1, I) from both shScramble and shTSC2 cells under 30 mM glucose conditions. This effect was not observed in cells cultured in 5.5 mM glucose (Fig. 3.1, F & Fig. 3.1, H).

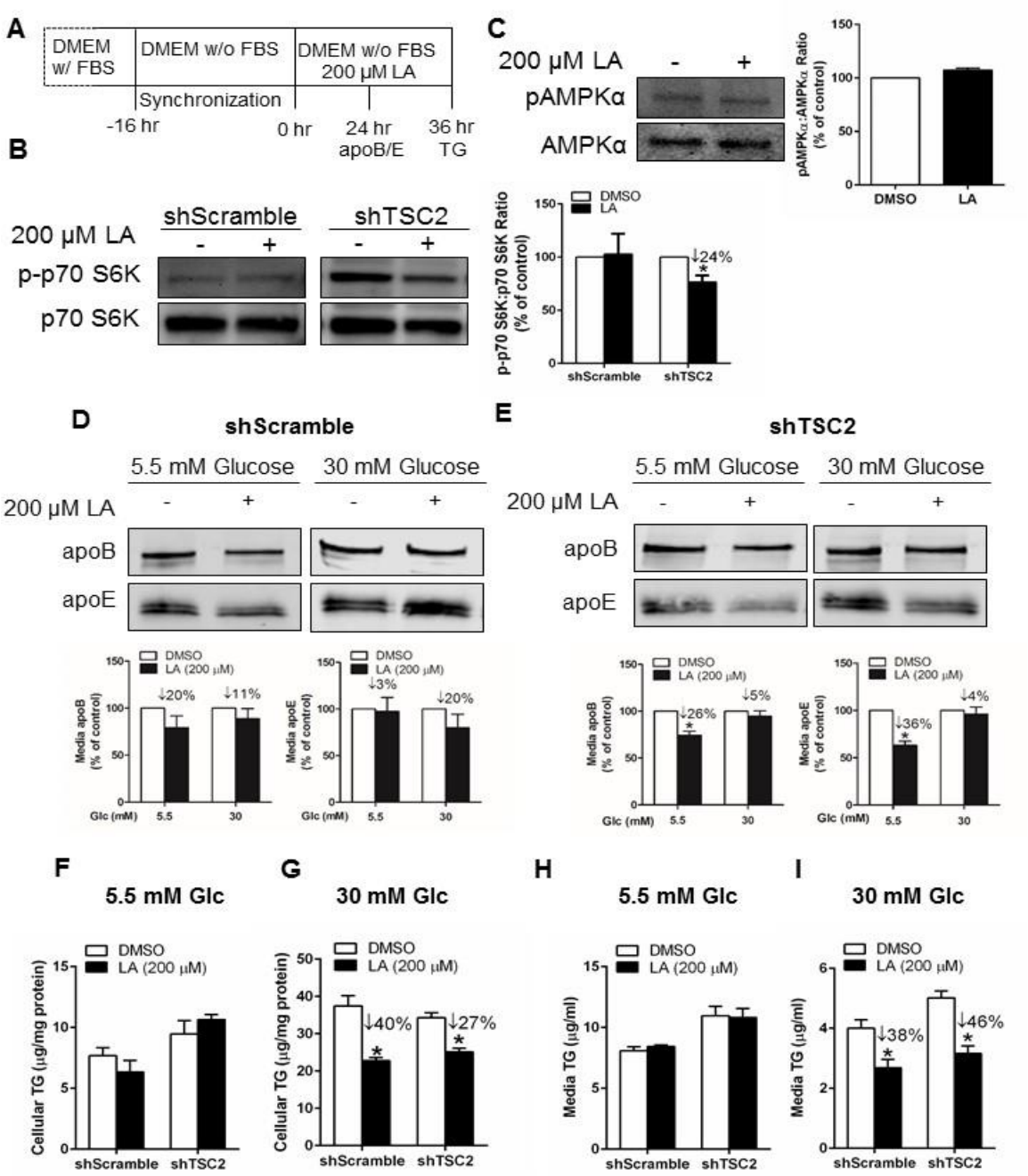

Fig 3.1. LA attenuates hyperactive mTORC1 activity and decreases secretion of lipoproteins and TG. Phospho-p70 S6K was normalized to total p70 S6K, and phospho-AMPKα to total AMPKα. Data represent mean ± SEM. (**A**) shScramble and shTSC2 cells were treated with 200 μM LA for 24 hrs to examine mTORC1 activity and lipoprotein secretion or 36 hrs to examine intracellular and secreted TG content. (**B**) LA (24 hrs) attenuates hyperactive mTORC1 signaling, but does not affect mTORC1 activity in shScramble cells in 30 mM glucose (n = 4). (**C**) LA (24 hrs) does not activate AMPK in shTSC2 cells (n = 4). (**D**) LA (24 hrs) did not alter apoB or apoE secretion from

shScramble (n = 7). (**E**) LA (24 hrs) significantly decreased the secretion of apoB and apoE from shTSC2 cells under 5.5 mM and 30 mM glucose conditions (n = 7). LA (36 hrs) supplementation did not alter (**F**) cellular TG or (**H**) secreted TG from shScramble or shTSC2 cells under 5.5 mM glucose (n = 6). Under 30 mM glucose conditions, LA (36 hrs) significantly decreased (**G**) cellular TG and (**I**) secreted TG from both cell lines (n = 6). Statistical significance was determined using Student's *t*-test, *P < 0.05 vs. vehicle control.

PBA decreases secretion of lipoproteins and TG without altering hyperactive mTORC1 signaling

Stably transduced shScramble and shTSC2 cells were treated with 8 mM PBA for 6 hrs to determine if PBA would attenuate mTORC1-driven secretion of lipoproteins and TG (Fig. 3.2, A). PBA treatment did not attenuate hyperactive mTORC1 signaling in shTSC2 cells; however, the SCFA markedly decreased mTORC1 activity in shScramble cells (Fig. 3.2, B). Next, we sought to determine whether PBA treatment would alter lipoprotein and TG secretion from the two cell lines. PBA significantly decreased apoB and apoE secretion from both shScramble and shTSC2 cells without affecting mTORC1 signaling (p-p70 S6K:p70 S6K) in shTSC2 cells (Fig. 3.2, C & F). Moreover, the decrease in lipoprotein secretion was similar between the two cell lines. PBA also significantly lowered the secretion of TG from shScramble and shTSC2 cells by 56% and 26%, respectively.

We next examined the cellular TG content of PBA treated cells to determine if PBA would represses TG synthesis. PBA did not alter the cellular concentration of TG in shScramble cells (Fig. 3.2, D); however, PBA significantly increased cellular TG (+76%) in shTSC2 cells (Fig. 3.2, G). This effect had no negative consequences on cell morphology (Fig. 3.2, I).

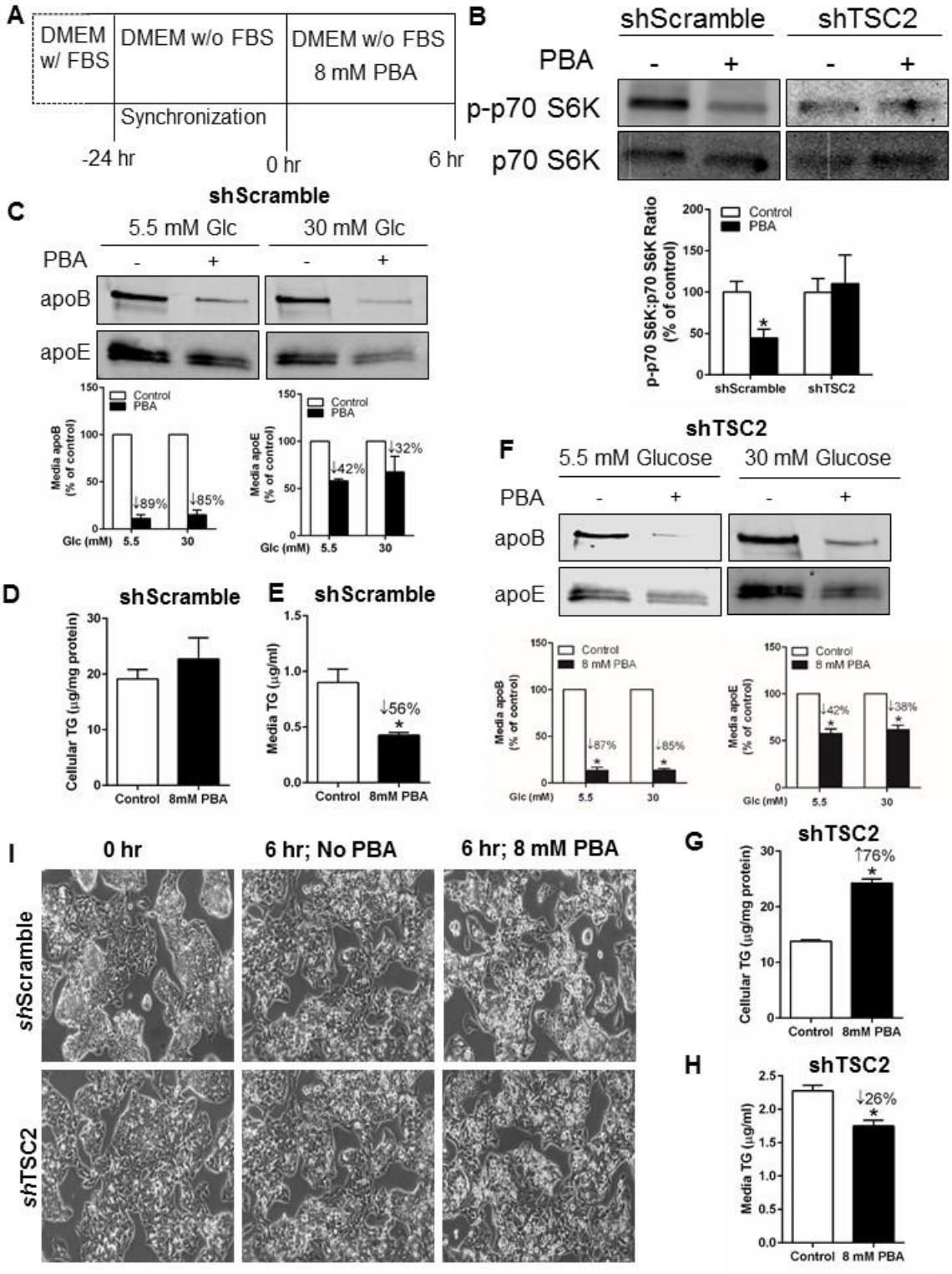
A
DMEM w/ FBS
DMEM w/o FBS
DMEM w/o FBS 8 mM PBA
Synchronization
-24 hr
0 hr
6 hr
B
shScramble
shTSC2
PBA
p-p70 S6K
p70 S6K
p-p70 S6K:p70 S6K Ratio (% of control)
Control
PBA
shScramble
shTSC2
C
shScramble
5.5 mM Glc
30 mM Glc
PBA
apoB
apoE
Media apoB (% of control)
Media apoE (% of control)
↓89%
↓85%
↓42%
↓32%
Glc (mM)
D
shScramble
Cellular TG (µg/mg protein)
Control
8mM PBA
E
shScramble
Media TG (µg/ml)
↓56%
F
shTSC2
5.5 mM Glucose
30 mM Glucose
8 mM PBA
↓87%
↓85%
↓42%
↓38%
G
shTSC2
↑76%
H
shTSC2
↓26%
I
0 hr
6 hr; No PBA
6 hr; 8 mM PBA
shScramble
shTSC2

Fig 3.2. PBA treatment decreases secretion of apolipoproteins and TG from cells with hyperactive mTORC1 signaling without altering mTORC1 activity. Phospho-p70 S6K was normalized to total p70 S6K. Data represent mean ± SEM. (**A**) shScramble and shTSC2 cells were treated with 8 mM PBA for 6 hours and then analyzed for mTORC1 activity, apolipoprotein and TG secretion, and cellular TG content. (**B**) PBA treatment did not alter hyperactive mTORC1 signaling in shTSC2 cells, but significantly decreased mTORC1 activity in shScramble cells. PBA treated shScramble cells secreted less (**C**) apoB, apoE, and (**E**) TG. (**D**) PBA did not alter cellular TG content in shScramble cells. PBA treated shTSC2 cells secreted less (**F**) apoB, apoE, and (**H**) TG. (**G**) There was significantly more intracellular TG (+76%) in PBA treated shTSC2 cells. (**I**) Six hour PBA treatment did not alter cellular morphology of shScramble or shTSC2 cells. Statistical significance was determined using Student's *t*-test, $*P < 0.05$ vs. no PBA control, n = 3-4.

DISCUSSION

Proper mTORC1 signaling is critical for many metabolic processes, especially in regards to anabolic metabolism. In states of overnutrition, insulin resistance, and obesity mTORC1 signaling becomes dysregulated and contributes to the development of hypertriglyceridemia. Pharmacological agents that directly target the mTORC1 complex are available; however, these drugs have negative side effects that include hypertriglyceridemia, hypercholesterolemia, and elevated plasma FFA [1]. This paradox has garnered interest in identifying potential bioactive agents that can counteract hyperactive mTORC1 through the induction of catabolism. The results presented in this study demonstrate that LA and PBA may be beneficial dietary aids in reversing mTORC1-driven secretion of lipoproteins and TG; however the SCFAs appear to impart their lipid lowering properties in distinct mechanisms.

AMPK senses intracellular energy status, promotes catabolic pathways, and inhibits mTORC1 activity [12,14]. LA has previously been shown to reduce mTORC1 signaling in the skeletal muscle of high fat diet fed rats in an AMPK dependent manner

[13]. Our data show that LA suppresses hyperactive mTORC1 signaling in hepatocytes without activating AMPK. This is consistent with our previous studies that suggest that LA imparts its health-promoting properties independently of AMPK in the livers of ZDF rats [5,15,16]. To our knowledge, this is the first report of the inhibitory action of LA on hyperactive mTORC1 in hepatocytes. Previous data from our group also demonstrated that dietary supplementation of LA induces the expression of PPARα target genes (e.g., *Cpt1β*, *Crat*, *Acot1*, *Acot2*) while repressing lipogenic genes (e.g., *Fas*, *Gpat1*, *Dgat2*) in livers of ZDF rats [5]. We postulate that LA suppressed mTORC1 signaling by inducing catabolism possibly through the action of PPARα; however, more research is needed to fully define the molecular mechanisms. Additionally, our data suggest that LA represses lipoprotein secretion under abnormally elevated mTORC1 signaling (shTSC2 cells) without altering lipoprotein secretion at baseline mTORC1 activity (shScramble cells). We also reported that LA supplementation can decrease cellular TG and secreted TG in shScramble and shTSC2 cells when cultured under hyperglycemic conditions. The decrease in lipoprotein and TG from LA-treated shTSC2 cells is similar, if not superior, to the reductions achieved with the pharmacological lipid-lowering agents Gemfibrozil (fibrate) [17] and Simvastin (statin) [18] in HepG2 cells.

PBA has previously been shown to decrease mTORC1 signaling in $TSC1^{-/-}$ and $TSC2^{-/-}$ MEFs [10,19]. Our data show that PBA can significantly repress mTORC1 signaling in control cells (shScramble), but not in shTSC2 HepG2 cells. Though mTORC1 signaling was unaffected by PBA in shTSC2 cells, we noted an abatement of lipoprotein and TG secretion that is consistent with in vivo studies [7,8]. For instance,

PBA (0.5 g/kg body weight; gavage) has been shown to suppress the secretion of apoB100 and lower serum TG content in *ob*/*ob* mice [8].

We observed a marked increase in intracellular TG content in PBA-treated shTSC2 cells. This effect was previously observed in the livers of PBA treated *db*/*db* mice, in which TG content increased despite elevated levels of phospho-ACC, a marker of decreased *de novo* lipogenesis [20]. *db*/*db* mice have upregulated hepatic mTORC1 signaling that parallels our model and may explain the observed similarities between *in vitro* and *in vivo* models [21]. Taken together, our data suggest that PBA promotes the degradation of apolipoproteins without inhibiting hyperactive mTORC1 activity. Consequently, *de novo* lipogenesis proceeds unabated and leads to TG deposition in intracellular lipid droplets. It is important to note that PBA treatment was previously shown to ameliorate hepatic steatosis in high-fructose-fed rats and decrease intracellular TG in 30 mM glucose treated HepG2 cells [7,12]. mTORC1 activity was not reported in the aforementioned studies. Further investigation is needed to unveil the mechanisms by which PBA decreases TG secretion but increases TG deposition in hepatocytes in relation to mTORC1.

In summary, findings presented in this study show that LA and PBA can repress the secretion of apoB, apoE, and TG from HepG2 cells in the context of mTORC1 hyperactivation. LA supplementation can attenuate hyperactive hepatic mTORC1 signaling. LA and PBA are used clinically to treat a variety of diseased states, yet their lipid lowering properties have recently been recognized. Our findings suggest that the

two SCFAs may be beneficial bioactive compounds in the treatment of mTORC1-driven hypertriglyceridemia.

REFERENCES

1. Kasiske BL, de Mattos A, Flechner SM, Gallon L, Meier-Kriesche H-U, Weir MR, et al. Mammalian target of rapamycin inhibitor dyslipidemia in kidney transplant recipients. Am J Transplant. 2008;8(7):1384–92.

2. Aggarwal D, Fernandez ML, Soliman GA. Rapamycin, an mTOR inhibitor, disrupts triglyceride metabolism in guinea pigs. Metabolism. 2006;55(6):794–802.

3. Pejic RN, Lee DT. Hypertriglyceridemia. J Am Board Fam Med. 2006;19(3):310–6.

4. Kim M-S, Park J-Y, Namkoong C, Jang P-G, Ryu J-W, Song H-S, et al. Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat Med. 2004;10(7):727–33.

5. Yi X, Pashaj A, Xia M, Moreau R. Reversal of obesity-induced hypertriglyceridemia by (R)-α-lipoic acid in ZDF (fa/fa) rats. Biochem Biophys Res Commun. 2013;439(3):390–5.

6. Weng H, Endo K, Li J, Kito N, Iwai N. Induction of Peroxisomes by Butyrate-Producing Probiotics. Lobaccaro J-MA, editor. PLoS One. San Francisco, CA USA: Public Library of Science; 2015 Feb 6;10(2):e0117851.

7. Ren LP, Song GY, Hu ZJ, Zhang M, Peng L, Chen SC, et al. The chemical chaperon 4-phenylbutyric acid ameliorates hepatic steatosis through inhibition of de novo lipogenesis in high-fructose-fed rats. Int J Mol Med. 2013;32(5):1029–36.

8. Ai D, Baez JM, Jiang H, Conlon DM, Hernandez-Ono A, Frank-Kamenetsky M, et al. Activation of ER stress and mTORC1 suppresses hepatic sortilin-1 levels in obese mice. J Clin Invest. 2012;122(5):1677–87.

9. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006;313(5790):1137–40.

10. Ozcan U, Ozcan L, Yilmaz E, Düvel K, Sahin M, Manning BD, et al. Loss of the Tuberous Sclerosis Complex Tumor Suppressors Triggers the Unfolded Protein Response to Regulate Insulin Signaling and Apoptosis. Mol Cell. 2008;29(5):541–51.

11. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37(8):911–7.

12. Li H, Min Q, Ouyang C, Lee J, He C, Zou MH, et al. AMPK activation prevents excess nutrient-induced hepatic lipid accumulation by inhibiting mTORC1 signaling and endoplasmic reticulum stress response. Biochim Biophys Acta - Mol Basis Dis. 2014;1842(9):1844–54.

13. Li Z, Dungan C, Carrier B, Rideout T, Williamson D. Alpha-Lipoic Acid Supplementation Reduces mTORC1 Signaling in Skeletal Muscle from High Fat Fed, Obese Zucker Rats. Lipids. Springer Berlin Heidelberg; 2014;49(12):1193–201.

14. Hardie DG. Minireview: The AMP-Activated Protein Kinase Cascade: The Key Sensor of Cellular Energy Status. Endocrinology. 2003. p. 5179–83.

15. Butler JA, Hagen TM, Moreau R. Lipoic acid improves hypertriglyceridemia by stimulating triacylglycerol clearance and downregulating liver triacylglycerol secretion. Arch Biochem Biophys. 2009;485(1):63–71.

16. Timmers S, de Vogel-van den Bosch J, Towler MC, Schaart G, Moonen-Kornips E, Mensink RP, et al. Prevention of high-fat diet-induced muscular lipid accumulation in rats by alpha lipoic acid is not mediated by AMPK activation. J Lipid Res. 2010;51(2):352–9.

17. Zhu D, Ganji SH, Kamanna VS, Kashyap ML. Effect of gemfibrozil on apolipoprotein B secretion and diacylglycerol acyltransferase activity in human hepatoblastoma (HepG2) cells. Atherosclerosis. 2002 Oct;164(2):221–8.

18. Itoh M, Abe Y, Iwama Y, Kimura F, Satoh M, Shoji M, et al. HPLC analysis of lipoproteins in culture medium of hepatoma cells: An in vitro system for screening antihyperlipidemic drugs. Biotechnol Lett. 2009;31(7):953–7.

19. Qin L, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy. 2010;6(2):239–47.

20. Tanis R. An Investigation of Mitochondrial Bioenergetics and the Turnover of Succinated Proteins in the Adipocyte during Diabetes. 2014.

21. Li S, Ogawa W, Emi A, Hayashi K, Senga Y, Nomura K, et al. Role of S6K1 in regulation of SREBP1c expression in the liver. Biochem Biophys Res Commun. 2011 Aug 26;412(2):197–202.

Chapter 4

CONCLUSIONS

CONCLUSION

The effects of constitutively active mTORC1 on the secretion of apoB-containing lipoproteins and TG from human hepatocellular carcinoma cells was examined in this thesis. Our study demonstrates that hyperactive mTORC1 increases intracellular TG content, with a concomitant increase in apoB and TG secretion (Figure 4.1). The observed increases in cellular and secreted TG are thought to result from TG synthesis, facilitated by hepatic DGAT1 and DGAT2. Subsequently, TG are used in the lipidation of apoB mediated by MTP, and secreted by hepatocytes as VLDL. By showing that constitutively active mTORC1 promotes the secretion of TG-rich apoB-containing lipoproteins, our studies provide new insight into the pathogenesis of hypertriglyceridemia.

Direct inhibition of mTORC1 is not a viable therapeutic strategy to treat mTORC1-driven hypertriglyceridemia as it increases TG synthesis and export. For example, patients treated with the mTORC1 inhibitor rapamycin develop hypertriglyceridemia, hypercholesterolemia, and elevated free fatty acids. An alternative approach is to stimulate catabolism to counteract mTORC1-driven anabolism. We found that two bioactive short chain fatty acids (SCFA) with purported catabolic properties, LA and PBA, can decrease the secretion of apoB-containing lipoproteins and TG in our model of mTORC1constitutive activation. However, the mechanism by which these two SCFAs impart their lipid lowering properties differ (Figure 4.1). Our data suggest that LA decreases the availability of cellular TG for apoB lipidation by repressing hepatic mTORC1 signaling. Since LA did not affect the secretion of apoB, we infer that LA led

to the secretion of poorly lipidated apoB particles. PBA, however, significantly repressed apoB and TG secretion, but did not influence mTORC1 signaling. Consequently, mTORC1-driven lipogenesis continued unabated, raising cellular TG synthesis. Over all, this work provides mechanistic insight into the lipid lowering properties of LA and PBA within the context of elevated mTORC1 signaling, and suggests that the two SCFAs have beneficial properties in the treatment of hypertriglyceridemia.

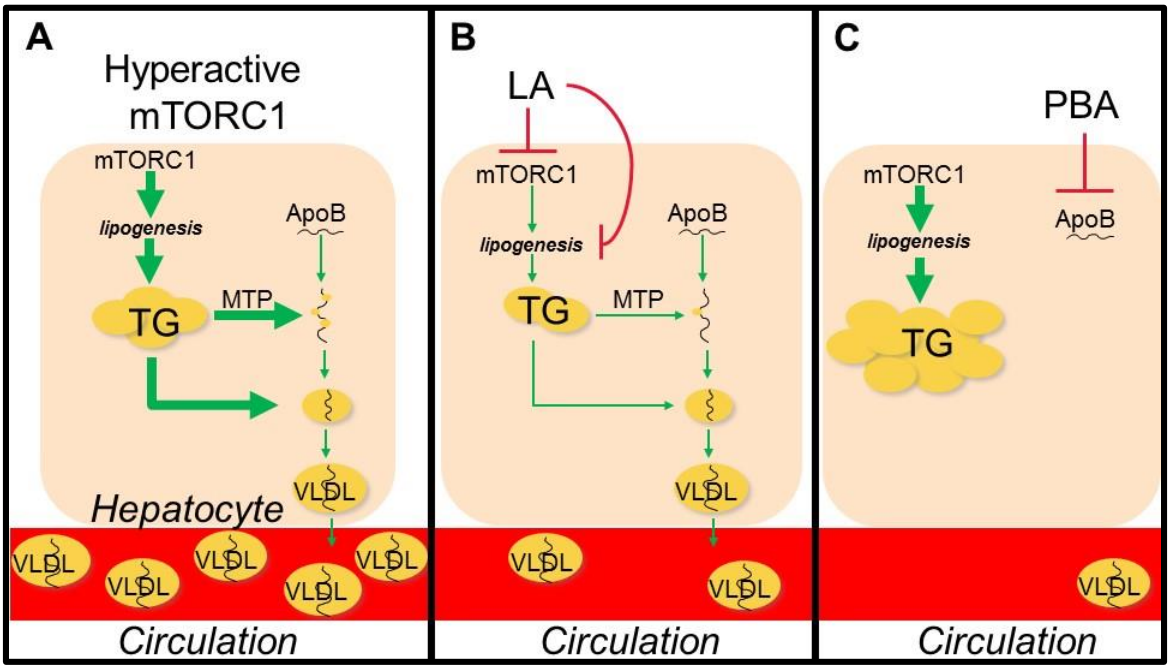

Figure 4.1. Proposed lipid-lowering mechanisms of LA and PBA in a model of constitutively active mTORC1. (**A**) Hyperactive mTORC1 increases lipogenesis and intracellular TG that is utilized by MTP to lipidate apoB. Fully lipidated apoB and TG are then secreted as VLDL. (**B**) LA attenuates hyperactive mTORC1 signaling and lipogenesis, without affecting the secretion of apoB. Consequently, secreted apoB is poorly lipidated and less TG is present within VLDL. (**C**) PBA does not affect hyperactive mTORC1 signaling or lipogenesis, but represses secretion of apoB-containing lipoproteins. Hindered lipid mobilization increases the TG content of the cell, stemming from increased deposition of TG in intracellular lipid droplets.