ROLE OF ATP-CITRATE LYASE AND AMP-ACTIVATED PROTEIN KINASE IN REGULATING LIVER LIPID SYNTHESIS

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LAY ABSTRACT

The dysregulation of cholesterol and triglyceride metabolism can manifest as risk factors for life-threating diseases such as atherosclerotic cardiovascular diseases (ASCVD), Type-2 diabetes (T2D), and nonalcoholic fatty liver disease (NAFLD). However, the underlying mechanisms controlling lipid homeoastasis in health and disease are not completely understood. ATP-citrate lyase (ACL) and AMP-activated protein kinase (AMPK) are emerging as key nodes in metabolism that integrate lipid metabolism with signals of nutrient availability and cellular energy status, respectively. These strategic positions in metabolism suggest that both these enzymes could play an important role in the underlying pathophysiology of lipid-related diseases, and are therefore, prime candidates for therapeutic intervention. In these studies, we expand our understanding of the role of AMPK in metabolism beyond energy sensing by identifying specific lipid metabolites as direct allosteric activators of kinase activity. We also evaluate the therapeutic utility of targeting both AMPK and ACL in novel models of hypercholesterolemia and metabolic disease, and demonstrate that ACL inhibition offers a promising strategy to address multiple unmet medical needs.

ABSTRACT

Cholesterol and fatty acid homeostasis is maintained by a complex network of regulatory mechanisms that control the biosynthesis and deposition of lipids over diverse physiological conditions. However, these processes can become dysregulated and uncoupled from energy metabolism by metabolic stress such as a hyper-caloric diet and physical inactivity; eventually manifesting as risk factors associated with atherosclerotic cardiovascular disease (ASCVD), Type 2 diabetes (T2D), and/or non-alcoholic fatty liver disease (NAFLD). AMP-activated protein kinase (AMPK) is a sensor of cellular energy status that promotes metabolic homeostasis by mediating effects on multiple cellular processes including cholesterol and fatty acid synthesis biosynthesis. However, the mechanisms linking AMPK to lipid metabolism under normal and pathological conditions, remain undefined. In these studies, we identify a novel nutrient sensing mechanism whereby the coenzyme A (CoA) activated esters of long-chain fatty acids (LCFA-CoA) directly activate AMPK via specific interactions within the β1-regulatory subunit involving a Ser108 residue previously shown only with synthetic activators. We demonstrate the physiological relevance for this mechanism in an acute setting by showing that fatty acid oxidation was attenuated in mice harboring an AMPKβ1-S108A knock-in mutation compared to WT mice. We then demonstrated that β1-selctive AMPK activation is mimicked by the CoA conjugated form of bempedoic acid, a synthetic small molecule lipid synthesis inhibitor in clinical development

for lowering elevated levels of low-density lipoprotein cholesterol (LDL-C). The importance of this mechanism was determined by assessing multiple disease outcomes in *Ampkβ1*$^{-/-}$/*Apoe*$^{-/-}$ double knockout (DKO) mice fed a high fat-high cholesterol (HFHC) diet ± bempedoic acid. In these studies, bempedoic acid treatment reduced plasma LDL-C and atherosclerosis in both *Apoe*$^{-/-}$ and DKO mice, while no differences in disease outcomes was detected between the two genotypes in response to HFHC feeding. Further mechanistic investigations in rodent and primary human hepatocytes, revealed that the CoA conjugate of bempedoic acid suppressed lipid synthesis via competitive inhibition of ATP-citrate lyase (ACL), which promoted LDL receptor upregulation and associated reductions in LDL-C. We then integrate these findings with published literature in a written synthesis aimed to evaluate the role of ACL in metabolism, and its potential utility as a therapeutic target to treat ASCVD and metabolic disorders in humans. Although several questions remain regarding the metabolic role of AMPK activation by LCFA-CoAs, these studies have expanded our understanding of how cells acutely integrate lipid and energy signals to maintain lipid homeostasis, and identified ACL as a promising strategy to treat hypercholesterolemia, ASCVD, and associated metabolic disorders.

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ABREVIATIONS

ABCA1	ABC transporter A1
ABCG1	ABC transporter G1
ACC	Acetyl-CoA carboxylase
ACL	ATP-citrate lyase
ACSS2	Acetyl-CoA synthetase 2
ACS	Acyl-CoA synthetase
ACSVL1	Very long-chain acyl-CoA synthetase 1
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
ASCVD	Atherosclerotic cardiovascular disease
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMDM	Bone marrow-derived macrophages
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
CBM	Carbohydrate binding module
CiC	Citrate transport protein
CMC	Carboxymethyl cellulose sodium salt

CoA	Coenzyme A
CPT-1	Carnitine palmitoyltransferase-1
DKO	Double knockout
DNL	De novo lipogenesis
ER	Endoplasmic reticulum
FASN	Fatty acid synthase
GTT	Glucose tolerance test
G6Pase	Glucose-6-phosphotase
G6PDH	Glucose-6-phosphate dehydrogenase
HCl	Hydrochloric acid
HDL	High-density lipoprotein
H & E	Hematoxylin and eosin
HFHC	High fat-high cholesterol
HMGR	3-hydroxy-3-methylglutarate-CoA reductase
HSL	Hormone-Sensitive lipase
Insig1	Insulin-induced genes 1
$KHCO_3$	Potassium bicarbonate
LCFA	Long-chain fatty acid
LCFA-CoA	Long-chain fatty acyl-CoA
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LDLR	Low-density lipoprotein receptor
LKB1	Liver kinase 1

mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NAS	Non-alcoholic fatty liver disease score
NASH	Non-alcoholic steatohepatitis
NEFA	Non esterified fatty acids
OAA	Oxaloacetate
OCT	Optimal cutting temperature compound
OGTT	Oral glucose tolerance test
ORO	Oil red O
p-ACC	Phospho- Acetyl-CoA carboxylase
p-AMPK	Phospho- AMP-activated protein kinase
PEPCK	Phosphoenolpyruvate kinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPP	Pentose phosphate pathway
RCT	Reverse cholesterol transport
RER	Respiratory exchange ratio
RT-PCR	Reverse transcription polymerase chain reaction
SAA	Serum Amyloid A
SCAP	SREBP cleavage activating protein

SREBP	Sterol-response element binding protein
t-ACC	Total- Acetyl-CoA carboxylase
t-AMPK	Total- AMP-activated protein kinase
TCA	tricarboxylic acid cycle
TNFα	Tumor necrosis factor α
T2D	Type 2 diabetes
WAT	White adipose tissue
WT	Wild Type
6-PGDH	6-phosphogluconate dehydrogenase

CHAPTER ONE

1 Introduction

1.1 Background and Rationale

Lipids comprise a large class of hydrophobic and amphiphilic molecules such as phospholipids, sterols, fatty acids, and associated esters, which are required for all organisms to survive, grow, and proliferate. Fatty acids and cholesterol are the two fundamental building blocks for the biosynthesis of more complex lipids that serve several functions in cell physiology including structural components of cellular membranes, bioactive signaling molecules, and substrates for post-translational modification and energy productions. However, the accumulation of either lipid species is cytotoxic so cells must closely monitor intracellular concentrations (Zhang and Kaufman 2003). During normal physiological conditions, such as the transition between fasting and feeding, cells constantly integrate nutrient and energy signals to maintain lipid homeostasis primarily by modulating the rates of 1) transport across the plasma membrane, 2) esterification for safe storage, 3) catabolism for energy production, and/or 4) *de novo* synthesis from non-lipid nutrients such as carbohydrates. However, owing to a number of genetic and environmental factors including a Western lifestyle, these processes can become dysregulated and often manifest as a number of life-threating conditions such as atherosclerotic cardiovascular disease (ASCVD), Type 2 diabetes (T2D), and non-alcoholic fatty liver disease (NAFLD) (Browning and Horton 2004; Ratziu, Bellentani et al. 2010; Cohen, Horton et al. 2011; Karlas,

Wiegand et al. 2013; Armstrong, Adams et al. 2014). While the specific underlying molecular defects linking defects in lipid homeostasis to disease in humans are still being investigated, aberrant de novo lipid biosynthesis has emerged as an important contributing factor. This is strongly supported by several studies showing that lipid synthesis is significantly increased in patients with confirmed NAFLD and/or risk factors including obesity, hypertriglyceridemia, and insulin resistance (Diraison, Dusserre et al. 2002; Schwarz, Linfoot et al. 2003; Donnelly, Smith et al. 2005; Tamura and Shimomura 2005). Moreover, therapies that target de novo fatty acid or cholesterol biosynthesis have demonstrated therapeutic utility for improving NAFLD (Stiede, Miao et al. 2017) and reducing cardiovascular (CV) risk (Silverman, Ference et al. 2016), respectively. Despite significant progress, ASCVD, T2D, and NAFLD are not adequately treated by currently available therapies and pose a significant health threat throughout the developed world (Mendis 2010; Ference and Mahajan 2013). As such, better insight into the molecular mechanisms utilized by cells to integrate energy and lipid metabolism and how they become dysregulated are required to develop novel treatment strategies.

Although cholesterol and fatty acid biosynthesis originate from the same pool of biosynthetic precursor, their pathways diverge early and are regulated by distinct mechanism (Horton, Goldstein et al. 2002). However, they are still co-dependent on the activities of two key enzymes, the AMP-activated protein kinase

(AMPK) and ATP-citrate lyase (ACL), each of which represent a key node in metabolism. ACL catalyzes the first committed step in synthesizing cholesterol and fatty acids from non-lipid macronutrients (e.g. carbohydrates) and serves as an important checkpoint where nutrient catabolism and lipid biosynthesis intersect. Early studies showed that its activity is largely under the control of insulin (Kornacker and Lowenstein 1965; Kim, Park et al. 1992; Moon, Park et al. 2002), and that its inhibition results in the suppression of cholesterol and fatty acid synthesis (Watson, Fang et al. 1969; Sullivan, Triscari et al. 1974). In mice, liver-specific ACL knockdown resulted in reduced lipid biosynthesis and plasma lipids, and improved steatosis and glycemic parameters (Wang, Jiang et al. 2009). By contrast, AMPK senses changes in cellular energy status and promotes metabolic homeostasis by mediating effects on multiple cellular processes (Beg, Stonik et al. 1987; Carling, Zammit et al. 1987; Arad, Seidman et al. 2007; Lage, Dieguez et al. 2008; Richter and Ruderman 2009; Steinberg and Kemp 2009; Viollet, Horman et al. 2010; Mihaylova and Shaw 2011; Carling, Thornton et al. 2012; Hardie, Ross et al. 2012). AMPK directly links lipid metabolism to energy homeostasis by catalyzing inhibitory phosphorylation of key fatty acid and cholesterol biosynthetic enzymes and transcription factors in response to energy deficit (Steinberg and Kemp 2009). The importance of AMPK in regulating lipid metabolism is supported by several preclinical studies demonstrating that genetic loss of AMPK promotes lipogenesis and reduces fatty acid oxidation, while over expression or

pharmacological activation of AMPK, inhibits lipid synthesis, increases fatty acid oxidation, reduces hepatic steatosis and improves insulin sensitivity (Seo, Park et al. 2009; Dzamko, van Denderen et al. 2010; Fullerton, Galic et al. 2013; Smith, Marcinko et al. 2016; Zhang, Yang et al. 2016; Woods, Williams et al. 2017).

However, many aspects of how AMPK and ACL are regulated and their role in health and disease are unknown. Moreover, no drug known to specifically target either enzyme has been tested in randomized clinical trials. Given the significant residual risks associated with ASCVD and associated metabolic disorders, and the apparent central role of lipid biosynthesis in their pathophysiology, a better understanding of how cells integrate lipid signals with energy metabolism to maintain homeostasis seems warranted. In particular, how these signals are transduced by enzymes that co-regulate cholesterol and fatty acid synthesis could provide key insight into the overlapping pathophysiology of lipid disorders, and towards the development of novel therapies aimed to address unmet medical needs.

1.2 Hepatic lipid synthesis in health and disease

1.2.1 Role of lipid synthesis in hyperlipidemia, ASCVD and NAFLD: pathophysiology and therapeutic perspectives

Although the first studies linking cholesterol to the pathogenesis of atherosclerosis date back to more than a century ago (Konstantinov, Mejevoi et al.

2006), progress towards elucidating the underlying molecular mechanisms leading to dyslipidemia and ASCVD have been historically hampered by a lack of preclinical models that recapitulate etiology in humans. Owing to several key differences in lipid and lipoprotein metabolism, rodents are highly resistant to atherosclerotic disease, even when fed diets rich in cholesterol and fat. The most notable difference is that rodents carry the majority of their circulating cholesterol in anti-atherogenic high-density lipoprotein (HDL) particles, which is in contrast to humans where the majority of circulating cholesterol is normally carried by apolipoprotein B (ApoB)-containing LDL particles. For the purposes of disease model development, this discrepancy has been addressed by generating mice with germline deletions of key genes in lipoprotein metabolism with the aim to better recapitulate a more human-like lipoprotein profile. Among the best studied models are the *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ knockout (KO) models, both of which exhibit pro-atherogenic lipoprotein profiles (elevated non-HDL-C), and given sufficient time and/or diet, develop significant atherosclerotic lesions (Zadelaar, Kleemann et al. 2007; Lee, Lin et al. 2017). Studies in these models have enhanced our understanding of the pathophysiology of atherosclerosis by revealing complex interactions among circulating lipids, inflammatory factors, immunity, and vascular smooth muscles and the endothelium (Lee, Lin et al. 2017).

While inflammation is clearly an important component of ASCVD in humans, a concept strongly supported by the demonstrated association between

high-sensitivity C-reactive protein (hsCRP) levels and CVD risk (Ridker, Danielson et al. 2009; Ridker, MacFadyen et al. 2010) and the apparent ASCVD benefit recently observed with anti-inflammatory intervention in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) (Tenenbaum and Fisman 2017), the totality of human data obtained from randomized trials of LDL-C-lowering therapies, Mendelian randomization studies, and prospective epidemiologic cohort studies, established a clear dominant and causal association between circulating LDL and ASCVD (Ference, Ginsberg et al. 2017). This insight has facilitated our understanding of the pathogenesis of ASCVD, which is now generally accepted to be a chronic and progressive disease whereby elevated levels of apolipoprotein B (ApoB) -containing cholesterol-rich lipoprotein particles (e.g. LDL and VLDL) accumulate within the arterial intima and promote the formation of macrophage-laden plaques that eventually occlude arteries and become inflamed and prone to rupture (Camejo, Lopez et al. 1975; Frank and Fogelman 1989; Schwenke and Carew 1989; Skalen, Gustafsson et al. 2002; Tabas, Williams et al. 2007). Statins (HMG-CoA reductase inhibitors) are among the most successful drugs in the world and lower elevated LDL-C levels by inhibiting hepatic cholesterol biosynthesis, thus promoting LDL particle clearance from the circulation. However, despite multiple decades of research and the development of highly effective LDL-C-lowering therapies, ASCVD remains the leading cause of death and disability in the western world (Mendis 2010; Ference and Mahajan

2013). While patient compliance is clearly a contributing factor, significant risk remains because many patients do not reach treatment goals with currently available treatment options, and/or the presence of inadequately controlled confounding comorbidities such as obesity, hypertension, T2D, and NAFLD.

Although statins have validated cholesterol synthesis inhibition as a mechanism to reduce ASCVD risk by reducing vascular LDL-C exposure (Lewington, Whitlock et al. 2007; Di Angelantonio, Sarwar et al. 2009; Ference and Mahajan 2013; Ference 2015; Ference, Majeed et al. 2015; Silverman, Ference et al. 2016; Ference, Ginsberg et al. 2017; Ference, Ginsberg et al. 2017), the direct impact of fatty acid synthesis inhibition in humans is less well understood (Miller, Stone et al. 2011; Helgadottir, Gretarsdottir et al. 2016). However, significant evidence suggest that fatty acid synthesis inhibition might indirectly provide CVD benefit by improving other risk factors and comorbidities (Browning and Horton 2004; Ratziu, Bellentani et al. 2010; Cohen, Horton et al. 2011; Karlas, Wiegand et al. 2013; Armstrong, Adams et al. 2014). One prime example is metabolic syndrome, which is not a disease in itself, but rather a cluster of ASCVD and T2D risk factors including hyperlipidemia, hypertension, insulin resistance, and obesity, which are thought to arise from imbalances in energy utilization and storage. In liver, metabolic syndrome manifests as an accumulation of ectopic lipid (steatosis) which is thought to be an initiating step for the onset of a spectrum of liver-related pathologies collectively referred to as NAFLD (Browning and Horton 2004;

Ratziu, Bellentani et al. 2010; Cohen, Horton et al. 2011; Karlas, Wiegand et al. 2013). Although the underlying pathophysiology of NAFLD is still under investigation, insulin resistance and subsequent deregulation of lipid biosynthesis appears to be a primary culprit in its pathogenesis (Anstee, Targher et al. 2013). The prevalence of NAFLD has sharply increased along with metabolic syndrome, and has recently emerged as the most common cause of chronic liver disease in the Western world and a leading cause of liver-related morbidity and mortality worldwide (Loomba and Sanyal 2013).

1.2.2 The pathways of de novo lipid synthesis

Under normal physiological conditions, most cell types acquire lipids by importing them from exogenous sources and therefore have low rates of *de novo* synthesis. By contrast, in hormone-sensitive lipogenic tissues such as liver, adipose, endometrium, and mammary glands, lipid synthesis can be highly induced by nutritional status, growth factors or hormones in a tissue specific manner (Horton, Goldstein et al. 2002; Anderson, Rudolph et al. 2007). The liver plays a key role in maintaining whole-body metabolism by serving as an important site for energy storage and conversion. When non-lipid nutrients (carbohydrates) are consumed in excess of calorie requirements, they are converted to long-chain fatty acids (LCFA) through a process called *de novo* lipogenesis. When LCFA are not immediately required for other biosynthetic processes, LCFAs are esterified for storage as triglycerides or cholesteryl esters, which can be subsequently mobilized

to meet biosynthetic and energy demands, or packaged and secreted as VLDL particles for utilization by other peripheral tissues.

The *de novo* biosynthesis of both cholesterol and fatty acids is dependent on cytosolic acetyl-CoA, which is primarily derived from the metabolism of pyruvate in the mitochondria (Figure1). However, carbon is not readily shuttled out of the mitochondrial in the form of acetyl-CoA and is instead converted to citrate by enzymes within the tricarboxylic acid cycle (TCA) before export by the mitochondrial citrate transport protein (CiC) to the cytosol. Citrate is then converted back to acetyl-CoA by the cytosolic enzyme, ATP-citrate lyase (ACL). Cytosolic acetyl-CoA then becomes committed to the pathway of fatty acid synthesis following conversion to malonyl-CoA by acetyl-CoA carboxylase (ACC), or to the mevalonate pathway via a series of condensation reactions followed by reduction to mevalonic acid by 3-hydroxy-3-methylglutarate-CoA reductase (HMGR), both rate-limiting steps in their respective pathways. The dependence of both cholesterol and fatty acid synthesis on cytosolic acetyl-CoA uniquely positions ACL at the nexus of mitochondria metabolism and *de novo* lipid biosynthesis. Acetyl-CoA is also required for the acetylation of many cellular proteins including histones, which promote broad influence over chromatin architecture and gene transcription (Wellen, Hatzivassiliou et al. 2009). Under some conditions, ACL can be bypassed by the direct CoA activation of acetate in the cytosol by the cytosolic acetyl-CoA synthetase2 (ACSS2); however, the

relevance of this pathway to lipid biosynthesis in human liver appears to be minor (Siler, Neese et al. 1999). Utilizing 7 malonyl-CoA molecules and one acetyl-CoA primer, the synthesis of palmitate (16:0 fatty acid) is completed by a four-step repeating cycle of condensation, reduction, condensation, and dehydration catalyzed by fatty acid synthase (FASN) (Wakil 1989; Kuhajda, Jenner et al. 1994). Importantly, the FASN reaction cycle is dependent on the reducing power of NADPH, and primarily relies on its generation from glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) from the pentose phosphate pathways (PPP) (Shimano, Yahagi et al. 1999; Luong, Hannah et al. 2000; Moon, Lee et al. 2000; Horton, Goldstein et al. 2002; Infantino, Iacobazzi et al. 2007). Palmitate is then stored in the form of triglyceride, utilized for protein post-translational modification, or further processed to meet other cellular biosynthetic demands such as membrane synthesis (Figure 1). The mevalonate synthesized by HMGR is a building block for the synthesis of several important biological intermediates and products including isoprenoids, CoQ10, dolichol, and cholesterol (for review, see (Goldstein and Brown 1990; DeBose-Boyd 2008)). Cholesterol can then be utilized for the production of steroids, or can be incorporated into the plasma membrane where it impacts fluidity and maintains proper signal transduction events. Other intermediate products of the mevalonate pathway support many biological activities required to facilitate signal transduction events and mitochondrial function (Figure 1).

Figure 1.

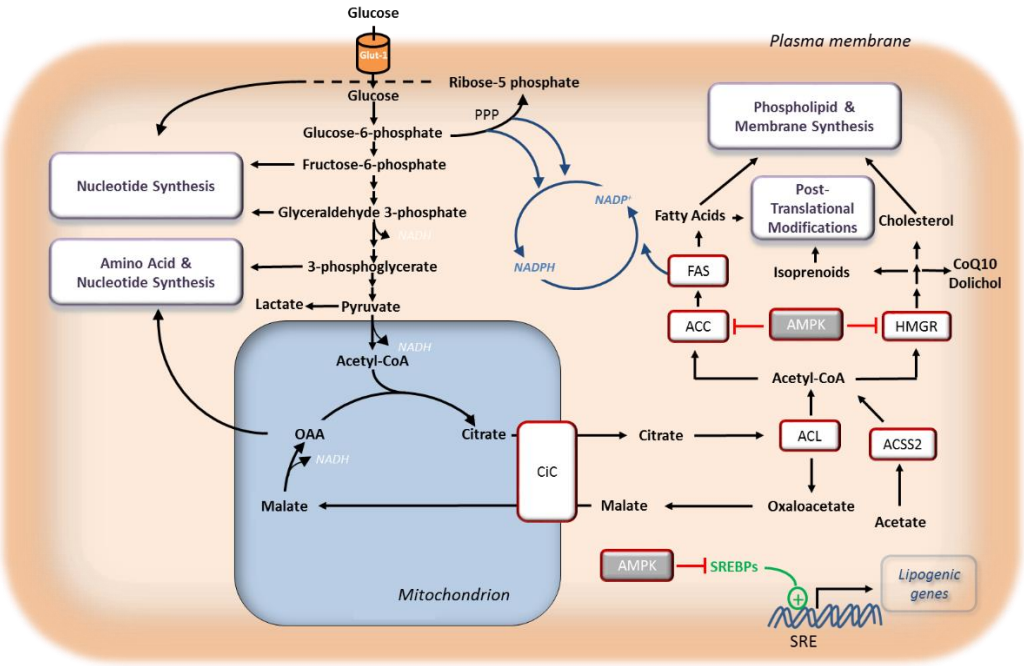

Figure 1. De novo cholesterol and fatty acid biosynthesis

When carbohydrate consumption exceeds energy requirements, glucose enters glycolysis where several intermediates can be shuttled for several biosynthetic processes including nucleotide and amino acid synthesis. The glycolysis intermediate, glucose-6-phosphate can be diverted to the pentose phosphate pathway (PPP) to generate NADPH form glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGDH), 5-carbon sugars and ribose 5- phosphate, the precursor nucleotide synthesis. Pyruvate, the product of glycolysis, can be reduced to lactate or shuttled to the mitochondrion and converted to acetyl-CoA where it enters a truncated TCA cycle. Acetyl-CoA combines with

oxaloacetate (OAA) to form citrate, which is transported out of the mitochondrion by the citrate transport protein (CiC) where it is then cleaved by ATP-citrate lyase (ACL) back to acetyl-CoA and OAA. OAA is converted back to malate and imported back to the mitochondria to replenish the TCA cycle. Acetyl-CoA is then utilized by both the fatty acid and cholesterol biosynthesis pathways which generates products and intermediates required for protein post-translational modification and structural components of the plasma membrane. *De novo* lipid synthesis and glycolysis are coupled by carbon flux and NADP+/NADP balance. Concomitant flux through both pathways maintains redox balance, and produces multiple metabolic precursors required for biosynthetic demands or energy storage.

1.2.3 Regulation of Lipid Synthesis

1.2.3.1 Transcriptional regulation

Transcriptional regulation of lipid synthesis is largely governed by a set of 3 transcription factors collectively called sterol-response element binding proteins (SREBPs). Two isoforms, SREBP-1a and SREBP-1c, are products of a single gene while SREBP-2 is produced from a separate gene (Ye and DeBose-Boyd 2011). All three SREBPs are synthetized as precursor forms that reside in the endoplasmic reticulum (ER) and must undergo subsequent processing before translocating to the nucleus (Brown and Goldstein 1999; Matsuda, Korn et al. 2001). Although

cholesterol and fatty acids are synthesized from a common substrate, their biosynthetic pathways are largely regulated by distinct SREBPs.

Cells maintain intracellular free cholesterol concentrations within a narrow concentration range primarily through an elegant and highly sensitive regulatory feed-back mechanism dependent on the transcription factor, SREBP-2 (for review, please see (Horton, Goldstein et al. 2002)). SREBP-2 appears to be constitutively expressed as an inactive precursor in the ER inhibited by insulin-induced genes 1 (Insig1). When cholesterol levels decrease, SREBP undergoes a maturation process involving several factors such as SREBP cleavage activating protein (SCAP), coat proteins that facilitate transport of the SREBP-SCAP complex to the Golgi, and multiple site-specific proteases that cleave SREBP into active SREBP, which facilitate translocation to the nucleus. The transcriptional activation of multiple SREBP-2 target genes, such as the LDL receptor and several cholesterol biosynthesis genes (e.g. HMG-CoA reductase) results in restored intracellular cholesterol levels. For review see (Ye and DeBose-Boyd 2011). Indeed, statins exploit this pathway by inhibiting liver HMG-CoA reductase, thereby reducing intracellular cholesterol levels and triggering SREBP-2-mediated LDLR upregulation. This results in a new homeostatic state where cells acquire more cholesterol from circulating LDL particles, thus reducing plasma LDL-C (Brown and Goldstein 1997; Brown and Goldstein 1999; DeBose-Boyd 2008). By contrast, SREBP-1a and 1c are primarily responsible for maintaining intracellular fatty acids

homeostasis. Their activity is subject to self-induction, and their maturation has been shown to be suppressed by increases in intracellular concentrations of unsaturated fatty acids and cholesterol (Yokoyama, Wang et al. 1993; Hua, Wu et al. 1995; Shimomura, Shimano et al. 1997; Hannah, Ou et al. 2001; Liang, Yang et al. 2002; Ye and DeBose-Boyd 2011). In liver, SREBP-1c-dependent transcriptional regulation of lipogenic enzyme expression (e.g. ACL, ACC, and FASN) is the primary means for regulating fatty acid synthesis rates, which is significantly increased in metabolic tissues in response to high carbohydrate diet, glucose and insulin (Shimomura, Shimano et al. 1998; Foretz, Guichard et al. 1999; Shimano, Yahagi et al. 1999; Shimomura, Bashmakov et al. 1999; Horton, Goldstein et al. 2002). Carbohydrate-response element binding protein (ChREBP) (Koo, Dutcher et al. 2001; Rufo, Teran-Garcia et al. 2001; Yamashita, Takenoshita et al. 2001; Iizuka, Bruick et al. 2004; Ishii, Iizuka et al. 2004; Ma, Robinson et al. 2006; Denechaud, Bossard et al. 2008; Poupeau and Postic 2011), and liver X receptors (LXR) (Schultz, Tu et al. 2000; Joseph, Laffitte et al. 2002; Kalaany, Gauthier et al. 2005; Cha and Repa 2007) also increase lipogenic gene expression in some tissues such as liver and adipose.

1.2.3.2 Other feedback mechanisms

Most allosteric and post-translational regulatory mechanism regulating de novo lipid synthesis appear localized to the rate-limiting enzymes of cholesterol and fatty acid synthesis, HMGR and ACC, respectively. For example, the

metabolically activated CoA conjugate of palmitate (palmitoyl-CoA), has been shown to be a potent inhibitor of both ACC (Nikawa, Tanabe et al. 1979) and HMGR (Roitelman and Shechter 1989). Furthermore, citrate allosterically activates ACC as part of a feed-forward loop to signal abundant lipogenic substrate availability (Martin and Vagelos 1962), an effect also observed with glutamate (Boone, Chan et al. 2000). Intriguingly, the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K/Akt) pathway may also directly regulate ACL by catalyzing the activating phosphorylation at Ser454 in response to insulin and isoproterenol (Berwick, Hers et al. 2002). Therefore, PI3K/Akt signaling promotes ACL activity via both post-translational and SREBP-1c-dependent transcriptional mechanisms, suggesting that it is an important point of regulation (Berwick, Hers et al. 2002; Bauer, Hatzivassiliou et al. 2005). It is noteworthy that long chain acyl-CoA ester (LCFA-CoAs) (e.g. palmitoyl-CoA) have been shown to be potent inhibitors of ACL in yeast (Boulton and Ratledge 1984); however, whether this regulatory mechanism is relevant in mammalian cells, is presently not known. Given the multiple potential points of direct allosteric regulation described, (e.g. ACL, HMGR and ACC), LCFA-CoAs appear to be important product feedback regulators of cholesterol and fatty metabolism.

1.2.3.3 Regulation of lipid synthesis by AMP-activated protein kinase

As discussed above, lipid synthesis is closely coupled to intracellular energy status through the actions of AMPK. As a sensor of energy metabolism, AMPK

exerts control over metabolism by catalyzing regulatory phosphorylation of multiple substrates including ACC and HMGR, the rate limiting enzymes in fatty acid and cholesterol synthesis, respectively (Beg, Stonik et al. 1987; Carling, Zammit et al. 1987; Arad, Seidman et al. 2007; Lage, Dieguez et al. 2008; Richter and Ruderman 2009; Steinberg and Kemp 2009; Viollet, Horman et al. 2010; Mihaylova and Shaw 2011; Carling, Thornton et al. 2012; Hardie, Ross et al. 2012). Under conditions of energetic stress, increases in the AMP/ATP and ADP/ATP ratio signal low energy status and promote AMPK phosphorylation and activation by upstream kinases including the tumor suppressor, liver kinase 1 (LKB1). Once activated, AMPK mediates inhibitory phosphorylation of ACC1 at Ser79, ACC2 at Ser 212, and HMGR Ser872, thus inhibiting *de novo* fatty acid and cholesterol synthesis (Figure 1). The net result is a switch from energy consuming (*e.g.* fatty acid synthesis) to energy producing (*e.g.* fatty acid β-oxidation) processes and restored energy balance (Carling, Mayer et al. 2011; Hardie, Carling et al. 2011; Oakhill, Scott et al. 2012). ACC acts as a critical metabolic checkpoint as it regulates the conversion of acetyl-CoA to malonyl-CoA which is the first committed step in fatty acid synthesis and a potent allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT-1), the rate limiting enzyme for the import of LCFA-CoA esters into mitochondria for β-oxidation (Bortz and Lynen 1963; McGarry, Leatherman et al. 1978; McGarry, Takabayashi et al. 1978; McGarry and Foster 1979; Faergeman and Knudsen 1997; Nakamura, Yudell et al. 2014). Moreover,

AMPK activation leads to the suppression of lipogenic gene expression by catalyzing the phosphorylation of Ser372/374 on SREBP1c and 2, which prevents proteolytic processing and transcriptional activity (Li, Xu et al. 2011) (Figure 1). The effects of AMPK on SREBP-dependent gene expression might also be linked to the transcription factor P53 as it has been shown to be induced and stabilized by AMPK (Fridman and Lowe 2003; Sanli, Storozhuk et al. 2012).

1.3 AMPK: Structure - function and mechanisms of activation

AMPK is expressed in all tissues as a heterotrimeric complex composed of a catalytic α subunit, and β and γ regulatory subunits. Multiple isoforms exist for each subunit where α (1 or 2), β (1 or 2), and γ (1, 2, or 3) can be expressed as up to 12 unique possible combinations with different heterotrimers preferentially expressed in different tissue types. Canonical activation of AMPK is achieved by an increase in the AMP : ATP ratio (energy deficit) which promotes the preferential competitive binding of AMP to the regulatory γ-subunit via three of four tandem repeats of sequence motifs known as cystathionine-β-synthase (CBS) repeats, which promotes a conformation change and increases basal activity (Hawley, Davison et al. 1996; Bateman 1997; Xiao, Heath et al. 2007; Zhu, Chen et al. 2011; Chen, Wang et al. 2012) (Figure 2). The kinase domain is adjacent to the autoinhibitory domain (α-AID) which suppresses kinase activity under certain conditions (Crute, Seefeld et al. 1998; Goransson, McBride et al. 2007; Pang, Xiong et al. 2007) and is linked to the α regulatory subunit interacting motif 1 (α-RIM1)

and α-RIM2, which binds the surface of the γ subunit at a critical AMP binding site. This interaction results in the observed allosteric activation that occurs in the presence of bound AMP (Xiao, Sanders et al. 2013) while also promoting Thr172 phosphorylation within the activation loop of the α- catalytic subunit. Together, these two effects result in synergistic activation of the kinase (Carling, Clarke et al. 1989; Davies, Helps et al. 1995; Cheung, Salt et al. 2000; Suter, Riek et al. 2006; Sanders, Grondin et al. 2007; Xiao, Heath et al. 2007; Oakhill, Steel et al. 2011) (Figure 2). AMPK α1/α2 are also subject to inhibitory phosphosporylation at Thr479 (Suzuki, Bridges et al. 2013) or Ser485/Ser491(Hurley, Barre et al. 2006; Cao, Meng et al. 2014) by glycogen synthase kinase β (GSKβ) and protein kinase A (PKA), respectively. PKB, PKD and S6 kinase have also been shown to phosphorylate Ser 485/ Ser491 (Horman, Vertommen et al. 2006; Ning, Xi et al. 2011; Dagon, Hur et al. 2012; Hawley, Ross et al. 2014; Kim, Figueroa-Romero et al. 2015; Coughlan, Valentine et al. 2016).

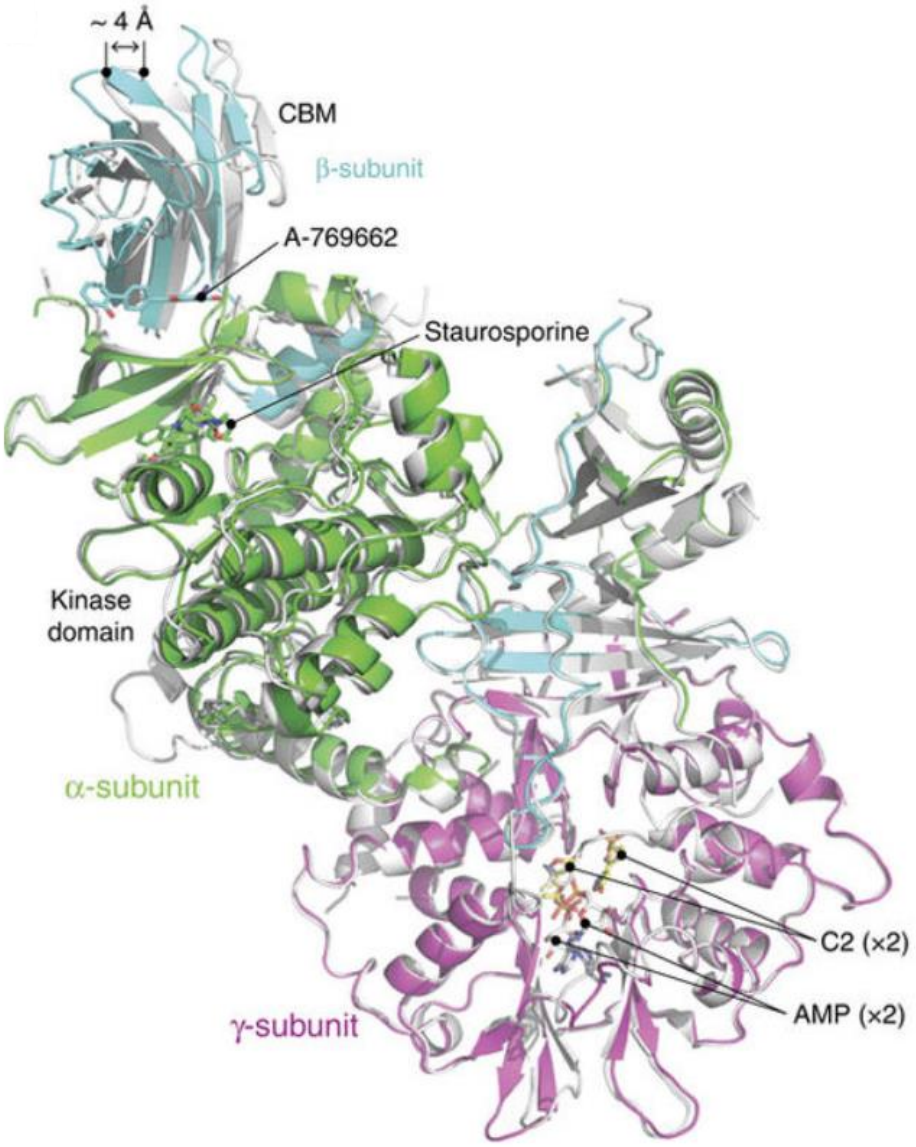

Figure 2. Structure of AMPK α2β1γ1 (Langendorf, Ngoei et al. 2016); (Reproduced with permission of the publisher). Structure of AMPK α2β1γ1 bound to Staurosporine, C2 (yellow sticks), A-769662, and AMP. α-subunit (green), γ-subunit (magenta) and β-subunit (cyan).

Until recently, the role of the β subunit has been thought to be largely limited to mediating glycogen binding and serving as a scaffold for the appropriate assembly and expression of the heterotrimer (Steinberg and Kemp 2009). However, recent studies have identified several synthetic β1- selective AMPK activators (Cool, Zinker et al. 2006; Giordanetto and Karis 2012; Hawley, Fullerton et al. 2012; Xiao, Sanders et al. 2013) that activate the kinase through a mechanism involving specific interactions with a S108 phosphorylation site within the carbohydrate binding module (CBM) of the β1 subunit (Sanders, Ali et al. 2007; Hawley, Fullerton et al. 2012) (Figure 2). This class of activators consists of a growing diverse set of molecules including A-769662, compound 991, PF-249, MK-8722, and salicylate, with a wide range of structural features (Sanders, Ali et al. 2007; Hawley, Fullerton et al. 2012; Xiao, Sanders et al. 2013; Cokorinos, Delmore et al. 2017; Myers, Guan et al. 2017). Until their discovery, pharmacological activation of AMPK had only been achieved indirectly by affecting mitochondrial energy production (i.e. increasing AMP: ATP ratios), or directly by synthetic analogues of AMP such as 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR). As such, these novel synthetic activators provide compelling evidence for a novel physiological role for the β1-subunit and the potential for an unidentified natural metabolite/ligand that binds it. Studies aimed to elucidate the molecular interactions of β1-selective AMPK activators revealed complex interactions with multiple subunits within the kinase that provide

important insight into its structure and function. Specifically, the co-crystallization of compound 991 with AMPK α2β1γ1 heterotrimers demonstrated this compound interacts with a large hydrophobic pocket created between the kinase domain within the α2-subunit and the CBM of the β1-subunit, and that its binding seemed to stabilize this interface. Moreover, the stabilization of this interface increased basal kinase activity and the prevention of protein phosphatase-dependent dephosphorylation of AMPKαT172 within the activation loop (Xiao, Sanders et al. 2013). This insight provides important details regarding the role of the β subunit in the structure and function of AMPK, and important clues for the unidentified natural ligand that potentially binds it.

It is noteworthy that a few reports suggest that the activity of AMPK could be directly regulated by lipid moieties (Carling, Zammit et al. 1987; Watt, Steinberg et al. 2006), although the underlying molecular mechanisms mediating this effect have not been carefully explored. Given the structural clues provided by the work conducted with synthetic agonists, it seems possible that AMPK might bind fatty acids via the β1 subunit as a mechanism to directly integrate lipid availibility with energy signals to maintain energy and lipid homeostasis. The identification of fatty acids as natural β1-dependent ligands could provide significant insight into the structure and function of AMPK, its role in metabolic disorders, and further guide efforts leading to next generation synthetic activators.

1.4 Role of AMPK in cardiovascular and metabolic disease

1.4.1 AMPK and energy metabolism in the liver

The liver facilitates whole-body energy homeostasis by serving as an important site for the storage and conversion of lipid and carbohydrate substrates for subsequent energy production, delivery to other peripheral tissues, or the biosynthesis of cellular components. Given the demonstrated mechanisms by which AMPK regulates lipid metabolism, changes in hepatic AMPK activity could play a role in the pathophysiology of cardiovascular and metabolic diseases. In rodent liver, AMPK is predominantly expressed as α1β1γ1 or α2β1γ1 heterotrimeric complexes with little β2 expression observed. Germline deletion of either the AMPK α1 or α2 subunit has minimal effect on hepatic AMPK activity (Viollet, Andreelli et al. 2003; Jorgensen, Viollet et al. 2004), which is apparently a result of compensatory upregulation of the alternant isoform. However, this compensatory mechanism is not seen in AMPKβ1 knock outs as these mice exhibit an approximately 95% reduction in total hepatic AMPK activity (Dzamko, van Denderen et al. 2010), an effect not observed in livers from AMPKβ2 knockout mice (Steinberg, O'Neill et al. 2010).

1.4.1.1 Hepatic Glucose Production

AMPK has been proposed to play a vital role in the regulation of carbohydrate metabolism, although the mechanisms by which this occurs have been

elusive. Initial enthusiasm surrounding potential links between glycaemia and AMPK were generated in part by the identification of AMPK as a molecular target of metformin (Zhou, Myers et al. 2001; Shaw, Lamia et al. 2005), a potent inhibitor of hepatic glucose production and the most prescribed diabetes drug in the world. Early studies looking at the effects of increased AMPK activity using other pharmacological activators such as AICAR (Lochhead, Salt et al. 2000; Bergeron, Previs et al. 2001) or constitutively expressed AMPKα2 (Foretz, Ancellin et al. 2005), suggested that increased AMPK activity repressed the expression of key gluconeogenic genes such as phosphoenolpyruvate kinase (PEPCK) and glucose-6-phosphatase (G6Pase), both of which have been shown to contribute to aberrant hepatic glucose production in metabolic disorders such as insulin resistance and T2D (O'Brien and Granner 1996). However, subsequent studies using liver-specific AMPKα1/α2 double knockout mice treated with metformin (Foretz, Hebrard et al. 2010; Miller, Chu et al. 2013) and AICAR (Hasenour, Ridley et al. 2014) were unable to corroborate a direct link between AMPK activity and hepatic glucose production, as these compounds continued to suppress hepatic glucose production in the absence of AMPK.

1.4.1.2 Fatty Acid Metabolism

While the potential for the suppression of hepatic glucose production via transcriptional regulation of gluconeogenic enzymes by AMPK require further investigation before it can be excluded, one other mechanistic link that has

continued to gain traction is AMPK-dependent improvements in insulin sensitivity; effects that might occur secondary to its effects on fatty acid metabolism. Although the molecular pathology of hepatic insulin resistance is not completely understood, significant evidence now suggests that it is linked to the accumulation of lipotoxic fatty acid / triglyceride metabolites that, owing to the bifurcation in the insulin signaling pathway in liver (Li, Brown et al. 2010), result in concomitant non-physiologic activation of gluconeogenesis and fatty acid synthesis (Li, Brown et al. 2010; Sachithanandan, Fam et al. 2010; Anstee, Targher et al. 2013). This pathological response to insulin signaling then becomes a self-perpetuating cycle where increased lipotoxicity resulting from insulin-induced fatty acid synthesis, further propagates insulin resistance and elevates blood insulin levels and fatty acid synthesis. When combined with insulin resistance in adipose tissue, which results in enhanced lipolysis and additional influx of fatty acids into the liver (Anstee, Targher et al. 2013), the liver becomes steatotic and prone to injury. When left untreated, this can promote endoplasmic reticulum (ER) stress and mitochondrial dysfunction (Puri, Mirshahi et al. 2008; Neuschwander-Tetri 2010; Neuschwander-Tetri, Clark et al. 2010), chronic pro-inflammatory signaling (Miao, Zhang et al. 2012), hepatocellular injury and apoptosis (Cusi 2012), and eventually NASH, cirrohosis and hepatocellular carcinoma (Michelotti, Machado et al. 2013). Interventions such as pharmacological inhibition of ACC (Harriman, Greenwood et al. 2016), have been shown to relieve the lipotoxic burden by suppressing fatty

acid synthesis, and reduce hepatic steatosis, improve insulin sensitivity, and lower blood glucose. However, recent evidence suggest that while targeting ACC reduces liver triglyceride levels, it promotes hypertriglyceridemia, which potentially limits the therapeutic utility of this approach (Kim, Addy et al. 2017).

The role of AMPK in regulating fatty acid metabolism in the context of insulin resistance and hepatic steatosis is supported by several preclinical studies assessing the phenotypes associated with both genetic over expression and loss of function models. For example, the effects of adenoviral-mediated AMPKα1 overexpression in hyperlipidemic type 2 diabetic rats showed that increased AMPK activity in vivo (as measured by ACC phosphorylation) resulted in the suppression of hepatic lipogenic gene (e.g. *Fas* and *Scd1*) expression and hepatic steatosis (Seo, Park et al. 2009). Similarly, in primary hepatocytes isolated from AMPKβ1 knock-out mice (which results in a >90% reduction in liver AMPK activity) reduced ACC phosphorylation, increased de novo lipogenesis, and reduced fatty acid β-oxidation rates were observed compared to wild type (WT) hepatocytes (Dzamko, van Denderen et al. 2010). These findings support the importance of AMPK-dependent ACC suppression in modulating fatty acid synthesis/oxidation, and are supported by studies showing that mice on a chow diet made refractory to AMPK-dependent regulation, by introducing point mutations in both ACC1 (S79A) and ACC2 (S212A), developed steatosis, hepatic insulin resistance and early signs of fibrosis (Fullerton, Galic et al. 2013). Importantly, these mice were also refractory to the

lipid lowering and insulin sensitizing effects of metformin in vivo (Fullerton, Galic et al. 2013). Furthermore, in studies conducted in primary hepatocytes, the direct AMPK activator, A769662, failed to suppress lipogenesis and improve insulin sensitivity in mice lacking AMPK β1 or the ability to phosphorylate ACC (Fullerton, Galic et al. 2013). In support of these findings, a recent study in mice with a liver-specific AMPKγ1-activating mutation (γD316A) fed a high-fructose diet, had increases in ACC phosphorylation and reduced rates of lipogenesis, improved insulin sensitivity, and were completely protected from hepatic steatosis (Woods, Williams et al. 2017). When taken together, these finding strongly suggest that increased hepatic AMPK activity should suppress fatty acid synthesis, attenuate hepatic steatosis and improve insulin sensitivity.

1.4.1.3 Cholesterol Metabolism

While the role of AMPK in regulating hepatic fatty acid metabolism has been well studied, its effects on cholesterol metabolism and its impact on the progression of atherosclerosis are less well known. As discussed above, the disruption of cholesterol homeostasis in humans can lead to hypercholesterolemia (elevated LDL-C, or non-HDL-C) and increased ASCVD, a finding recapitulated in genetic hypercholesterolemic murine models[38,39]. However, the role of AMPK in regulating cholesterol metabolism in liver and its impact on circulating lipoproteins have not been well studied. Given that the activation of AMPK catalyzes the inhibitory phosphorylation on HMGR (Ser872), it could be

anticipated that the corresponding suppression of cholesterol synthesis would promote effects on cholesterol metabolism that resembles inhibition via statin therapy. However, statins promote their LDL-C reductions primarily by upregulating LDL receptor expression via SREBP-2 activation, a transcription factor shown to be suppressed by AMPK (Li, Xu et al. 2011). Therefore, the impact of AMPK activation on LDL-C metabolism is uncertain. While several studies investigating germline deletion of AMPKα1 or α2 on atherogenic backgrounds (i.e. *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$) demonstrate that mice deficient in AMPK are more susceptible to atherosclerosis, the underlying mechanism(s) and tissue type mediating this effect is not clear. Moreover, the absence of whole-body AMPK α1 or α2 did not significantly impact plasma cholesterol (Dong, Zhang et al. 2010; Dong, Zhang et al. 2010; Wang, Zhang et al. 2011; Cai, Ding et al. 2016; Ding, Zhang et al. 2016), and the involvement of direct anti-inflammatory effects in the vessel wall cannot be ruled out in these models (Dong, Zhang et al. 2010; Dong, Zhang et al. 2010; Wang, Zhang et al. 2011; Cai, Ding et al. 2016; Ding, Zhang et al. 2016). This genetic evidence calls into question the role of AMPK in modulating LDL-C metabolism, and is further supported by studies showing that a variety of AMPK activators such as A-769662, AICAR, and metformin did not significantly impact plasma LDL-C levels in *Apoe*$^{-/-}$ mice despite activating liver AMPK (Ma, Wang et al. 2017). Although LDLR expression was not specifically measured in any of these studies, the lack of impact on plasma LDL-C upon AMPK

deletion or activation, suggests that activating liver AMPK might not result in statin-like LDL-C reductions; however, this requires further investigation.

Just as mammals have a mechanism for delivering cholesterol to peripheral tissues via LDL particles, they also have a mechanism for removing cholesterol (cholesterol efflux) from these tissues for delivery back to the liver via HDL particles by a process known as reverse cholesterol transport (RCT) (Rader, Alexander et al. 2009). Tight coordination of these two processes is required to maintain cholesterol balance and prevent its accumulation in the vasculature and peripheral tissues. The efflux of cholesterol from the cell to HDL particles is largely mediated by ATP-binding cassette transporters, ABCA1 and ABCG1, or scavenger receptor class B type 1 (SR-B1). Once effluxed from the cell, HDL transfers cholesterol to LDL particles in exchange for triglycerides for subsequent delivery back to the liver via LDL receptor-mediated uptake, or directly to the liver via specific interactions with SR-B1. Indeed, some reports suggest that the activation of AMPK in the liver and in macrophages could exert anti-atherogenic effects by enhancing RCT both via efflux from macrophages, due to upregulation of ABCA1 and ABCG1 (Fullerton, Ford et al. 2015) and by SR-B1-mediated hepatic delivery (Ma, Wang et al. 2017). However, further investigation is required.

1.4.2 Role of AMK in Inflammation

As discussed above, atherosclerosis and NAFLD are progressive diseases marked not only by the accumulation of ectopic lipids, but also by immune cell infiltration and chronic low-grade inflammation within the vessel wall and liver, respectively. Multiple studies have reported that pro-inflammatory signals such as LPS, TNFα, or high-fat diet, suppress AMPK activity in a variety of cell types including liver (Woo, Xu et al. 2014), and macrophages (Yang, Kahn et al. 2010; Galic, Fullerton et al. 2011). Although the specific molecular mechanisms are not yet clear, the suppression of AMPK activity could be linked to the modulation of phosphorylation status mediated by increasing phosphatase-dependent dephosphorylation of AMPKα1/α2 Thr172 within the activation loop (Steinberg, Michell et al. 2006; Wu, Song et al. 2007), or by promoting inhibitory AMPKα1/α2 Thr 479 phosphorylation by a variety of upstream kinases (Dagon, Hur et al. 2012; Suzuki, Bridges et al. 2013). Nonetheless, the suppression of AMPK activity by pro-inflammatory signals could contribute to the onset of multiple diseases such as ASCVD and NASH through a variety of downstream effects on multiple cellular processes.

While the accumulation of lipid-laden macrophages in the vessel wall and liver can be relatively benign and reversible in the absence of inflammation, concomitant induction of acute pro-inflammatory signaling pathways promotes a switch in macrophage polarization resulting in the propagation of inflammation,

lipid retention, tissue destruction, and disease progression (Ganeshan and Chawla 2014; Kelly and O'Neill 2015). This switch from an anti-inflammatory phenotype (M2) to a pro-inflammatory phenotype (M1) is also accompanied by reprograming of metabolism that results in a switch from fatty acid oxidation to glycolysis. Studies have clearly shown that multiple compounds such as A-769662, metformin, AICAR, and palmitoleate, reduce macrophage inflammation in an AMPK-dependent manner; however, the underlying mechanisms are not known (Yang, Kahn et al. 2010; Chan, Pillon et al. 2015; Kelly, Tannahill et al. 2015; Kemmerer, Finkernagel et al. 2015; Vasamsetti, Karnewar et al. 2015; Zhou, Tang et al. 2016). Although other mechanisms mediated by regulatory phosphorylation of effectors involving cell stress response and pro-inflammatory signaling likely contribute to the anti-inflammatory activity of AMPK (Day, Ford et al. 2017), multiple studies have reported that the close association between energy metabolism and inflammatory phenotype in macrophages could be a significant contributor. This is evidenced by in vitro studies demonstrating that AMPK activation promotes concomitant polarization toward an anti-inflammatory M2 phenotype and increased fatty acid oxidation (Sag, Carling et al. 2008; Galic, Fullerton et al. 2011; Mounier, Theret et al. 2013; Cao, Cui et al. 2016). Although the underlying molecular mechanism by which AMPK links macrophage metabolism to inflammatory status are not yet clear, it might be associated with increased mitochondrial function by enhancing mitochondrial biogenesis (Galic, Fullerton et al. 2011), fission (Wang,

Zhang et al. 2017) or via direct suppression of ACC via Ser79 phosphorylation (Galic, Fullerton et al. 2011). As discussed above, inhibition of ACC results in simultaneous suppression of fatty acid synthesis and enhanced fatty acid β-oxidation by reducing its product, malonyl-CoA, and alleviating the allosteric inhibition of CPT-1-dependent import of LCF-CoAs into the mitochondria for oxidation. The mechanisms linking fatty acid oxidation and suppressed inflammatory signaling are the subject of ongoing investigations.

Significant in vitro evidence supports the inhibition of inflammation by AMPK activity can impact cellular processes in metabolic tissues such as adipose and liver. For example, AMPK α1 gain and loss of function studies in macrophages demonstrate that AMPK activity is reciprocally associated with NF-κB signaling, and that suppression of its activity was associated with adipocyte insulin resistance in cultured adipocytes (Yang, Kahn et al. 2010). Moreover, this was further supported in vivo where genetic loss of hematopoietic AMPK activity promoted the accumulation of pro-inflammatory macrophages in adipose and liver, and increased insulin resistance in response to a high-fat diet (Galic, Fullerton et al. 2011; Cao, Cui et al. 2016). Within the context of atherosclerosis, the importance of AMPK activity is supported by double knockout studies showing that germline deletions of AMPK α1 or α2 in *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice resulted in accelerated progression of vascular lesions independently of changes in plasma lipids (Dong, Zhang et al. 2010; Dong, Zhang et al. 2010; Cai, Ding et al. 2016); however, a

variety of cell types including endothelial cells and smooth muscle cells have been implicated in these effects making the specific contribution of macrophages difficult to determine. The specific role of immune cells in atherosclerosis was addressed by a study in *Ldlr*$^{-/-}$mice which showed that myeloid-specific deletion of AMPKα1 increased the progression of atherosclerosis (Cao, Cui et al. 2016); however, these mice also displayed elevated levels of LDL-C and VLDL-C, which could not be excluded as a contributing cause.

1.4.3 Role of AMPK in Adipose Tissue

For many years, adipose tissue was largely regarded as a depot for energy storage that was utilized when caloric consumption exceeded metabolic requirements; however, over the last two decades adipose tissue has emerged as a central mediator of endocrine function and site of inflammation that when dysregulated, can play a role in promoting insulin resistance (Lowell, V et al. 1993; Chondronikola, Volpi et al. 2014; Blondin, Labbe et al. 2015). There are two general classes of adipose tissue, white (WAT) and brown (BAT); and while WAT primarily serves as a primary site for the storage of energy substrate in the form of triglycerides, BAT plays a role in energy expenditure by maintaining body temperature. Early studies showed that AMPK activity is reciprocally associated with adiposity in both types of adipose tissue (Lindholm, Ertel et al. 2013; Ruderman, Carling et al. 2013; Fritzen, Lundsgaard et al. 2015), which raises the possibility that AMPK might be an effector of adipocyte function. Given that the

dysregulation of adipocyte metabolism has been implicated in the onset of insulin resistance, the role of AMPK has been intensely studied in this tissue.

Initial investigations into the role of AMPK in adipose tissue largely focused on its presumed enery-sparing role in metabolism by preventing the potential for fatty acid futile cycling through the suppression of lipolysis. This was supported by studies using AICAR, which showed that AMPK activation was associated with increased phosphorylation of hormone-sensitive lipase (HSL) at Ser565 and suppressed β-adernergic –induced lipolysis. However, subsequent investigations yielded paradoxical findings such as physiologic conditions that increase lipolysis in adipose tissue, including exercise (Park, Kaushik et al. 2002; Watt, Holmes et al. 2006) or fasting (Daval, Diot-Dupuy et al. 2005) seem to increase AMPK activation. Although the mechanism mediating this effect is not clear, studies showing that pharmacologic or genetic inhibition of lipolysis also prevents the activation of AMPK, suggesting its activation occurs as consequence rather than an effector of lipolysis (MacPherson, Dragos et al. 2016), and call into question its regulatory role in lipolysis. AMPK is expressed primarily as α1, β1/β2, and γ1 /γ2 in adipose tissue (Daval, Diot-Dupuy et al. 2005; Mottillo, Desjardins et al. 2016) and studies in mice with germline deletion of AMPKα1 or adipose-specific α1α2 knock out mice, suggest that AMPK is required for basal lipolysis, an effect mediated by an activating phosphorylation of adipose triglyceride lipase (ATGL) at Ser406 (Daval, Diot-Dupuy et al. 2005; Kim, Tang et al. 2016). Some

of these studies also supported early findings with AICAR suggesting that AMPK might also suppress lipolysis in response to β-adrenergic signals by catalyzing the phosphorylation of Ser565 of HSL (Kim, Tang et al. 2016). However, other studies investigating the effects of adipose-specific deletion in adult mice (iβ1β2AKO mice), showed that the absence of AMPK did not affect ATGL Ser406 or HSL Ser565 phosphorylation, or basal or isoproterenol-stimulated lipolysis (Mottillo, Desjardins et al. 2016). Therefore, additional investigation is required to uncover the role of AMPK in regulating adipose tissue lipolysis and to understand the apparently conflicting results obtained in different models.

In addition to its potential effects on lipolysis, AMPK might also be important for maintaining BAT metabolic function and influencing whole body energy expenditure. This could be achieved by either promoting the development of BAT, preserving existing BAT function, or facilitating the "browning" of WAT (Day, Ford et al. 2017). The importance of AMPK in maintaining BAT function is supported by studies showing that adipose-specific knockout mice exposed to acute cold stress have an impaired ability to maintain body temperature or respond to β-adrenergic challenge, an effect likely linked to impaired mitochondrial content and function (Mottillo, Desjardins et al. 2016; Yang, Liang et al. 2016). When fed a HFD, defects in BAT in these mice accelerate the onset of NAFLD and dysglycemia. AMPK also plays a critical role in promoting the browning of WAT a process whereby WAT adopts phenotypic traits of BAT such as increased

expression of uncoupling protein 1 (UCP1) and the appearance of multiple mitochondria (Alvarez-Crespo, Csikasz et al. 2016; MacPherson, Dragos et al. 2016). The role of AMPK in the browning of WAT appears to be linked in part, to maintaining PGC-1α activity (Wan, Root-McCaig et al. 2014; MacPherson, Dragos et al. 2016). Increased AMPK is also linked BAT function by findings investigating the effects of pharmacologic activators such as resveratrol, metformin (Wang, Liang et al. 2015; Li, Zhang et al. 2016; Wang, Liang et al. 2017), corosolic acid (Yang, Leng et al. 2016), betaine (Zhou, Chen et al. 2015), and galic acid (Doan, Ko et al. 2015). Consistent with the findings from the genetic loss of function models, these studies showed that compounds that activate AMPK promote increases in PGC-1α, makers of mitochondria content, and improved mitochondrial function. Taken together, these findings suggest that pharmacological activation of BAT potentially provides an attractive approach to treat NAFLD and T2D by increasing energy expenditure, thus reducing adiposity and improving insulin resistance.

1.5 Summary

Lipid metabolism plays a critical role in maintaining whole-body energy homeostasis which influences multiple cellular processes in several tissues types. A hyper-caloric diet promote imbalances in energy metabolism, the dysregulation of lipid synthesis, accumulation of ectopic lipids, and pro-inflammatory signaling in multiple cell types; promoting the onset of ASCVD, T2D and/or NASH. AMPK

tightly links de novo cholesterol and fatty acid biosynthesis rates to energy metabolism, and AMPK activity is suppressed by pro-inflammatory signals associated with metabolic disease. In preclinical studies, methods aimed to increase AMPK activity result in reduced lipid synthesis and inflammation, and provide beneficial effect on multiple disease outcomes. However, many aspects of AMPK structure and function remain unknown and could have important implications in the pathogenesis of diseases with a metabolic origin. Specifically, whether fatty acids or their metabolites modulate AMPK activity via specific interactions within the β1-subunit would provide key insight into how cells integrate lipid and energy metabolism. Although the downstream effects of AMPK activation can be multifactorial (e.g. modulation of insulin sensitivity) many beneficial effects appear to be linked to the suppression of cholesterol and fatty acid synthesis. The specific contribution of lipid synthesis inhibition to the beneficial effects associated with AMPK activation should be further characterized using tissue specific HMGR (Ser871) and ACC (Ser79) knock-in models.

Other enzymes also play a central role in regulating both cholesterol and fatty acid synthesis, and could also prove to be important links between energy and lipid metabolism. ACL is one promising candidate as it is uniquely located at the first committed step toward converting excess energy substrate to lipid for energy and substrate storage. Moreover, as the primary source of cytosolic and nuclear acetyl-CoA, ACL could transduce nutrient availability signaling by influencing

protein acetylation and gene transcription. Therefore, additional investigations are required to improve our understanding of the molecular pathology underpinning the links between lipid metabolism, AMPK, and ACL function, and the potential therapeutic benefits associated with modulating their activities.

1.6 Main objective

Identify and characterize novel mechanisms that link lipid biosynthesis and energy metabolism and investigate their importance and therapeutic potential in a preclinical disease model.

1.7 Thesis Aims

Investigate novel sensing mechanism by which cells integrate energy and lipid metabolism and their involvement in regulating lipid biosynthesis. Specifically, determine whether natural or synthetic lipid moieties directly modulate AMPK or other potential metabolic nodes such as ACL, and elucidate their molecular mechanisms. Establish the physiological relevance of this regulatory mechanism and investigate its importance for the onset of metabolic outcomes, hyperlipidemia, or disease progression in a model of atherosclerosis. Finally, investigate whether this lipid sensing mechanism can be pharmacologically mimicked or exploited in a manner likely to improve disease outcomes in humans.

CHAPTER TWO

2 Long-chain fatty acid metabolites as AMPKβ1-selective ligands: A novel nutrient-sensing mechanism

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This manuscript aims to characterize the potential for several lipid and lipid-related metabolites to directly regulate AMPK activity through novel interactions with active heterotrimeric complexes, and establish its physiological relevance in vivo. AMPK is classically regarded as an energy sensor that promotes energy homeostasis by coordinating metabolic adaptions in response to changing intracellular energy status. AMPK directly responds to changes in the relative concentrations of ATP, ADP, and AMP by mediating competitive binding by specific interactions with the regulatory γ-subunit. However, AMPK is also expressed with another regulatory β-subunit with a poorly defined role. It has been recently shown that AMPK responds to a class of synthetic AMPK activators that bind and activate the kinase through a distinct mechanism involving a S108 residue within the β1-subunit. These findings suggested that a previously unidentified natural ligand regulates AMPK by interacting with this drug binding site. The identification of this ligand could expand our understanding of the physiological role of AMPK and its importance in disease. In these studies, we screened several ligand candidates and identified LCFA-CoA esters as the first natural AMPKβ1-selective activators. We demonstrate the physiological relevance of this mechanism by showing that the oral administration of lipid emulsion (Intralipid®) promotes fatty acid oxidation in WT mice, but not in mice with a single AMPK β1-S108A knock-in mutation. These data indicate that AMPK integrates lipid and energy signals by distinct mechanisms to promote metabolic homeostasis, and suggest that dysregulation of this mechanism might contribute to the pathogenesis of metabolic disease.

S.L.P, G.R.S, B.E.K, J.W.S, designed the experiments. S.L.P., J.W.S, R.J.F, K.R.W.N., E.A.D., E.M.D. performed the experiments. S.L.P., G.R.S., B.E.K wrote the manuscript, and all other authors reviewed and edited the manuscript.

Experiments that I conducted include: Fig 1A, B, C, D, E, F, and G; Fig 2A and C; Fig 3A, C, and D; Figure 4A and B; and Supplementary Figure 1.

Long-chain fatty acid metabolites as potential AMPKβ1-S108 dependent natural ligands for nutrient-sensing

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Abstract

Long-chain fatty acids (LCFA) have emerged as important energy signals that coordinate metabolic adaptations through a variety of mechanisms. The AMP-activated protein kinase (AMPK) is a metabolic sensor that maintains energy homeostasis via several mechanisms including the regulation of fatty acid metabolism. AMPK acutely regulates fatty acid metabolism by catalyzing inhibitory phosphorylations of acetyl-CoA carboxylase (ACC) which leads to concomitant suppression of LCFA synthesis and enhanced mitochondrial β-oxidation. Here, we provide evidence for a novel regulatory feedback mechanism by which LCFA-coenzyme A (CoA) (LCFA-CoA) esters, the metabolically activated form of LCFAs, directly activate AMPK. We demonstrate that similar to the synthetic activator, A-769662, activation of AMPK by palmitoyl-CoA is β1-specific and markedly attenuated by a single alanine knock-in mutation at S108 within the AMPKβ1 carbohydrate binding module (AMPKβ1-S108A KI). To assess the physiological significance of this mechanism, we generated AMPKβ1-S108A KI mice and show that they have reduced metabolic flexibility in response to acute fatty acid exposure. These findings indicate that LCFA-CoAs can act as physiological AMPKβ1-dependnet ligands for AMPK.

Introduction

Long-chain fatty acids (LCFAs) are the predominant energy substrate for most tissues and are essential for maintaining cellular energy homeostasis. Despite the importance of LCFAs for survival, their first intracellular metabolite following uptake by the cell, LCFA-coenzyme A (LCFA-CoA) esters, exert toxic effects on numerous cellular processes. As such, many mechanisms have developed to maintain intracellular LCFA-CoA concentrations within a narrow range despite changing metabolic conditions and wide variations in circulating concentrations of free fatty acids. The AMP-activated protein kinase (AMPK) is an evolutionarily conserved metabolic sensor that is an essential regulator of fatty acid metabolism; effects that are mediated through phosphorylation of numerous substrates including acetyl-CoA carboxylase (ACC) (1-9). The net result is a switch from energy consuming (*e.g.* fatty acid synthesis) to energy producing (*e.g.* fatty acid β-oxidation) processes and restored energy balance (10-12). ACC acts as a critical metabolic checkpoint, as it regulates the conversion of acetyl-CoA to malonyl-CoA which is the first committed step in fatty acid synthesis and a potent allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT-1), the rate limiting enzyme for the import of LCFA-CoA esters into mitochondria for β-oxidation (13-18). Thus, ACC activity is tightly linked to cellular energy status through the actions of the AMPK.

Canonical activation of AMPK is triggered by energy deficit as a rise in the intracellular AMP: ATP ratio promotes the exchange of ATP for AMP or ADP bound to the regulatory γ-subunit (19-23). Binding of AMP increases kinase activity by inducing a conformational shift that results in allosteric activation and enhanced Thr172 phosphorylation within the α- catalytic subunit (22, 24-29). In addition to activation by adenine nucleotides, some evidence suggests that AMPK may also respond to variations in energy substrate availability. While the mechanisms by which AMPK senses changes in carbohydrate availability have been intensely studied (30-35), surprisingly only a few reports have indicated a potential role for lipid moieties to control enzyme activity (1, 36) and the potential mechanisms explaining these observations remain unclear. It is noteworthy that recent studies have identified a structurally diverse class of synthetic β1- selective AMPK activators, including A-769662, salicylate, compound 991, PF-249, MK-8722, and compound 7 (37-43) that activate the kinase through a distinct mechanism involving S108 within the CBM (40, 44). Co-crystalization of AMPK α2β1γ1 heterotrimers with compound 991 revealed specific interactions within a large hydrophobic pocket created between the kinase domain within the α2-subunit and the CBM of the β1-subunit. Binding of compound 991 was shown to stabilize the interaction between these two subunits resulting in allosteric activation and protection of AMPKαT172 from dephosphorylation (39). While these findings have advanced our understanding of AMPK structure-function and have revealed

important information to guide drug development, they also provide compelling evidence for an unidentified regulatory role of the β-subunit under normal physiological conditions. Furthermore, the natural metabolite/ligand that is mimicked by these synthetic activators, and the physiological importance of the S108 site, is not known.

In these studies, we aimed to better characterize the mechanism by which LCFA activate AMPK, and determine its physiological importance in regulating metabolism. By utilizing a series of enzymatic and genetic in vitro and in vivo models, we provide first evidence for a novel direct interaction between LCFA-CoA esters and AMPK, and establish the importance of the AMPKβ1-S108 site in mediating this activity. These findings expand the allosteric regulation of AMPK beyond adenine nucleotides, and suggest that AMPK also integrates changes in the concentration of high-energy LCFA-CoA esters to maintain energy homeostasis.

Materials and Methods

Materials

Dulbecco's Modified Eagle Media (DMEM), non-essential amino acids, HEPES, phosphate buffered saline (PBS), sodium pyruvate and penicillin/streptomycin were obtained from Invitrogen® (Logon, Utah). Fetal bovine serum (FBS) was obtained from Hyclone® (Grand Island, New York). Bovine albumin, fraction V,

insulin, hydrocortisone, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate were acquired from Sigma Chemical Company (St. Louis, MO). Biocoat® type I collagen-coated plates were purchased from Becton Dickinson Labware (Bedford, MA). Phospho-AMPKα (T172) ELISA and antibodies to AMPKα (threonine 172), t-AMPKα (total), ACC (Serine 79), t-ACC (total), AMPKβ1/2, phospho-AMPKβSer108, and β actin, were obtained from Cell Signaling Technologies (Beverly, MA). Acyl-CoA lithium salts, high-performance liquid chromatography (HPLC) grade reagents and solvents, and active AMPKα1β1γ1 and AMPKα1β2γ1 complexes were obtained from Sigma-Aldrich (St. Louis, MO). ULight-Acetyl-CoA Carboxylase (Ser79) Peptide (SAMS peptide) and europium-anti-phospho-Acetyl-CoA Carboxylase (Ser79) antibody was obtained from PerkinElmer (Waltham, MA).

AMPK Activity and PP2Cα Protection Assay

AMPK activity was determined by phosphorylation of the U*Light*-SAMS peptide. Briefly, 0.5 nM active recombinant full-length human AMPK heterotrimers (isolated from Sf9 cells obtained from Sigma-Aldrich. St. Louis, MO) was pretreated with the indicated activators +/- 0.5 nM PP2Cα (protein phosphatase-2Cα) in 30 μL kinase buffer containing 50 mM HEPES pH 7.5, 1mM EGTA, 2mM DTT, 0.01% Tween, in white opaque 96-well microplates at 37˚C for 15 minutes. Reactions were returned to room temperature on an orbital plate shaker for 5

minutes before a 10 μL addition of a mixture containing 4X ATP (30μM final) and Ulight SAMS (50 nM final). Plates were briefly centrifuged at 2000rpm and placed back on the plate shaker at room temperature for 15 minutes. Reactions were stopped by the addition of 40 μL of detection mix containing 40 mM EDTA and 8 nM Eu-anti-phospho ACC antibody. SAMS phosphorylation was determined by TR-FRET (Lm1 Ex = 330 nm, Em = 668 nm (630 nm Co); Lm2 Ex = 330 nm, Em = 620 nm, (570 nm Co). The 668 /620 nm fluorescence emission ratio was calibrated to standardized active AMPKα1β1γ1 enzyme with reported activity of 685nmole/min/mg.

AMPK activity assay using radiolabelled ^{32}P-ATP was conducted as described previously (Scott et al (2008) Chem Biol). AMPK heterotrimers were expressed and purified from either E.coli expression system or transfected COS7 cells. Specifically for AMPK from COS7 cells, AMPK heterotrimers were immunoprecipitated (with respective tag antibodies) and washed extensively with assay buffer (50mM HEPES pH 7.4, 1mM DTT and 0.1% Tween-20) prior to kinase reaction. Activity assay was conducted in presence of 100μM SAMS peptide, 5mM $MgCl_2$, 200μM [γ-^{32}P] ATP for 10 min at 30^{o}C in the presence or absence of ligand, palmitoyl-CoA (10μM) or AMP (100μM) as a positive control. Phosphotransferase activity was quenched by spotting onto P81 phosphocellulose paper (Whatman, GE Healthcare) followed by repeated washes in 1% phosphoric

acid (Glass et al. 1978 Anal. Biochem). The level of ^{32}P transfer to the SAMS peptide was quantified by liquid scintillation counting (Perkin Elmer).

PP2Cα Protection Assay

AMPKαT172 phosphorylation was determined under conditions described above for TR-FRET detection with the following exceptions. Following pretreatment with indicated activators +/- 0.5 nM PP2Cα (protein phosphatase-2Cα) in 30 μL kinase buffer containing 50 mM HEPES pH 7.5, 1mM EGTA, 2mM DTT, 0.01% Tween, in white opaque 96-well microplates at 37˚C for 15 minutes, reactions were stopped with 40 mM EDTA and AMPKαT172 phosphorylation determined by ELISA following the manufactures directions (cell signaling technologies).

Cell Culture

Mouse primary hepatocytes were obtained from C57Bl/6 mice by collagenase digestion as previously described (45). Briefly, murine hepatocytes were suspended in William's Media E containing 10% FBS and 10% antibiotic-antimicotic and plated in 6- or 12-well collagen coated plates, and allowed to adhere for 4-5 hours. Cells were then washed with PBS and media re-added for overnight incubation. All experiments were performed the following morning after cells were serum starved for 2-3 hours. For western blots, cells were pretreated with serum free media containing 1% fatty acid-free BSA containing the indicated concentrations of fatty

acids or control AMPK agonists. Media was rapidly removed and cell lysis buffer applied to cells rapidly on ice and plates snap frozen on liquid nitrogen. Lysates were stored at -80oC for further analyses.

Immunoprecipitation of AMPK complexes form HEK 293 Cells

HEK-293 cells stably expressing WT or R531G mutant γ2 have been described. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml hygromycin B. Cells were lysed and and active MPK complexes were immunoprecipitated using EZview Red ANTI-FLAG M2 Affinity Gel per the manufactures instructions.

Immunoblotting

Cell lysates were prepared using 1X lysis buffer containing, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM, sodium pyrophosphate,1 mM β glycerophosphate,1 mM Na3VO4, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X phosphatase inhibitor cocktail (Sigma). Total lysate protein concentrations were determined using the BCA Protein Assay (BioRad Laboratories, Hercules, CA). Protein concentrations were adjusted and diluted in 4X LDS (lithium dodecyl sulfate gel sample buffer) containing 50 mM DTT. Proteins were separated using

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4%-12%) Bis/Tris, MOPS running buffer (Invitrogen® Logon, UT). Separated proteins were electrophoretically transferred to polyvinyl difluoride (PVDF) membranes. Nonspecific binding was blocked and membranes were probed with antibodies against β-actin P/T-AMPKα, P/T-ACC, P/T-AMPKβ1/2, and P/T-raptor.

In Vivo Studies

Germline AMPKβ1-S108A KI mice were generated by Taconic and provided by Kei Sakamoto (Nestle, Switzerland). Analysis of respiratory exchange ratio (RER) was performed using the Oxymax Columbus Instruments Comprehensive Lab Animal Monitoring System; mice were acclimatized to the system for 48 hrs prior to data collection. AMPKβ1-S108A KI and WT mice were fasted overnight and allowed access to food at 07:00. After a re-feeding period, mice received intralipid (10mg/kg) or vehicle (saline) via oral gavage, at 09:00. After an oral gavage, mice were denied access to food, and respiratory measurements were continued until 19:00.

Study Approval

All animal procedures were approved by the McMaster University Animal Ethics Research Board (AUP #:16-12-41).

Results

Long-chain fatty acyl-CoA esters mediate allosteric activation of recombinant human AMPK heterotrimers

We initiated our investigation into the potential mechanism by which fatty acids activate AMPK by determining whether long chain fatty acids (LCFAs) or their respective metabolically activated CoA (LCFA-CoA) esters, directly affected enzyme activity. To this end, we measured the effects of a wide concentration range (0.1 to 100 μM) of palmitate, palmitoyl-CoA, myristate, or myristoyl-CoA on the activity of AMPKα1β1γ1 preparations from Sf9 cells, and found that both acyl-CoA esters activated the kinase ~3-fold while their respective free acid forms were inactive (Figure 1A). Importantly, activation was observed at concentrations known to be physiologically relevant and below the critical micelle concentration for LCFA-CoAs (14), with EC_{50} value of approximately 1 μM for both palmitoyl-CoA and myristoyl-CoA. To characterize the specificity of AMPK for the chain length of saturated LCFA-CoA esters, we measured AMPK activity in the presence of multiple acyl-CoAs ranging from C2 (acetyl-CoA) to C18 (stearoyl-CoA) and found that LCFA-CoAs ≥ C12 activated the kinase (Figure 1B). Palmitoleoyl-CoA, oleoyl-CoA, and linoleoyl-CoA also increases AMPK activity to a similar magnitude, suggesting that AMPK also responds to the desaturated forms of LCFA-CoAs (Figure 1B). We further established the specificity of AMPK for LCFA-

CoAs by showing that several other coenzymes, precursors, and vitamins that share structural features with CoA also failed to allosterically activate AMPK (Figure 1 C). Moreover, other metabolites involved in LCFA-CoA biosynthesis or catabolism such as malonyl-CoA, free coenzyme A (CoASH), palmitoylcarnitine, and pantothentic acid, also failed to stimulate the kinase (Figure 1 D), further demonstrating the specificity of AMPK for LCFA-CoAs. To establish whether the fatty acid or CoA moiety were individually sufficient to prevent binding and activation by the acyl-CoA conjugate, we pre-incubated AMPKα1β1γ1 complexes with 10 μM palmitoyl-CoA alone or in combination with increasing concentrations of free CoA or palmitic acid. As previously observed, palmitoyl-CoA (10 μM) alone increased AMPK activity by approximately 3-fold, and was unaffected by the addition of up to 100 μM free CoA (CoASH) or palmitic acid (Supplementary figure 1), indicating that neither unconjugated free acid nor free CoA prevented palmitoyl-CoA-dependent activation of AMPK. We then tested whether LCFA-CoA esters also activated α2-containing complexes. Similar to AMP, palmitoyl-CoA activated α2-containing complexes in a concentration-dependent manner (EC_{50} = 1.1 μM and 2.1 μM, respectively) with maximum activation for both activators reaching ~6-fold (Figure 1E). These findings demonstrate that activation of AMPK by LCFA-CoA esters was present in trimeric complexes containing both α1- and α2- subunit isoforms, and similar to previous observation with AMP (39), activation was enhanced in α2 containing AMPK complexes.

Palmitoyl-CoA does not prevent PP2Cα –dependent AMPK T172 de-phosphorylation

It has been previously shown that binding by both AMP (γ-regulatory subunit) and A-769662 (β-regulatory subunit) results in a conformational shift which protects phosphorylated T172 from protein phosphatase-dependent dephosphorylation and enzyme deactivation (39). To determine whether LCFA-CoA esters also protect T172 from protein phosphatase activity, we measured AMPKα1β1γ1 activity following pretreatment with protein phosphatase-2Cα (PP2Cα). As expected, the treatment of AMPKα1β1γ1 with PP2Cα alone resulted in a concentration-dependent and proportional reduction in both αT172 phosphorylation and activity (Figure 1F). However, in contrast to AMP, palmitoyl-CoA did not attenuate PP2Cα-dependent inhibition of AMPK activity (Figure 1G). Moreover, allosteric activation of AMPK by palmitoyl-CoA was significantly inhibited in the presence of PP2Cα. These findings suggest that although palmitoyl-CoA directly interacts with AMPK heterotrimers, its interactions are distinct from other allosteric activators (e.g. AMP and A-769662) and might require αT172 phosphorylation for full activation.

LCFA-CoAs activate AMPK in a manner distinct from AMP

Given the structural similarities between AMP and the adenine moiety of CoA, we conducted studies to determine whether activation of AMPK by palmitoyl-CoA was additive when co-incubated with saturating concentrations of AMP. Consistent with a distinct mechanism of activation, palmitoyl-CoA activated AMPK to a similar degree in the presence or absence of 100 μM AMP (Figure 2A). Further distinguishing AMPK activation between AMP and palmitoyl-CoA, studies using an AMPKα1β1γ1 phospho-mimetic mutant where the threonine 172 residue within the activation loop of the kinase domain has been substituted with aspartic acid (T172D), showed that while AMP activated the T172D mutant by ~7-fold, activation by palmitoyl-CoA was only ~1.5-fold (Figure 2B). Next, we treated active AMPK complexes immunoprecipitated from isogenic cell lines expressing wild type AMPK (WT) or the AMP- insensitive AMPKγ2 R531G mutants (RG). Both palmitoyl-CoA (10 μM) and A-769662 (0.5 μM) significantly increased AMPK activity isolated from both WT and RG cells, while AMP activated WT AMPK only (Figure 2C). These findings indicate that LCFA-CoAs activate AMPK complexes independently of interactions with the AMP binding site within the AMPKγ regulatory subunit (Figure 2C).

We next investigated whether palmitoyl-CoA mediated allosteric activation via the α catalytic subunit. Consistent with our findings comparing α1 to α2 trimeric complexes preparations from Sf9 cells, palmitoyl-CoA directly stimulated the

activity of α2(2-279) (kinase domain) fragments from bacterial preparations, while no apparent activation was observed on α1(1-392) (kinase domain + autoinhibitory domain) fragments (Figure 2D). Neither free palmitic acid, free CoA, A-769662, nor AMP activated α2(2-279), indicating a specific and unique mechanism of action for palmitoyl-CoA (Figure 2E). We confirmed that activation of AMPK by palmitoyl-CoA was not due to unanticipated palmitoylation of the α2 subunit resulting from potential acyl transferase contamination in the enzyme preparation as S-hexadecyl-CoA (a LCFA-CoA anaologue modified to prevent its trans-acylation) also efficiently stimulated kinase activity (Figure 2F). Furthermore, we show that activation of AMPK by palmitoyl-CoA is not a result of a non-specific detergent effects as determined by performing serial dilution. Enzyme activity measured in the presence of 0.1% Tween was strictly linear with enzyme dilution with or without palmitoyl-CoA indicating that the stimulation obtained with palmitoyl-CoA was not due to it blocking non-specific loss of enzyme activity due to detergent effects (Supplementary Figure 2)(46).

LCFA-CoAs mediate β1 (S108)-dependent AMPK activation

Despite identifying a novel allosteric interaction between palmitoyl-CoA and the α2(2-279) fragment, this activity does not address the mechanism by which palmitoyl-CoA activated α1β1γ1 complexes. Therefore, we investigated potential interactions with the β-regulatory subunit by first determining whether palmitoyl-

CoA also activated β2-containing complexes. Similar to A-769662, palmitoyl-CoA and myristoyl-CoA, when tested up to 100 μM, did not activate AMPK α1β2γ1 complexes prepared from Sf9, while AMP significantly increased activity (Figure 3A). We then tested whether β1-specific activation by palmitoyl-CoA was also observed in bacterial preparations. These studies showed that consistent with our findings from Sf9 preparations, activation of β2-containing complexes by palmitoyl-CoA was markedly attenuated when compared to β1-containing complexes, suggesting a critical role of the β-regulatory subunit in mediating the activation of AMPK by palmitoyl-CoA (Figure 3B), It has been previously shown that binding and activation of AMPK by β1-selective synthetic activators A-769662, 991, and salicylate are attenuated in cells that contain a single S108A KI mutation within the CBM of the β1-subunit (39, 40, 44). These functional data in combination with the co-crystalization of AMPK α2β1γ1 heterotrimers with compound 991, revealed a binding site within a large hydrophobic pocket created between the kinase domain and the CBM of the β1-subunit (39). Intriguingly, when we tested whether activation of AMPK by palmitoyl-CoA was also affected by the S108A mutation, we found that activation was markedly attenuated in the AMPKα1β1(S108A)γ1 mutant (Figure 3B). As expected, AMP efficiently stimulated kinase activity in AMPKα1β1γ1, AMPKα1β2γ1, and AMPKα1β1(S108A)γ1 complexes to a similar degree (Figure 3B). While these findings establish an important role for the β1 S108 site in mediating the activation

of AMPK by palmitoyl-CoA in the context of the α1β1γ1-heterotrimer, clearly this activity involves complex interactions between the α-catalytic and β1-subunit that may vary depending the isoform combination expressed.

Long-chain fatty acids mediate β1-S108 selective AMPK activation in primary hepatocytes

We next investigated whether LCFA-CoA esters mediated β1(S108)-dependent activation of AMPK in intact cells. To this end, we generated mice harboring the AMPKβ1-S108A KI mutation and measured AMPK activation by palmitate in primary hepatocytes isolated from WT and AMPKβ1-S108A KI mice. Similar to A-769662 (10 μM), palmitate increased ACC (S79) phosphorylation in a concentration dependent manner in WT hepatocytes, an affect that was markedly blunted in hepatocytes from AMPKβ1-S108A KI mice (Figure 3C). By contrast, AMPKγ-subunit-dependent activation by AICAR was observed in both WT and AMPKβ1-S108A KI hepatocytes (Figure 3B). No difference in AMPKβ expression was observed between genotypes (Figure 3C). We next investigated whether metabolic adaptation in response to acute exposure to fatty acids in vivo was affected by the AMPKβ1-S108A KI mutation by measuring the respiratory exchange ratio (RER) in mice orally administered Intralipid®, a lipid emulsion primarily composed of linoleic, oleic, palmitic, α-linolenic, and stearic acids. No statistically significant differences in the RER were observed between WT and

AMPKβ1-S108A KI mice during normal fasting or refeeding. However, following a 12 hour fast and 2 hour refeed, oral administration of Intralipid® immediately suppressed the RER in WT mice compared to vehicle treatment (Figure 4A) Importantly, Intralipid®-induced RER suppression was significantly attenuated in AMPKβ1 S108A KI mice (Figure 4A). We then measured liver ACC S79 phosphorylation in WT and AMPKβ1-S108A KI mice 1 hour post Intalipid® administration and found that while Intalipid® significantly increased ACC S79 phosphorylation in WT mice, no difference between vehicle or Intralipid® could be detected in livers from AMPKβ1-S108A KI mice (Figure 4B). AMPK T172 and Raptor phosphorylation also trended higher in Intralipid® treated WT mice only, although this did not reach statistical significance (Figure 4B). To determine whether adipose AMPK activation contributes to the suppression of RER in response to Intralipid®, we repeated the experiment in mice with adipose-specific deletion of AMPK β1/β2 (iβ1β2AKO) mice. These studies showed that Intralipid® did not activate AMPK signaling in adipose tissue and effectively suppressed RER in both WT and iβ1β2AKO mice, suggesting that adipose AMPK was not a significant contributor (Supplementary Figure 3). These in vivo findings support the critical role for the S108 site within the AMPKβ1-subunit in coordinating metabolic adaptations in response to chronic and acute exposure to dietary fatty acids under normal physiological conditions.

Discussion

AMPK is a metabolic sensor composed of an α catalytic, and β and γ regulatory subunits. The underlying molecular mechanisms governing post-translational modification of the α-subunit (T172 phosphorylation) and nucleotide sensing by the γ-regulatory subunit have been carefully studied and have clearly identified an important role for these subunits in cell physiology (for review see (2)). Until recently, the β-subunits have been largely regarded as a scaffold that facilitates glycogen binding and specific interactions between the α-catalytic and γ-regulatory subunits. However, the identification of β1- selective synthetic AMPK activators (e.g. A-769662, salicylate, compound 991, PF-249, MK-8722, and compound 7 (37-43)), has significantly advanced our understanding of the structure-function of the β1-subunit, including its interactions with the α-catalytic subunit and the importance of the S108 phosphorylation site in regulating AMPK activity. Furthermore, these findings raised the intriguing possibility that this drug binding site may have developed to bind a previously unidentified natural ligand critical for the regulation of AMPK activity.

In the present studies using AMPK preparations from Sf9 and bacterial sources, and two distinct methods for measuring kinase activity (TR-FRET SAMS and 32P SAMS assay methods), we demonstrate that palmitoyl-CoA is a previously unidentified allosteric activator of AMPK. Our investigations have uncovered a

novel mechanism by which LCFA-CoAs directly activate AMPK activity via interactions with the α2-catalytic and β1-regulatory subunits. Similar to the synthetic activator A-769662, we show that activation by palmitoyl-CoA is attenuated in β2-containing complexes, and by a single S108A KI mutation within the β1-subunit. We establish that this interaction is specific for LCFA-CoAs and that short-, medium, and very long- chain acyl-CoAs, free fatty acids or free CoA (CoASH) do not activate AMPK complexes. We also show that LCFA-CoAs do not promote the protection of T172 from PP2Cα-mediated de-phosphorylation that is observed for A-769662 and AMP. We then generated mice that were insensitive to the AMPK-activating properties of LCFA-CoAs by introducing a single alanine knock-in mutation at S108 within the β1-subunit (AMPKβ1-S108A KI), and clearly establish an important regulatory role for this mechanism in vivo by demonstrating impaired metabolic adaptions (fuel switch) in response to acute fatty acid exposure.

Some aspects of the LCFA-CoA activation mechanism remain perplexing. Although the LCFA-CoA activation is sensitive to mutation of the β1-S108 site in the context of the AMPK heterotrimer, LCFA-CoA stimulation does not require the β-subunit as the α2(2-279) fragment is stimulated. In order to investigate the binding of LCFA-CoA to AMPK we carried out crystallization trials with bacterial AMPK α2β1γ1. Successful crystallization of the AMPK heterotrimer requires the presence of detergent such as Cocamidopropyl betaine (CAPB) (47). We found

that palmitoyl-CoA can replace CAPB detergent in supporting crystallization at stoichiometric concentrations with AMPK indicating a single binding site. However, we have not detected any electron density attributable to palmitoyl-CoA or CAPB in the structures.

Despite many remaining questions, these studies provide the first direct evidence suggesting that LCFA-CoA are a natural direct ligand for AMPK, and provide important clues for the physiological importance of the AMPKβ1-S108 site. Although the specific interactions between adenine nucleotides and LCFA-CoAs, and the interactions between the α and β subunits in controlling AMPK activity should be further explored, these findings suggest that AMPK not only senses changes in cellular energy status, but integrates these signals with changes in the concentration of high-energy LCFA-CoAs to intimately match cellular energy requirements with substrate availability.

Figure 1.

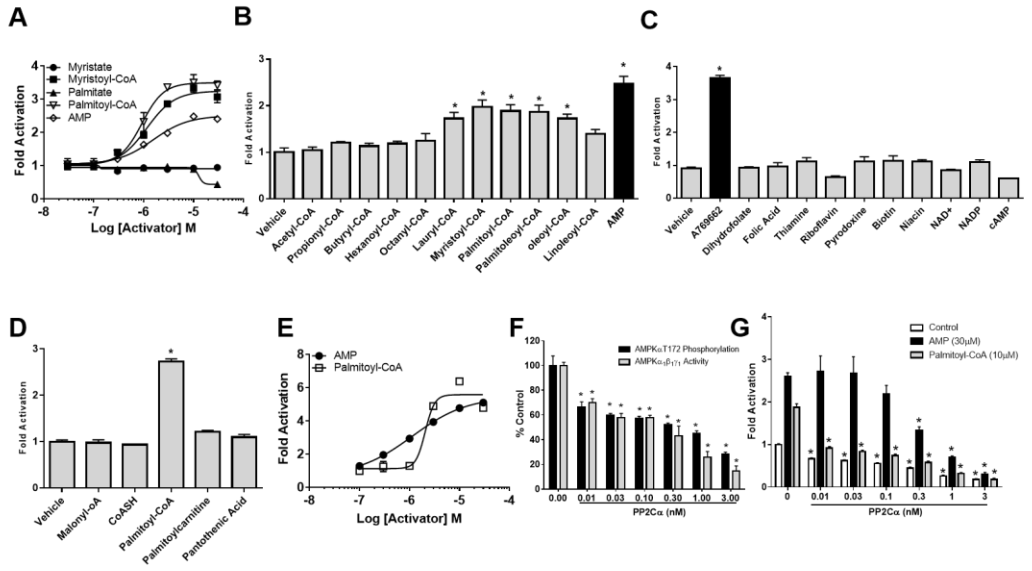


Figure 1. LCFA-CoA directly activate β1-containing AMPK complexes. (A) Activity of AMPKα1β1γ1 preparations from Sf9 cells (basal activity = 1680.48 pmol/mg/min.) determined by TR-FRET in the presence of (A) increasing concentrations of myristic acid and palmitic acid, and their respective coenzyme A (CoA) thioester conjugates. AMP (30 μM) and A-769662 (1 μM) was used as a positive control (B) various acyl-CoAs with chain lengths ranging from C2 to C18. (C) coenzymes (100 μM), cofactors (100 μM) or vitamins (100 μM) (D) metabolites or biosynthetic precursors important for palmitoyl-CoA synthesis or catabolism (E) AMPKα2β1γ1 activity in the presence of the indicated concentration of AMP or palmitoyl-CoA. (F) AMPKα1β1γ1 activity and αT172

phosphorylation in the presence of the indicated concentration of PP2Cα (G) AMPKα1β1γ1activity was determined in ± the indicated concentration of PP2Cα, palmitoyl-CoA or AMP (30 μM). Data are representative of multiple independent experiments performed in duplicate or triplicate. Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test; * = $p < 0.05$ versus vehicle

Figure 2.

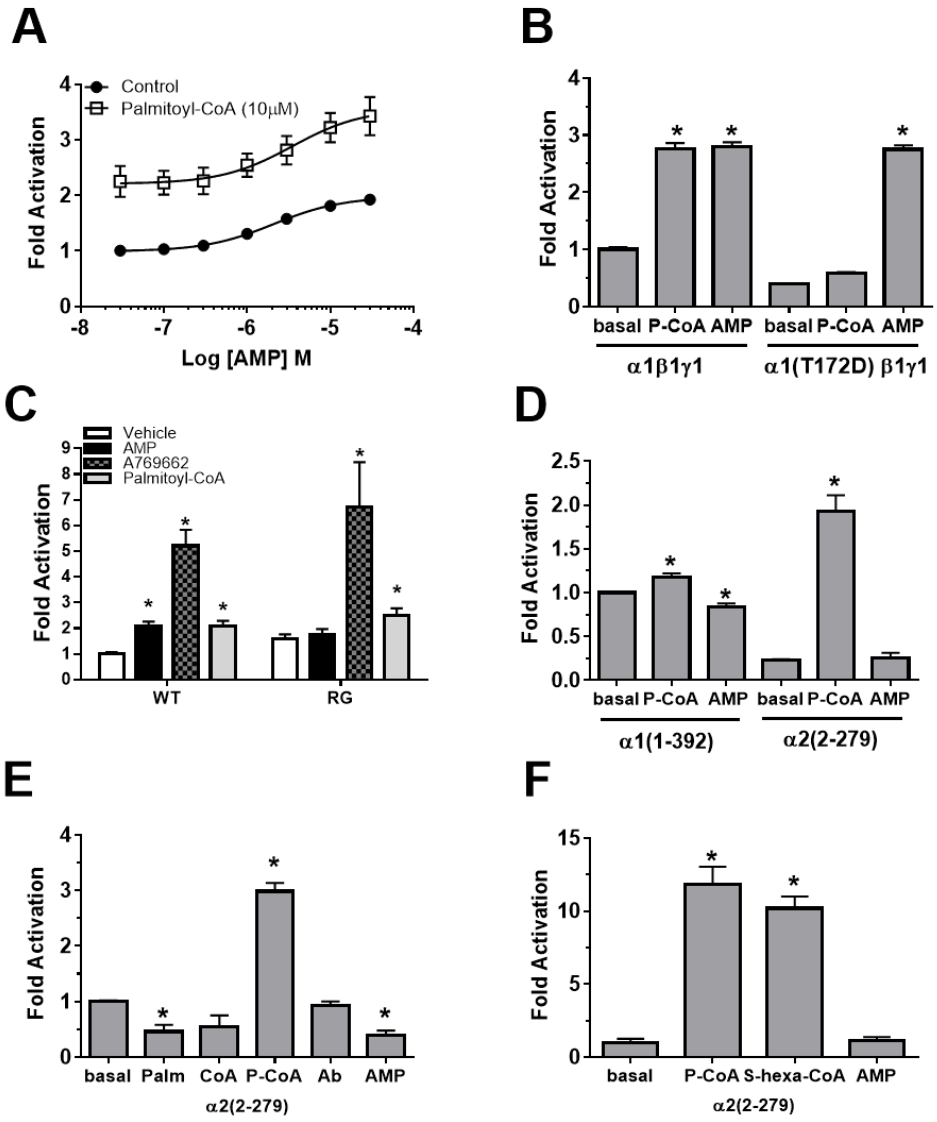


Figure 2. Palmitoyl-CoA activates AMPK in a manner distinct from AMP. (A) Activation of AMPKα1β1γ1 activity by palmitoyl-CoA (10 μM) in the presence of the indicated concentration of AMP. (B) Bacterial AMPKα1β1γ1 and

AMPKα1(T172D)β1γ1 activity in the presence of palmitoyl-CoA (10μM) or AMP (100 μM) measured by 32P SAMS assay. (C) AMPK complexes immunoprecipitated from isogenic cell lines expressing wild type AMPK (WT) or the AMP-and AMP- insensitive AMPKγ2 R531G mutants (RG) were treated with AMP (10 μM), A769662 (0.5 μM), or palmitoyl-CoA (10 μM) and kinase activity measure by TR-FRET. (D) Bacterial AMPKα1(1-392) and AMPKα2(2-279) fragment activity in the presence of palmitoyl-CoA (10μM) or AMP (100 μM) measured by 32P SAMS assay. (E) AMPKα2(2-279) fragment activity in the presence of 10 μM palmitoyl-CoA or S-hexadecyl-CoA (S-hexa-CoA), or 100 μM AMP. (A, B, D, and E) are representative of multiple independent experiments performed in duplicate or triplicate measures; (C) n = 2, performed in triplicate. Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test; * = $p < 0.05$ versus vehicle

Figure3

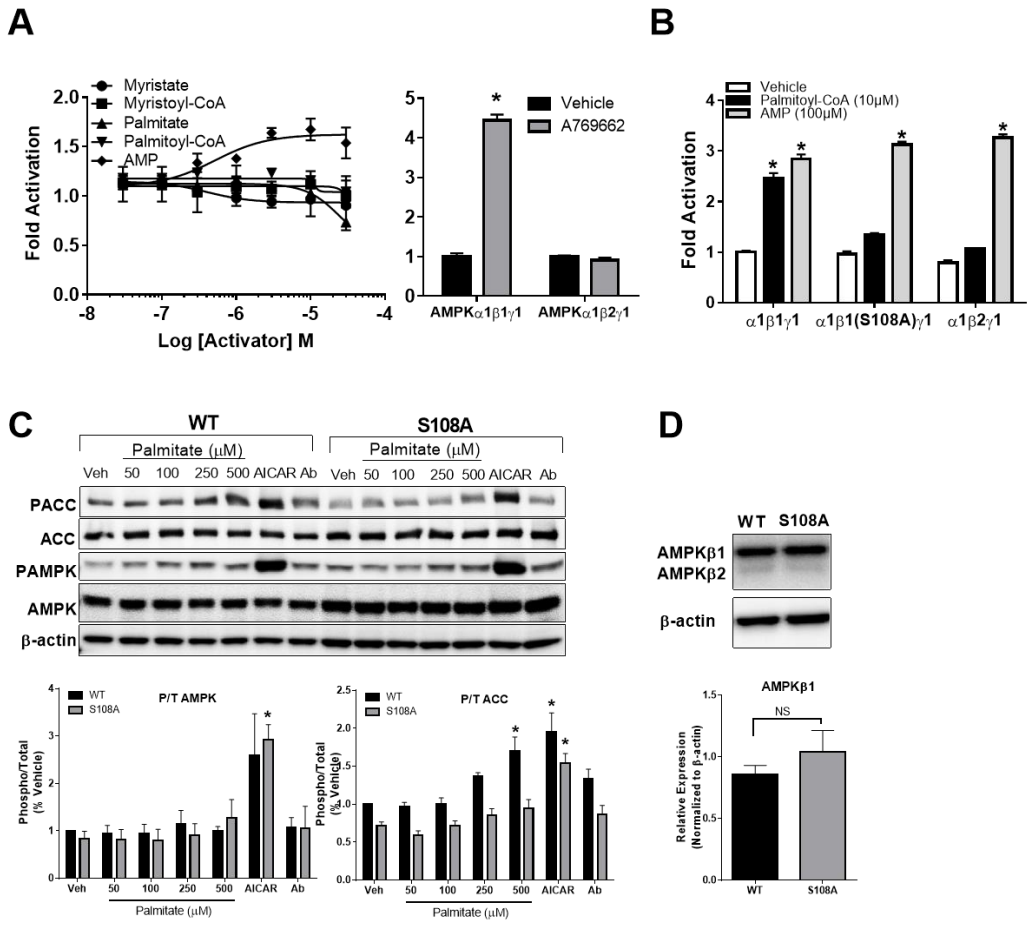


Figure 3. Long-chain fatty acids mediate β1-(S108) dependent AMPK activation. (A) AMPKα1β2γ1 (Sf9) activity in the presence of the indicated concentrations of palmitoyl-CoA and AMP. Treatment of AMPKα1β1γ1 and AMPKα1β2γ1 complexes with A-769662 (0.5 μM) served as a positive control for

β1-dependence. (B) Bacterial AMPKα1β1γ1, AMPKα1β1(S108A)γ1and AMPKα1β2γ1 activity was determined in the presence of palmitoyl-CoA (10 μM) or AMP (100 μM). (C) Primary mouse hepatocytes were isolated from WT or AMPK β1-(S108A) KI mice and treated with the indicated concentration of palmitate, AICAR (100 μM), or A-769662(Ab) (10 μM), and AMPKα172, and ACC(S79) phosphorylation measured by western blotting. (D) AMPKβ1/2 expression was measure in primary mouse hepatocytes isolated from WT and AMPKβ1-(S108A) KI mice. Data are presented as mean ± SEM from multiple independent experiments (A and B), or ate the mean of n = 3 independent experiments performed in triplicate. Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test; * = $p < 0.05$ versus vehicle

Figure 4.

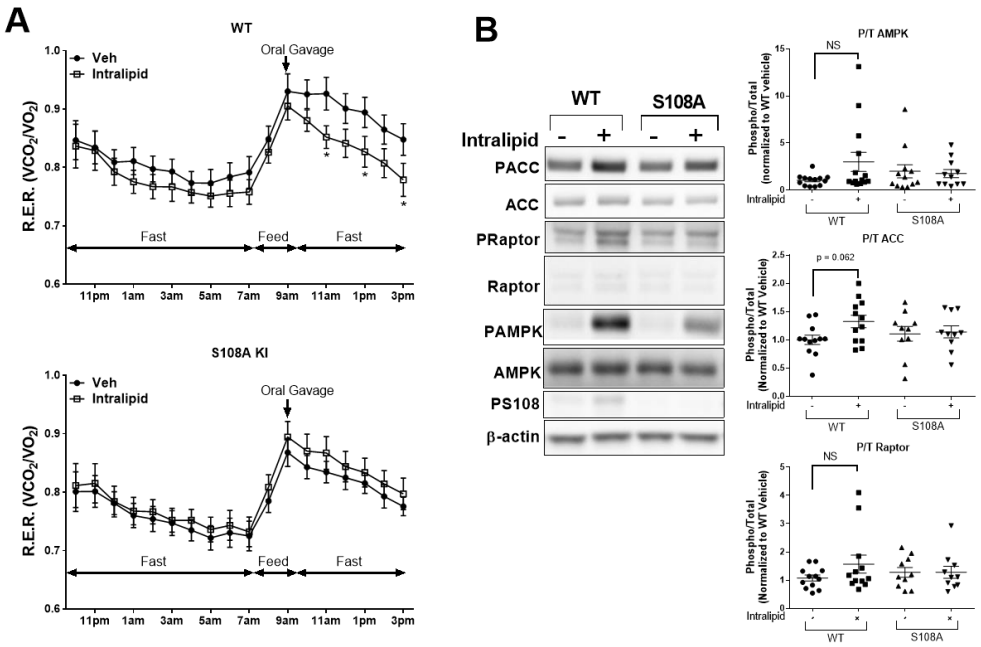
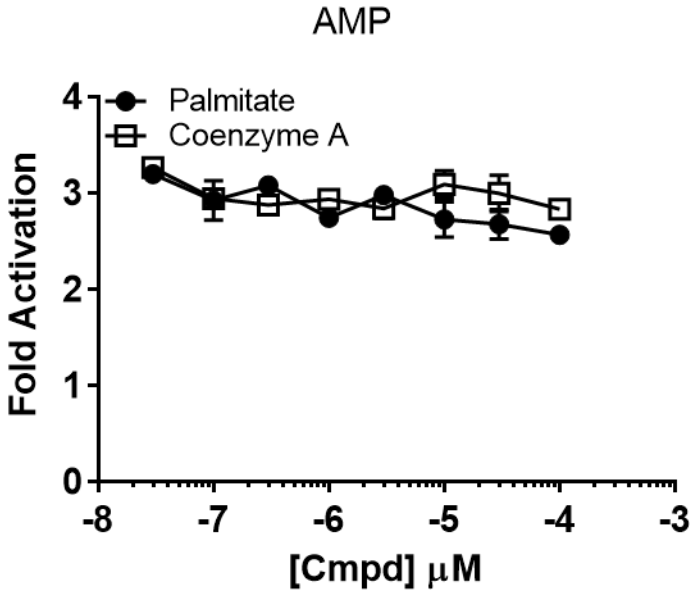


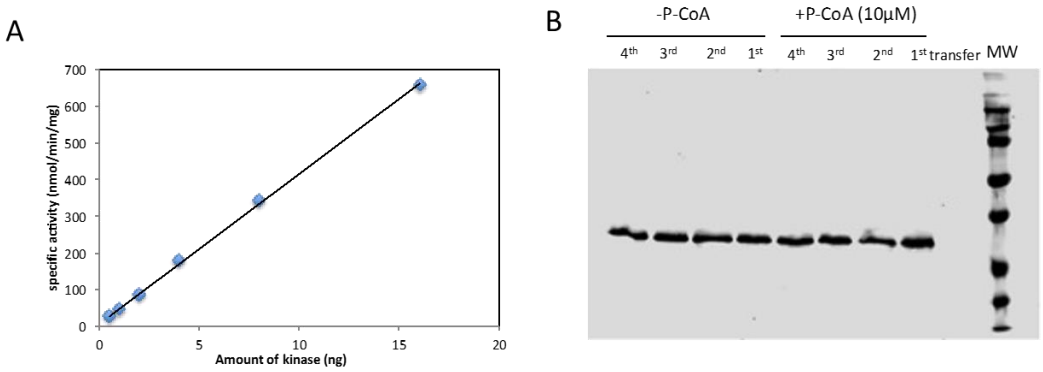
Figure 4. AMPK activity and metabolic response to dietary fat are attenuated in AMPKβ1 S108A KI mice. WT and AMPKβ1 S108A KI mice on normal chow diet were fasted for 12 hours and refed for two hours prior to oral administration of saline or intralipid. (A) Respiratory exchange ratio was monitored for an additional 6 hours, or (B) liver AMPK signaling was measured one hour post administration of Intralipid®. (A and B) n =12, (C) n = 10 to 15, and (D) n= 5 to 8. Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test or Student's t test; * = $p < 0.05$

Supplementary Figure 1.



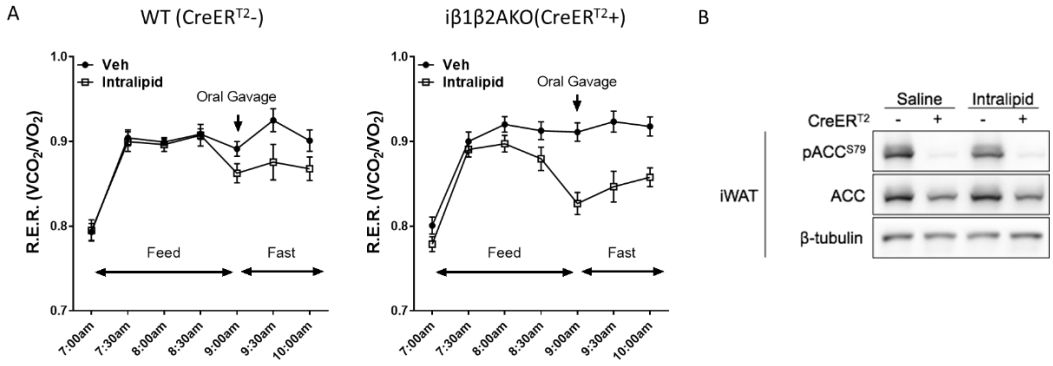
Supplementary Figure 1. AMPKα1β1γ1 Activation by Palmitoyl-CoA is Not Affected by Co-incubations with Palmitate or Coenzyme A. AMPKα1β1γ1 complexes were pre incubated in the presence of palmitoyl-CoA (10μM) ± the indicated concentration of palmitate or free Coenzyme A for 15 minutes prior to running the activity assay. Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test; * = $p < 0.05$ versus vehicle

Supplementary Figure 2.



Supplementary Figure 2. AMPK activation by palmitoyl-CoA is not due to detergent effects. Serial dilution experiments were conducted to determine whether apparent palmitoyl-CoA activation was linked to a detergent effect associated with the prevetion of AMPK loss due to tube binding during dilution. (A) Serial 1:2 dilutions of bacterial AMPK α1β1γ1 heterotrimer in assay buffer + 0.1% Tween-20 showed that the complex does not bind the tube. (B) Serial 1:2 diution of bacterial AMPK α2(2-279) ± palmitoyl-CoA show no loss of enzyme after subsequent transfers in both samples. Assay buffer: 50mM HEPES pH 7.4 + 0.1% Tween-20.Amount of kinase: 50ng AMPKα2 (2-279)/lane ± P-CoA (10µM). Ab: pT172 (Cell Signaling).

Supplementary Figure 3



Supplementary Figure 3. Intralipid Effectivly Supresses RER in WT and iββAKO mice. WT and AMPK iββAKO mice on normal chow diet were fasted for 12 hours and refed for two hours prior to oral administration of saline or Intralipid®. (A) Respiratory exchange ratio (R.E.R.) was monitored, or (B) liver AMPK signaling was measured one hour post administration of Intralipid®. n =7-8, Comparisons were made using one-way ANOVA, Bonferroni's multiple comparison's test or Student's t test; * = $p < 0.05$

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CHAPTER THREE

3 Liver Specific ATP-Citrate lyase inhibition by bempedoic acid lowers LDL-C and attenuates atherosclerosis

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Our studies in chapter two described how we identified a novel mechanism by which liver AMPK senses dietary fat via specific interactions involving a S108 residue within the CBM of the β1-subunit, and how this mechanism facilitates acute metabolic adaptation in vivo. In this manuscript, we study whether mice with germline deletion of AMPKβ1 on an atherogenic *Apoe*$^{-/-}$ background (i.e. *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ double knockout mice), are more susceptible to the onset of metabolic disease and/or atherosclerosis in response to HFHC-feeding. We also explore whether the β1-selective activation of AMPK by natural LCFA-CoA esters can be mimicked by the CoA ester of bempedoic acid, and if this mechanism contributes to the anticipated beneficial effects of this compound in *Apoe*$^{-/-}$ mice. Because bempedoic acid is in late-stage clinical development for the treatment of hypercholesterolemia, the potential involvement of this regulatory mechanism in mediating its pharmacodynamic effects could provide key information regarding its translation to humans. These studies showed that while HFHC feeding accelerated the severity of several disease outcomes such as bodyweight gain, hypercholesterolemia, hyperglycemia, and the progression of atherosclerosis, these outcomes were not further exacerbated in *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ mice. Consistent with clinical investigations, bempedoic acid reduced plasma LDL-C in *Apoe*$^{-/-}$ mice, which was associated with reductions in atherosclerosis; however, these outcomes were also not sensitive to AMPKβ1 deletion. Additional mechanistic studies identified that the CoA ester of bempedoic acid mediated its LDL-C lowering effects by suppressing cholesterol synthesis via ACL inhibition and upregulating liver LDL receptor activity. These findings demonstrate that germline deletion of AMPKβ1 did not contribute to the severity of metabolic disease in response to HFHC feeding, nor the pharmacodynamics of bempedoic. Our subsequent investigations into the mechanism of action of bempedoic acid identified key links

between ACL activity and LDL metabolism with potentially important implications for the role of ACL in metabolism and as a target treat hypercholesterolemia in humans.

S.L.P., R.S.N., R.C.A., S.F., P.H.G., N.D.L and G.R.S. designed the experiments. S.L.P., E.A.D., R.J.F., S.L., C.M.B, and B.K.S. performed experiments and testing. S.L.P., E.A.D., R.J.F., S.L., C.M.B., and B.K.S, provided technical expertise and performed data analyses. S.L.P. and G.R.S. wrote the manuscript

Experiments that I was a primary contributor include: Fig 1A, B, C, D, E, & F; Fig 2A, B, C, D, E, F, G, H, I, & J; Fig 3A, B, H, I & N; Fig 4D & C; Fig 5A, B, C, D, E, F, G, H, I, J & K; Supplementary Fig 1A, B, C, D, & E; Supplementary Fig 2A, B, C, & D; Supplementary Fig 3A, B, C, &D; Supplementary Fig 4A & B; Supplementary Fig 5A, B, C,& D; Supplementary Fig 6A, B, C, D, E, F, G, H, I; and Supplementary Fig 7A.

Liver specific ATP-citrate lyase inhibition by bempedoic acid decreases LDL-C and attenuates atherosclerosis

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Abstract:

Despite widespread use of statins to reduce low-density lipoprotein cholesterol (LDL-C) and associated atherosclerotic cardiovascular risk, many patients do not achieve sufficient LDL-C lowering due to muscle-related side-effects, indicating novel treatment strategies are required. Bempedoic acid (ETC-1002) is a small molecule intended to lower LDL-C in hypercholesterolemic patients, and has been previously shown to modulate both ATP-citrate lyase (ACL) and AMP-activated protein kinase (AMPK) activity in rodents. However, its mechanism for LDL-C lowering, efficacy in models of atherosclerosis, and relevance in humans, are unknown. Here we show that ETC-1002 is a prodrug that requires activation by very long-chain acyl-CoA synthetase-1 (ACSVL1) to modulate both targets, and that inhibition of ACL leads to LDL receptor upregulation, decreased LDL-C and attenuation of atherosclerosis, independently of AMPK. Furthermore, we demonstrate that the absence of ACSVL1 in skeletal muscle provides a mechanistic basis for ETC-1002 to potentially avoid the myotoxicity associated with statin therapy.

Introduction

An elevated level of plasma low-density lipoprotein cholesterol (LDL-C) is a significant risk factor for atherosclerotic cardiovascular disease (ASCVD), the leading cause of death and disability in the western world.[1,2] Statins are the standard of care for controlling elevated LDL-C and are proven to reduce cardiovascular risk and prevent progression of coronary heart disease.[3-5] Statins lower LDL-C by inhibiting hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in the cholesterol biosynthesis pathway.[6] This inhibition leads to reduced hepatic cholesterol levels which triggers the up-regulation of LDL receptors resulting in increased LDL particle clearance from the blood.[7-9] Despite the proven benefits of statins, many patients remain at risk for ASCVD due to their inability to tolerate the statin dose required to achieve recommended LDL-C goals. Myalgia (muscle pain, cramping, and/or weakness) constitutes the most common adverse effect associated with statin treatment and often results in dose limitations, poor compliance, or even discontinuation.[10,11] An estimated 2 to 7 million patients in the U.S. have stopped statin treatment due to muscle complaints despite being at risk for CVD.[12,13] Although the underlying pathophysiology of statin-induced myalgia is not completely understood, significant evidence supports that it is linked to HMG-CoA reductase inhibition in skeletal muscle resulting in reduced production of one or more biological intermediates important to maintain normal muscle cell function.[14-19] This insight

has attracted interest in identifying novel treatment strategies capable of complementing the LDL-C lowering effects of statins without blocking the biosynthesis of key products required for normal skeletal muscle function.

ATP-citrate lyase (ACL) is a cytosolic enzyme upstream of HMG-CoA reductase in the lipid biosynthesis pathway that catalyzes the cleavage of mitochondrial-derived citrate into oxaloacetate and acetyl-CoA, the latter serving as common substrate for *de novo* cholesterol and fatty acid synthesis. Although ACL is not rate limiting, its strategic position at the intersection of lipid and carbohydrate metabolism, and its potential to regulate lipoprotein metabolism, attracted early interest as a drug target to treat dyslipidemia.[20-23] Early discovery strategies that focused on synthesizing citrate and citryl-CoA analogues yielded compounds that were highly potent in cell-free systems; however, they did not advance to clinical development largely due to poor cell permeability and insufficient bioavailability.[24,25]

ETC-1002, (bempedoic acid; 8 hydroxy-2,2,14,14 tetramethylpentadecanedioic acid) is a first-in-class, oral, small molecule cholesterol synthesis inhibitor, that appears to circumvent these issues by exploiting the activity of an unknown endogenous acyl-CoA synthetase (ACS) to mediate its intracellular CoA activation to the active ACL inhibitor, ETC-1002-CoA.[26] Consistent with inhibition of

cholesterol synthesis, ETC-1002 significantly lowers elevated levels of LDL-C in hypercholesterolemic patients by 30% as monotherapy, up to an additional 24% when added on to stable statin therapy, and up to 50% when combined with ezetimibe, suggestive of a distinct mechanism for LDL lowering.[27-30] We have previously shown that the hypolipidemic effects of ETC-1002 are consistent with the inhibition of hepatic ACL by the CoA thioester form of ETC-1002 (ETC-1002-CoA) which results in the suppression of metabolic intermediates downstream of ACL and a reduction in the rates of *de novo* cholesterol and fatty acid synthesis. However, we have also demonstrated that ETC-1002 treatment increased AMP-activated protein kinase (AMPK)[26], a metabolic sensor capable of catalyzing regulatory phosphorylation of numerous substrates that affect inflammatory signaling and lipid metabolism.[31-39] This activation of AMPK in the liver of mice was not associated with changes in cellular energy charge suggesting that ETC-1002 may directly increase AMPK activity via an allosteric mechanism. Despite the importance of these studies, the mechanism by which ETC-1002 lowers LDL-C, modulates ACL and AMPK activity, and the potential importance of these pathways, remain undefined. Furthermore, it is currently unknown whether LDL-C lowering by ETC-1002 is sufficient to reduce the progression of atherosclerosis.

In the present study, we establish ETC-1002 as prodrug which requires the activity of very long chain acyl-CoA synthease-1(ACSVL1) for conversion to an active

modulator of ACL and AMPK activity. Using genetic and pharmacologic methods to suppress ACL *in vitro,* and by testing the effects of ETC-1002 in a novel APOE/AMPK β1 double knockout mouse model, we establish ACL inhibition as the primary mechanism leading to reduced LDL-C and atherosclerosis. Furthermore, we provide a mechanistic basis for the differentiation of ETC-1002 from the myotoxic effects associated with statins by demonstrating the absence of ACSVL1 expression in skeletal muscle from mice and humans, and showing that ETC-1002 does not suppress the cholesterol biosynthesis pathway in this tissue nor promote the associated myotoxicity.

Results

ETC-1002-CoA directly modulates ACL and AMPKβ1 activity

To investigate the mechanism by which ETC-1002 inhibits ACL and increases AMPK activity, we tested whether ETC-1002 or ETC-1002-CoA directly modulated recombinant human ACL and AMPK$\alpha_1\beta_1\gamma_1$ heterotrimeric complexes in cell-free systems. We first conducted kinetic analyses of ETC-1002 and ETC-1002-CoA against multiple ACL substrates and coenzymes to better understand the molecular mechanism of inhibition. These studies showed that ETC-1002-CoA exhibited competitive inhibition kinetics with respect to CoA (Figure 1A) (Ki = 2 μM), while non-competitive inhibition was observed for citrate (Figure 1B) and ATP (Figure 1C), suggesting that ETC-1002-CoA competes for CoA binding.

Importantly, ETC-1002 free acid was confirmed to be inactive against recombinant human ACL (Figure 1D).

We next investigated how ETC-1002 activated AMPK. Using purified enzyme preparations of AMPK$\alpha_1\beta_1\gamma_1$, we found that ETC-1002-CoA dose dependently activated the kinase, while ETC-1002 free acid had no effect (Figure 1E). As anticipated, A-769662 (a direct β1-specific activator of AMPK [40]) and AMP increased enzyme activity (Figure 1E). We then completed the same assays in AMPK α1β2γ1 complexes and found that while AMP continued to activate the complex, both ETC-1002-CoA and A769662 did not increase enzyme activity (Figure 1F). These findings in cell-free enzyme assays 1.) Establish that ETC-1002-CoA is a competitive ACL inhibitor with respect to free CoA, and 2.) Demonstrate that ETC-1002-CoA is the active form that directly interacts with AMPK, and not the free acid as previously proposed[26], and 3.) Show that ETC-1002 can only inhibit ACL and activate AMPK β1 in tissues that are capable of catalyzing the CoA activation of ETC-1002 depicted in (Figure 1G).

Identification of the ETC-1002 synthetase

Given the requirement for CoA activation of ETC-1002 to inhibit ACL and activate AMPK, we performed a series of studies aimed to identify the specific ACS isoform that catalyzes this reaction to better define the tissue specificity for ETC-1002

activity. Previous findings demonstrated the presence of ETC-1002-CoA in rodent liver [26]; therefore, we confirmed ETC-1002-CoA formation in human liver and demonstrate that the enzyme is almost exclusively present in the microsome enriched fraction, while negligible activity was detected in the mitochondrial or cytosol enriched fractions (Supplementary Fig. 1A). We then characterized ACS kinetics in human liver microsomes to establish optimum assay conditions (Supplementary Fig. 1B), and demonstrated consistent ETC-1002-CoA synthesis among individual human donors (784 ± 124 SEM pmol/mg/min. n = 8).

Although the specific physiological roles of many ACS isoforms remain unknown, their respective substrate specificities, subcellular localization, and tissue expression profiles have been extensively characterized.[41,42]. To define the natural substrate profile of the ACS responsible for synthesizing ETC-1002-CoA, we measured [^{14}C]-ETC-1002 synthesis in the presence of a 3-fold molar excess of multiple unlabeled natural short-, medium-, long-, and very long-chain saturated fatty acids. These studies clearly show that fatty acids with C12 to C20 carbon chain lengths were most competitive for ETC-1002, with C22 fatty acid and larger also showing competition (Figure 2A). When similar studies were carried out using aliphatic dicarboxylic acids as competitive substrates, near complete inhibition of ETC-1002-CoA synthesis was observed with C16 and C18 carbon lengths (Figure 2B). As shown by Lineweaver-Burk plots, representative substrates for both C16

saturated mono and dicarboxylic acids (*i.e.* palmitic and hexadecanedioic acids) demonstrated competitive inhibition kinetics for ETC-1002-CoA synthesis (Supplementary Fig. 1C). Furthermore, the known ACSVL1 substrates, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), also markedly competed for ETC-1002-CoA synthesis, while bile acid ligase activity, indicative of the liver-specific ACSVL6/FATP5 activity, was ruled out as cholate and chenodeoxycholate (CDCA) were shown to be inactive (Supplementary Fig. 1D). Members of the thiazolidinedione (TZD) class of drugs, which have been shown to be specific ACSL4 inhibitors [43], also did not compete for ETC-1002-CoA synthesis (Supplementary Fig. 1D). We have previously shown that triacsin C inhibited ETC-1002-CoA formation by approximately 40 to 50% in primary rat hepatocytes, and attenuated the inhibitory effects of ETC-1002 treatment on lipid synthesis to a similar degree.[26] Triacsin C is a potent inhibitor of ACSL1 and 4, and a weak inhibitor of ACSVL1[43-47] Therefore, we tested whether triacsin C directly inhibited ETC-1002-CoA synthesis in human liver microsomes. Consistent with the effects observed in hepatocytes, triacsin C (10 μM) inhibited ETC-1002-CoA formation by 40 to 50% (Supplementary Fig. 1D). When taken together, these competitive substrate and ACS inhibitor findings indicate a profile most consistent with the activities described for ACSVL1 (a.k.a. FATP2; gene *Slc27a2*). This was further supported by showing that ACSVL1 protein levels correlated (r^2 = 0.984) to the rate of ETC-1002-CoA synthesis in various subcellular liver fractions

(Supplementary Fig. 1E). To establish that ACSVL1 was the specific ACS isoform that catalyzes the CoA activation of ETC-1002 we used siRNA-mediated gene silencing in McArdle cells. ACSVL1 (*Slc27a2*) siRNA treatment reduced protein levels by more than 80% which was associated with an 85% reduction in ETC-1002-CoA formation (Figure 2C). Importantly, ACSVL1 gene silencing blocked ETC-1002 but not atorvastatin-dependent inhibition of *de novo* cholesterol synthesis, thus establishing the high specificity of ACSVL1 for the CoA activation of ETC-1002 (Figure 2D). These studies demonstrate that that the prodrug ETC-1002 is converted to ETC-1002-CoA by ACSVL1 and that this is required to suppress cholesterol synthesis in hepatocytes.

The established requirement for CoA activation suggests that ETC-1002 only modulates ACL and AMPK activities in cell types that express ACSVL1. Importantly, the tissue expression profile of ACSVL1 has been independently studied in mice and shown to be restricted to liver and kidney while absent in other tissues including skeletal muscle.[48] Consistent with these previous observations, we found that ETC-1002-CoA was detected in liver, but not skeletal muscle or adipose tissue from ETC-1002-treated mice (Supplementary Table 1). Investigations in microsome preparation from human tissues demonstrated that while ACSVL1 was highly expressed in liver, it was only minimally detected in kidney, and was undetectable in skeletal muscle (Figure 2E). In contrast to

observations in liver microsomes where ACSVL1 was highly expressed, the absence of ACSVL1 in human skeletal muscle corresponded to a lack of ETC-1002-CoA synthetase activity (Figure 2F); however, CoA activation of palmitate was still observed in human skeletal microsomes indicating the activity of other ACS isoforms was present (Figure 2F). Importantly, consistent with the absence of ACSVL1 expression in primary human skeletal muscle myotubes (Figure 2G), ETC-1002 did not suppress cholesterol synthesis (Figure 2H) or induce signs of muscle apoptosis or cytotoxicity, a finding that was in stark contrast to that seen with simvastatin or atorvastatin (Figure 2I & J; and Supplementary Fig. 5). Similar observations were also made in rodent immortalized L6 myotubes (Supplementary Fig. 2, 3, & 4). Collectively, these findings indicate that the CoA activation of ETC-1002 and subsequent suppression of cholesterol synthesis requires ACSVL1, and since ACSVL1 is not expressed in skeletal muscle, ETC-1002 is unlikely to cause the associated myotoxicity.

ETC-1002 reduces atherosclerosis independent of AMPKβ1

Given that ACSVL1 is predominately expressed in the liver and that modulation of both ACL and AMPK activity could affect blood levels of atherogenic lipoproteins, we next aimed to investigate the relative importance of these pathways for mediating the cholesterol lowering effects of ETC-1002, and whether these effects lead to reduced vascular lesion development. To this end we crossed APOE

deficient (*Apoe*$^{-/-}$) mice with mice lacking AMPK β1 (*Ampkβ1*$^{-/-}$) to generate double knockout (DKO) mice. We have previously shown that AMPK β1 null mice have dramatic (>90%) reductions in liver AMPK activity [49]; an effect also observed when crossed onto the APOE deficient background as detected by significant reductions in activating phosphorylation at AMPK αT172 and phosphorylation of its downstream substrate acetyl-CoA carboxylase (ACC) (Figure 3A). Consistent with previous results, we observed that ETC-1002 increased liver AMPK activity in *Apoe*$^{-/-}$ mice, but this effect was not observed in DKO mice lacking AMPK β1 (Figure 3A). We also confirmed ACSVL1 expression in liver using skeletal muscle as a negative control (Figure 3B).

While mice fed a high fat-high cholesterol (HFHC) diet displayed significant increases in body weight, adiposity, fasting glucose, and diminished glucose tolerance compared to chow-fed mice, no statistically significant genotype or ETC-1002 (30 mg/kg/day) treatment effect on these parameters were observed (Supplementary Fig. 6A through H). HFHC-feeding also increased VLDL (Figure 3C and D), LDL (Figure 3C and E), and total cholesterol (Figure 3C and G) by > 2.5-fold in both *Apoe*$^{-/-}$ and DKO mice; however, no differences in plasma lipoprotein protein profiles were observed between genotypes. Importantly, ETC-1002 treatment reduced LDL-C in both *Apoe*$^{-/-}$ and DKO mice to a similar degree (38% and 44%, respectively) (Figure 3C and E), and had no effect on other

lipoprotein fractions (Figure 3C, D and F). Although ETC-1002 markedly reduced LDL-C, because *Apoe*$^{-/-}$ mice carry the majority of their plasma cholesterol in the VLDL fraction, this effect corresponded to only a modest reduction in total plasma cholesterol of 18% in *Apoe*$^{-/-}$ mice and 13% in DKO, although the effect did not reach statistical significance in DKO mice (Figure 3G). We then assessed the effects of HFHC-feeding and ETC-1002 treatment on hepatic lipids and showed that HFHC-feeding increased hepatic cholesterol mass by ~2-fold in both *Apoe*$^{-/-}$ and DKO mice, an effect which was almost completely blocked by ETC-1002 treatment (Figure 3H). HFHC-feeding also increased hepatic triglycerides by approximately 2-fold. Remarkably, ETC-1002 treatment completely prevented the increase in triglycerides in both genotypes, showing a 74% and 69% reduction in *Apoe*$^{-/-}$ and DKO mice, respectively (Figure 3I).

Changes in the rates of liver lipogenesis and fatty acid oxidation are important determinants in controlling liver lipid content.[50] Consistent with decreased lipogenesis and increased fat oxidation, ETC-1002 treatment suppressed the respiratory exchange ratio (RER) during the dark (feeding) cycle in both *Apoe*$^{-/-}$ and DKO mice (Figure 3J & K), while no treatment effect was observed during the light (fasted) cycle (Figure 3J & L). To further investigate the mechanisms contributing to AMPK-dependent and independent effects on lipid metabolism *in vivo*, we conducted experiments in primary mouse hepatocytes from AMPK β1 KO

mice. We found that consistent with the AMPK independent reductions in liver lipids and RER observed *in vivo*, ETC-1002 suppressed total lipid synthesis in both WT and AMPK β1 KO hepatocytes (Figure 3M). These data indicate that ETC-1002 reduces LDL-C as well as liver triglycerides and cholesterol through AMPK-independent pathways.

We then investigated the mechanism by which ETC-1002 reduces LDL-C. It has been shown that statins reduce LDL-C by triggering a well-characterized feedback mechanism whereby inhibition of cholesterol synthesis results in reduced cellular cholesterol levels which activates sterol response element binding protein-2 (SREBP2)-dependent LDL receptor transcription. Upregulation of the LDL receptor results in a new homeostatic state where more cellular cholesterol is derived from blood LDL particles, thus reducing blood LDL-C.[51-53] Given the effects of ETC-1002 treatment on lipid synthesis and hepatic cholesterol, we investigated whether ETC-1002 treatment also increased hepatic SREBP – dependent gene expression, and whether this was affected by the absence of AMPK β1. Indeed, AMPK signaling has been shown to regulate some SREBP isoforms through phosphorylation.[32] As expected, HFHC feeding significantly suppressed the expression of numerous genes in *Apoe*$^{-/-}$ mice known to be under SREBP2-dependent transcriptional regulation including *Srebf2*, *Ldlr*, *Pcsk9*, and *Hmgr*, while a slight increase in *Srebf1c* expression was observed (Supplementary Fig. 6I).

Interestingly, *Srebf2* expression was elevated in DKO mice compared to *Apoe*$^{-/-}$ mice; however, consistent with the absence of a genotype effect on plasma LDL-C levels, this did not result in changes in *Ldlr* expression. No differences in *Acly* expression between genotypes were observed. Importantly, ETC-1002 treatment increased *Srebf2* and *Ldlr* expression by > 2-fold in both *Apoe*$^{-/-}$ and DKO mice when compared to their respective HFHC-fed controls (Figure 3N), an effect that was further supported by an increase in plasma membrane-associated LDLR in liver sections from ETC-1002-treated mice (Figure 3O). ETC-1002 also increased *Pcsk9*, *Acly* and *Srebf1c* in both genotypes, although this effect did not achieve statistical significance in DKO mice. Similar to *Srebf2*, *Slc27a2* (ACSVL1) expression was increased by ETC-1002 treatment and in DKO mice compared to *Apoe*$^{-/-}$ mice (Figure 3N), suggesting that *Slc27a2* may also be subject to transcriptional regulation by SREBP2. Although some ACS isoforms have been shown to be regulated by SREBP2[54], other effects on lipid metabolism resulting from loss of AMPK β1 or ETC-1002 treatment cannot be ruled out. These findings in *Apoe*$^{-/-}$ / AMPK β1 double knockout mice and primary hepatocytes demonstrate that the mechanism by which ETC-1002 lowers LDL-C is consistent with compensatory SREBP2- dependent LDL receptor upregulation in response to AMPK independent suppression of cholesterol synthesis.

We then evaluated whether the reductions in plasma cholesterol observed with ETC-1002 treatment translated to reductions in vascular lesion size. Morphological assessments of lesions from H & E-stained sections from the aortic sinus of these mice revealed attenuated lesion development in chow-fed *Apoe*$^{-/-}$ mice whereas HFHC-fed *Apoe*$^{-/-}$ and DKO mice developed significantly larger lesions (Figure 4A). However, no difference in lesion size was observed between *Apoe*$^{-/-}$ and DKO mice fed a HFHC diet suggesting that the absence of AMPK β1 did not accelerate atherosclerosis. Despite the modest reductions in total plasma cholesterol by ETC-1002 treatment, quantitation of sections from the aortic sinus of ETC-1002 treated *Apoe*$^{-/-}$ mice showed a marked reduction (21%; $p < 0.05$, one-way ANOVA and Bonferonni's multiple comparisons test) in lesion size (Figure 4A). Similar to our observations on plasma total cholesterol, aortic lesions from ETC-1002-treated DKO mice trended lower than the DKO control group; however, the reduction was attenuated compared to *Apoe*$^{-/-}$ mice and did not reach statistical significance. Analysis of whole aorta cholesterol levels showed that increases in response to HFHC-feeding (> 2-fold) was markedly attenuated by ETC-1002 treatment in both *Apoe*$^{-/-}$ (-38%) and DKO (-31%) mice (Figure 4B), suggesting AMPK independence. Investigations in bone marrow-derived macrophages from WT and AMPK β1 KO mice showed that consistent with the absence of ACSVL1 expression (Supplementary Fig. 7A), ETC-1002 did not activate AMPK (Supplementary Fig. 7B) nor affect *de novo* lipid synthesis rates in macrophages

(Supplementary Fig. 7C) suggesting that the effects of ETC-1002 are not mediated by direct effects on lesion macrophages. To assess whether the effects of ETC-1002 on hepatic lipid metabolism was associated with reductions in low-grade systemic inflammatory status, we measured plasma serum amyloid A (SAA), a liver-derived acute phase protein that has been shown to be elevated by HFHC feeding. Plasma SAA levels were reduced by >30% by ETC-1002 treatment in both *Apoe*$^{-/-}$ and DKO mice, indicating a reduction in diet-induced low-grade inflammation (Figure 4C). These findings suggest that the anti-atherosclerotic effects of ETC-1002 are primarily mediated by AMPK-independent effects on hepatic lipid metabolism resulting in reduced low-grade inflammation and plasma levels of atherogenic LDL-C.

ETC-1002 up-regulates LDLR activity in human hepatocytes

To examine whether the effects on LDL metabolism observed in mice were linked to cholesterol synthesis inhibition in humans, we treated primary human hepatocytes (PHH) with ETC-1002 and found that it reduced the incorporation of [^{3}H]-H_2O into sterol and fatty acid fractions by 59% and 50%, respectively (Figure 5A). Concentration response studies demonstrated that the half maximal effect concentration for inhibition of *de novo* cholesterol synthesis was approximately 10 μM (Figure 5A). Following a 36 hour treatment, both ETC-1002 and atorvastatin reduced total intracellular cholesterol mass by 21% and 42% respectively (Figure 5B), which corresponded to a 31% and 32% reduction in media ApoB

concentrations (Figure 5C). Assessments of media apoB levels at earlier time points demonstrated that ETC-1002 did not affect secretion rates (Figure 5D), which was supported by a lack of an effect on [^{14}C]-oleate-derived triglyceride secretion (Figure 5E). However, a significant reduction in media apoB levels was observed following 24 hours of treatment, suggesting a potential increase in LDL receptor-mediated apoB uptake. To further validate this observation, we first measured whether ETC-1002 treatment increased the expression of sterol-responsive genes consistent with activation of SREBP-2 –dependent gene transcription in humans as observed in mice *in vivo.* Similar to atorvastatin treatment, ETC-1002 increased the expression of *SREBF2, HMGR, PCSK9, and LDLR* mRNA with maximum effects reaching 1.4, 1.3, 1.5, and 2.3-fold, respectively (Figure 5F). Furthermore, ETC-1002 and atorvastatin treatment also increased DiI (3,3′-dioctadecylindocarbocyanine iodide)-LDL association, confirming that like statins, ETC-1002 treatment up-regulates LDLR activity in human liver cells. Interestingly, A-769662 was inactive, suggesting that β1-selective AMPK activation was not sufficient to increase LDLR activity in primary human hepatocytes. These findings further implicate the role of ACL inhibition in the LDL-C lowering effects of ETC-1002 (Figure 5G).

ACL suppression increases LDLR activity

As we had shown that the lipid lowering effects of ETC-1002 were AMPK independent, we lastly wanted to establish whether suppression of ACL activity by ETC-1002 was sufficient to trigger LDL receptor up-regulation. We first demonstrated model suitability by confirming that our hepatocytes (McArdle cells) were sensitive to pharmacological inhibition of cholesterol synthesis activity by ETC-1002 and atorvastatin treatment. Consistent with our observations in both primary mouse (Figure 3L) and human hepatocytes (Figure 5A), ETC-1002 elicited concentration-dependent inhibition of [^{14}C]-lactate incorporation into cholesterol, cholesteryl ester, and triglycerides (IC_{50} = 9.7, 8.4, 17.8 µm, respectively) (Figure 5H). Atorvastatin treatment also decreased cholesterol and cholesteryl ester synthesis (Figure 5H). These effects of ETC-1002 treatment were associated with a 3.2-fold (EC_{50} = 15 µM) increase in LDL receptor activity (Figure 5I), and in support of our clinical findings, the addition of ETC-1002 to atorvastatin increased LDL receptor activity above atorvastatin treatment alone[29], supporting that the co-suppression of ACL and HMG-CoA reductase activity is complementary (Figure 5I). Transfection of hepatocytes with *Acly* siRNA resulted in an 80% reduction in ACL protein (Figure 5J) which corresponded to more than a 50% increase in LDL receptor protein (Figure 5J) and a 4-fold increase in LDL receptor activity (Figure 5K). These findings support the critical requirement of ACL activity to supply substrate for cholesterol biosynthesis and demonstrate an important regulatory link

between ACL activity and LDL receptor regulation, thus demonstrating that ACL inhibition is a novel molecular target to reduce LDL-C.

Discussion

ETC-1002 is a small molecule cholesterol synthesis inhibitor being developed to lower elevated levels of LDL-C that has been previously shown to inhibit hepatic ACL and promote AMPK signaling. We have further investigated the underlying mechanism for these activities and demonstrate the requirement for CoA activation of ETC-1002 to directly modulate the activities of both enzymes. Importantly, we identify ACSVL1 as the specific ACS isoform responsible for catalyzing the CoA activation of ETC-1002 and demonstrate the requirement of this activity to directly inhibit ACL and mediate β1-dependent AMPK activation. Due to the known tissue expression profile of ACSVL1, these findings demonstrate that modulation of ACL and AMPK activities by ETC-1002-CoA is almost exclusively restricted to liver. We then extrapolate these findings to provide a mechanistic basis for differentiation from the myotoxic effects of statins by showing that the absence of ACSVL1 expression in human skeletal muscle precludes ETC-1002 from inhibiting the cholesterol biosynthesis pathway, a critical source of important biological intermediates essential to maintain normal muscle cell function.[14-18]

Using primary human hepatocytes and a novel *Apoe*$^{-/-}$ / *Ampk β1*$^{-/-}$ double knockout mouse model, we exclude the involvement of AMPK signaling in the mechanism for LDL-C lowering by ETC-1002 and establish ACL as the molecular target. ACL is a cytosolic enzyme upstream from HMG-CoA reductase that catalyzes the first committed step in the utilization of citrate derived from the mitochondrial oxidation of carbohydrates for lipid synthesis. Suppression of ACL activity leads to a reduction in cytosolic acetyl-CoA, a required precursor for cholesterol and fatty acid synthesis[26], and subsequent compensatory LDL receptor upregulation.[20,24,25,55,56] We show that similar to statins, inhibition of ACL by ETC-1002 leads to reduced cholesterol biosynthesis and the upregulation of LDL receptor expression in primary human hepatocytes, and *Apoe*$^{-/-}$ and DKO mice *in vivo*. Furthermore, we demonstrate the therapeutic utility of this mechanism by showing that the resulting reductions in plasma LDL-C were associated with a proportionate reduction in atherosclerosis. ETC-1002 treatment markedly reduced whole aortic cholesterol and lesion size within the aortic sinus. The absence of ACSVL1 expression in macrophages precludes the contribution of direct effects of ETC-1002 on lesion macrophages and suggests that its anti-atherosclerotic activity is primarily driven by reduced systemic inflammation and LDL-C secondary to inhibition of hepatic ACL.

Pharmacological inhibition of cholesterol biosynthesis leads to effective lowering of elevated LDL-C, a validated biomarker for ASCVD risk reduction in hyperlipidemic patients.[3-5] Despite the proven benefits of statins, many patients remain at risk for CVD due to muscle related adverse effects which prevent them from tolerating a statin dose required to achieve recommended LDL-C goals. As such, the management of LDL-C levels can also be achieved by other mechanisms such as inhibition of cholesterol absorption in the gut (*i.e.* ezetimibe), or preventing LDL-receptor degradation (*i.e.*, PCSK9 inhibitors).[57-59] Importantly, each of these mechanisms primarily reduces LDL-C by upregulating the activity of the LDLR, a mechanism proven to reduce CV events.[60-62] These strategies have significantly influenced further expectations for cardiovascular risk reduction in patients with high risk co-morbidities, which has led to the combination of statins with other existing LDL-C lowering agents and the need for novel therapies with mechanisms that complement the effects of statins without increasing adverse effects in skeletal muscle.[57-59] Our findings establish ACL as a viable target for therapeutic intervention by reducing hepatic cholesterol synthesis and increasing LDL receptor activity. Furthermore, characterization of the underlying mechanism leading to the CoA activation of the prodrug, ETC-1002, provides a mechanistic basis for differentiation from statin therapy by improving the liver specificity for cholesterol biosynthesis inhibition and thereby decreasing the potential for muscle-related adverse effects (Figure 6).

Methods

Materials

Dulbecco's Modified Eagle Media (DMEM), non-essential amino acids, HEPES, phosphate buffered saline (PBS), sodium pyruvate, penicillin/streptomycin, DiI-LDL were obtained from Invitrogen® (Logan, Utah). Fetal bovine serum (FBS) was obtained from Hyclone® (Grand Island, New York). Bovine albumin, fraction V, insulin, hydrocortisone, simvastatin, and atorvastatin were acquired from Sigma Chemical Company (St. Louis, MO). Succinic acid, octadecanedoic acid, and arachidic acid were obtained from TCI Chemical (Portland, OR). All remaining fatty acids were obtained from Sigma Chemical Company (St. Louis, MO). Triacsin C, pioglitazone, rosiglitazone, troglitazone, and ACSVL1 antibody were obtained from Abcam (Cambridge, MA). Radiochemicals [^{3}H]-H_2O, [(U)^{14}C]-glucose [1-^{14}C]-acetic acid, [^{14}C]-citrate and [^{14}C]-oleate were obtained from American Radiolabeled Chemicals, Inc (St. Louis, MO) or PerkinElmer (Waltham, MA). Biocoat® type I collagen- coated plates were purchased from Becton Dickinson Labware (Bedford, MA). Primary antibodies to P/T(23A3), AMPK (#2531, #2603), P/T(C83B10) ACC (#3661, #3676), AMPKβ1 (#12063), ACL (#4332), and β-actin(13E5) (#4970) were diluted 1:1000, and secondary antibody (#7074) was diluted 1:10,000, and were all obtained from Cell Signaling Technologies (Beverly, MA). Primary antibodies to LDLR(EP1553Y) (#Ab52818)

diluted 1:1000, and ACSVL1 diluted to 1 µg/mL (#ab83763), were purchase from Abcam. ApoB and ApoAI ELISA were obtained from ALerChek (Springvale, Main). HPLC grade reagents, solvents, and Ultima Gold™ scintillation cocktail were obtained from Sigma-Aldrich (St. Louis, MO). Microscint O was purchased from PerkinElmer (Waltham, MA).

ETC 1002 formulation

For *in vitro* assays, ETC 1002 was formulated using aseptic technique at 30 and 100 mM in sterile dimethylsulfoxide (DMSO) and stored in sterile microcentrifuge tubes at 4ºC for up to 4 weeks. Working solutions of ETC 1002 were prepared in serum-free medium containing 12 mM HEPES, 10,000 U/mL penicillin, and 100 µg/mL streptomycin.

Cell Culture and siRNA Transfection

Authenticated McA-RH7777 cells were purchased from ATCC® (CRL-1601) free of mycoplasma and used for no more than 3 passages. Cells were grown and treated in DMEM containing 1 g/L D glucose, supplemented with 10% FBS. Reverse transfections were performed in 6 well culture plates at 2.5 X 10^5 cells/well using Lipofectamine 2000 Invitrogen® (Logan, Utah). Cells were incubated for 48 hours with 10 nM silencer® small interfering ribonucleic acid (siRNA) for ACSVL1 or ACL or negative control Invitrogen® (Logon, Utah).

Primary Human Myotubes

Cryopreserved primary human myocytes (Lonza CC-2561) were thawed, centrifuged, and resuspended in complete media (SkGM-2 Bullet kit, Lonza) following the manufacturer's directions. Cells were plated in Laminin-coated plates (Corning) and allowed to adhere and grow to 90% confluence. Media was changed to basal media containing 2% horse serum (Life Technologies, Inc.) and 5 μg/mL insulin, and cells continued in culture until myotubes formed. For sterol synthesis assays, fresh basal media containing [^{14}C]- glucose, and vehicle, ETC-1002, simvastatin, or atorvastatin was added to myotube cultures for an additional 12 hours. Viability after 12 hours was assessed in parallel cultures using MTT assay, and non-saponifiable lipids were extracted as described below.. Morphology was determined by visual assessment of merged images from bright field and fluorescent capture (20X) of Hoechst-stained cultured primary human myotubes treated with vehicle, statins (+/- 500 μM mevalonate), or ETC-1002 (100 μM) for 48 hours. Viability/cytotoxicity was determined after 48 hours treatment by measuring GF-AFC/bis-AAF-R110 cleavage, and Caspase 3, 7 activity luciferase-based DEVD cleavage (ApoTox-Clo, Promega).

Primary Human Hepatocytes

Cryopreserved primary human hepatocytes (Triangle Research Labs, LLC) were thawed, centrifuged, and resuspended in complete William's E medium containing

10% FBS. Cells were plated in collagen-coated plates and allowed to adhere for 4 hours. For [^{3}H] H_2O incorporation studies, cells were switched to serum-free medium and incubated for an additional 18 to 20 hours in the presence of [^{3}H]-H_2O +/- indicated compounds prior to lipid extraction. For [1-^{14}C]-acetate incorporation studies, cells incubated overnight in serum-free medium. Cells were washed and preincubated with fresh medium +/- compounds for 1 hour prior to the addition of [^{14}C]-acetate. Cells incubated for an additional 3 hours prior to lipid extraction described below.

***De Novo* Lipid Synthesis Assay**

Rates of lipid synthesis were assessed in cultured McArdle hepatoma cells, primary human hepatocytes, and skeletal muscle myotubes using [(U)^{14}C]-Lactate, [1-^{14}C]-acetate, or [^{3}H]-H_2O. Experiments were performed in DMEM with 4.5 g/L glucose. Cells were treated with compound or vehicle (0.1% DMSO) for up to 4 hours followed by lipid isolation. After metabolic labeling, cell extracts were processed for thin layer chromatography (TLC), or saponified and non-saponified lipids were extracted from cells. Briefly, cells were scraped in 1M KOH/EtOH and transferred to glass vials. Wells were washed with 1M KOH/EtOH and added to the same glass vial. Samples were vortexed and heated for 2 hours with occasional vortexing, and then cooled to room temperature. For the isolation of the sterol fraction, 1 part H_2O and 2 parts n-hexanes were added per sample, and vials were capped, vortexed, and

centrifuged for 5 minutes at room temperature. The top organic layer was transferred to a new tube and sample was extracted again as above in 2 parts n-hexanes. Each sample was heated to 50ºC under N_2 gas until all solvent was evaporated. Samples were re-suspended in toluene and diluted in scinillant. For the fatty acid fraction, 1 part 2N HCL and 2 parts petroleum ether was added to the remaining aqueous layer of each sample. Samples were extracted twice in petroleum ether as described above and transferred to a new glass vial. Samples were heated to 50ºC under N_2 gas until all solvent was evaporated. Samples were re-suspended in toluene and diluted in scinillant.

AMPK Activity and PP2Cα Protection Assay

AMPK activity was determined by measuring the phosphorylation of the ULight-SAMS peptide (Sequence: CHMRSAMSGLHLVKRR synthetic peptide derived from residues 73-85 of rat acetyl-CoA carboxylase in which Ser77 was mutated to Ala; phosphorylation site: Ser79; #TRF0208, PerkinElmer) using TR-FRET. Briefly, 0.5 nM active recombinant full-length human AMPK heterotrimers (isolated from Sf9 cells obtained from Sigma-Aldrich. St. Louis, MO) was pretreated with the indicated activators in 30 µL kinase buffer containing 50 mM HEPES pH 7.5, 1mM EGTA, 2mM DTT, 0.01% Tween, in white opaque 96-well microplates at 37°C for 15 minutes. Reactions were returned to room temperature

on an orbital plate shaker for 5 minutes before a 10 µL addition of a mixture containing 4X ATP (30µM final) and Ulight SAMS (50 nM final). Plates were briefly centrifuged at 2000rpm and placed back on the plate shaker at room temperature for 15 minutes. Reactions were stopped by the addition of 40 µL of detection mix (CR97-100, PerkinElmer) containing 40 mM EDTA and 8 nM Eu-anti-phospho ACC antibody (#TRF0118-D, PerlinElmer). SAMS phosphorylation was determined by TR-FRET (Lm1 Ex = 330 nm, Em = 668 nm (630 nm Co); Lm2 Ex = 330 nm, Em = 620 nm, (570 nm Co). The 668 /620 nm fluorescence emission ratio was calibrated to standardized active AMPKα1β1γ1 enzyme with reported activity of 685nmole/min/mg.

ACL Enzyme Activity Assay

The activity of recombinant human ACL was carried out essentially as described in[63]. Briefly, 7.5X compounds were added to a 96 well PolyPlate containing 60 µL of Buffer (87 mM Tris, pH 8.0, 20 µM MgCl2, 10 mM KCl, 10 mM dithiothreitol [DTT]) per well with substrates CoA (200 µM), adenosine triphosphate (ATP) (400 µM), and [14C]-citrate (specific activity: 2 µCi/µmol)(150 µM). Reaction was started with 4 µL (300 ng/well) ACL and the plate incubated at 37°C for 3 hours. The reaction was terminated by the addition of 3.5 µL 500 mM ethylenediaminetetraacetic acid (EDTA). 200 µL MicroScint O was then added to the reaction mixture and incubated at room temperature overnight with gentle

shaking. The [^{14}C] acetyl- CoA signal was detected (5 minutes/well) in a TopCount NXT liquid scintillation counter (Perkin-Elmer, Waltham, MA).

LDLR Activity Assay

Cells were seeded in 12 well collagen-coated plates at approximately 60% confluence in complete DMEM 10% FBS and allowed to grow overnight. Cells were switched to 5% FBS media +/- compounds and incubated overnight. Cells were washed and serum-free DMEM 0.2% FA-free BSA was added +/- compound and 10 μg/mL DiI-LDL. Cells incubated between 2 and 6 hours before placed on ice and extensively washed with ice-cold PBS. Cholesterol was extracted into 500 μL IPA for 15 minutes on plate shaker, transferred to an Eppendorf tube and centrifuged for 5 minutes at 10,000 x *g*. 300 μL of each sample was transferred to a black 96 well plate and fluorescence measured. Standard DiI-LDL solutions were prepared in IPA, and μg DiI-LDL per mg cell protein was calculated. LDLR-specific LDL association was determined by subtracting fluorescence from cells treated with LDLr neutralizing antibody.

ETC 1002-CoA Synthetase Activity Assay

Human liver sub-cellular fractions and pooled (n = 50) microsomes were obtained from XenoTech , and individual human donor microsomes were obtained from LifeTech. 25 μL of [^{14}C]-ETC 1002 (20X; 10 μM final) and 25 μL blank or

competitive (20X) substrate was added to 425 µL 1X ACS buffer containing 175 mM Tris HCl, 0.1% triton, 32 mM MgCl, 20 mM DTT, 40 mM ATP, 4 mM coenzyme in each reaction vial. Vials were placed on oscillating 37°C water bath for 15 minutes prior to the addition of 25 µL (50 µg) of microsomes to each reaction vial. Following a 15 minute incubation, reactions were stopped by the addition of 200 µL of 2N HCl to each reaction vial. ETC 1002-CoA was separated by 4, 2 mL diethyl ether extractions and 150 µL aqueous from each reaction mixture was added to 5 mL of scintillation fluid and DPM determined.

RT-qPCR

Cryopreserved primary human hepatocytes (Triangle Research Labs, Charlottesville, VA) were seeded in 6 well collagen-coated plates and allowed to attach for 4 hours. Cells were exposed to compounds at the indicated concentration overnight. Cells or mouse liver tissue were lysed in TRIzol reagent (ThermoFisher) to remove lipid, and the aqueous phase was applied to an RNeasy kit (Qiagen, CA, USA) column for subsequent purification. Relative gene expression was calculated using the comparative Ct (2-ΔΔCt) method where values were normalized to a housekeeping gene. Taqman® primers *Slc27a2* (Mm00449517-m1), *Ldlr* (Mm01177349-m1), *Hmgr* (Mm01282499-m1), *Pcsk9* (Mm01263610-m1), *Acly* (Mm01302282-m1), *Srebf2* (Mm01306292-m1), were purchased from ThermoFisher.

Western Blots

Hepatocyte or RH7777 cell lysates were prepared using approximately 150 to 400 μL 1X lysis buffer containing, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM, sodium pyrophosphate,1 mM β glycerophosphate,1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X phosphatase inhibitor cocktail (Sigma). Total lysate protein concentrations were determined using the BCA Protein Assay (BioRad Laboratories, Hercules, CA). Protein concentrations were adjusted and diluted in 4X LDS (lithium dodecyl sulfate gel sample buffer) containing 50 mM DTT. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4%-12%) Bis/Tris, MOPS running buffer (Invitrogen® Logon, UT). Separated proteins were electrophoretically transferred to polyvinyl difluoride (PVDF) membranes. Nonspecific binding was blocked and membranes were probed with antibodies against β-actin, total and phosphorylated forms of AMPK (PT722), and ACC (S79), and LDLR, ACL, ACSVL1, and AMPK β1. Cropped gel images are shown in main figures and uncropped gels are shown in Supplementary Fig. 8.

Animals

All animal procedures were approved by the McMaster University Animal Ethics Research Board (AUP #:12-12-44).

Apoe$^{-/-}$ / *Ampkβ1*$^{-/-}$ double knockout mice

Male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ mice (C57Bl/6) were maintained on a 12-h light dark cycle (lights on at 7.00 am) and housed in a pathogen-free facility at 23°C with bedding enrichment. At 8 weeks of age, mice were allocated to treatment groups to achieve matched mean body weight, and either continued on a normal chow diet (Harland 8640) or were put on a high fat high-cholesterol (HFHC) (TD.09821) ± ETC-1002 targeted to achieve a 30 mg/kg/day dosage for 12 weeks. Lesion histology and lipoprotein profile analyses were performed blinded, while the other study endpoints were not.

Metabolic studies

After 10 weeks of treatment, glucose tolerance tests (GTTs) were performed on 6 hour fasted mice by intraperitoneal injection of glucose (1.2 g/kg body weight) and blood glucose measured by Aviva blood glucose monitor (Roche) from a small tail vein nick at the time points indicated. After 11 weeks of treatment, respiratory exchange ratio (RER) was assessed using a Columbus Laboratory Animal Monitoring System[64], and whole body adiposity was measured by TD-NMR using a Bruker minispec. Fasting (12 h) and fed blood samples were collected by tail vein bleed for serum insulin measurements. Commercially available ELISA kits were

used to measure plasma serum amyloid A (SAA) (Tridelta Development Ltdl Kildare Ireland) and insulin (ALPCO Diagnostics, Salem, NH)

Plasma Lipids and Lipoproteins

At the end of study, fresh EDTA plasma (50 µl) from fasted (6 hours) mice was separated by Fast Performance Liquid Chromatography (FPLC) using an AKTA purifier and a Superose 6 column. A constant flow rate of 0.4 ml/min was used to collect 700ul fractions. An 125ul aliquot of each fraction was used to measure total cholesterol enzymatically in samples on a 96 well microtitre plate with 75 µl of two times concentrated reagents (triglyceride, RocheDiagnostics, Laval, Quebec; cholesterol, WAKO Diagnostics, Richmond, VA; standards, Randox, Crumlin, Co. Antrim, UK)

Tissue Histology

Following perfusion–fixation with 10% neutral buffered formalin[65,66], hearts (including the aortic roots) were cut transversely and embedded in paraffin. Serial sections, 4 µm thick, were cut starting from the aortic root origin and collected for measurement of lesion size (hematoxylin/eosin staining) and immunohistochemical analyses.[67] Lesions were traced manually and measured using computer-assisted image analysis equipment (Olympus BX41 microscope, Olympus DP70 CCD camera, and ImagePro Plus software), and mean lesion size calculated from the first

section of each animal. LDLR IHC was performed on two sections from OCT frozen liver samples from two mice per treatment group. Sections were incubated with diluted (1:20) goat anti-LDLR primary antibody (R&D Systems), followed by diluted (1:200) Alexa 488 donkey anti-goat secondary antibody. Blocking was performed in 5% normal donkey serum. Images were captured at 20x magnification at identical exposure times.

Tissue lipids

Lipids were extracted from approximately 50 mg of frozen liver directly homogenized in 1.0 mL chloroform/methanol (2:1) or whole aortae with connective tissue removed, essentially described by Folch et. al.[68] Samples were dried then solubilized in isopropanol and analyzed for triglyceride (WAKO, Diagnostics, Burlington, Canada 11877771 216) or, total cholesterol (WAKO, Cholesterol E, #439-17501) as per manufacturer's instructions.

Statistical Analysis

All values are reported as mean ± S.E.M. Data were analyzed using Student's t tests or one-way ANOVA or repeated measures ANOVA with Bonferroni post-hoc tests where appropriate. Differences were considered significant when $P<0.05$. Sample sizes were selected based on experience from our previous publications. Data were

excluded only in the case where a technical error occurred in sample preparation or analysis.

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Competing financial interests

Stephen L. Pinkosky, Roger S. Newton, Carolyn M. Birch, Sergey Filippov[1], Pieter H. E. Groot[1], Gregory R. Steinberg, and Narendra D. Lalwani

Author Contributions

S.L.P., R.S.N., R.C.A., S.F., P.H.E.G., N.D.L and G.R.S. designed the experiments. S.L.P., E.A.D., R.J.F., S.L., C.M.B, and B.K.S. performed experiments and testing. S.L.P., E.A.D., R.J.F., S.L., C.M.B., and B.K.S, provided technical expertise and performed data analyses. S.L.P. and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments.

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Metabolism and Obesity and the J. Bruce Duncan Endowed Chair in Metabolic Diseases at McMaster University.

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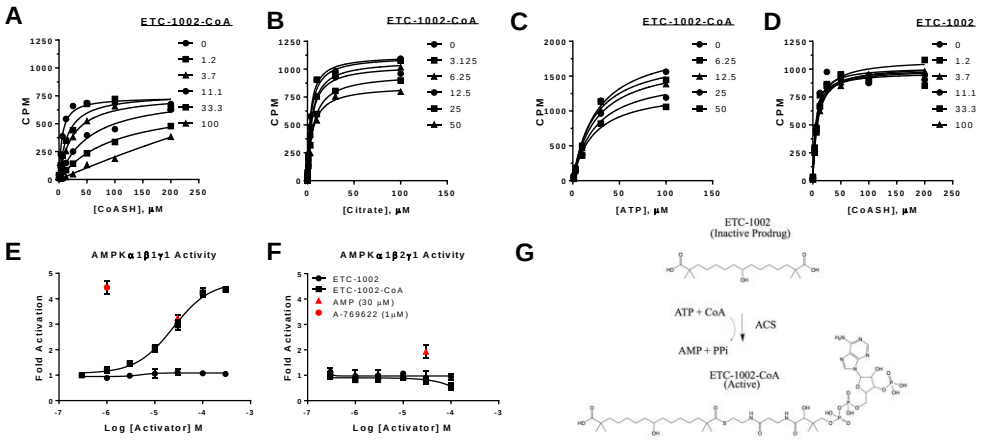


Figure 1. ETC-1002-CoA inhibits ACL and mediates β1-selective AMPK-activation. Recombinant human ACL was incubated in the presence of the indicated concentrations of ETC-1002-CoA (A through C) or ETC-1002 (D), and (A) coenzyme A (CoA), (B) Citrate, or (C) ATP. Conversion of [^{14}C]-citrate to [^{14}C]-acetyl-CoA was measured. Recombinant human (E) AMPKα1β1γ1 and (F) AMPKα1β2γ1 complexes were incubated in the presence of the indicated concentrations of ETC-1002, ETC-1002-CoA, AMP or A-769662. (G) Structure of ETC-1002 and the biochemical reaction that generates ETC-1002-CoA. ETC-1002-CoA/CoASH enzyme ACL kinetic analyses are single measures, and representative results from n = 3 independent experiments shown; AMPK activity was determined by TR-FRET expressed as mean ± SEM of triplicate measures. (A) Ki calculated by Michaelis-Menten kinetic analysis.

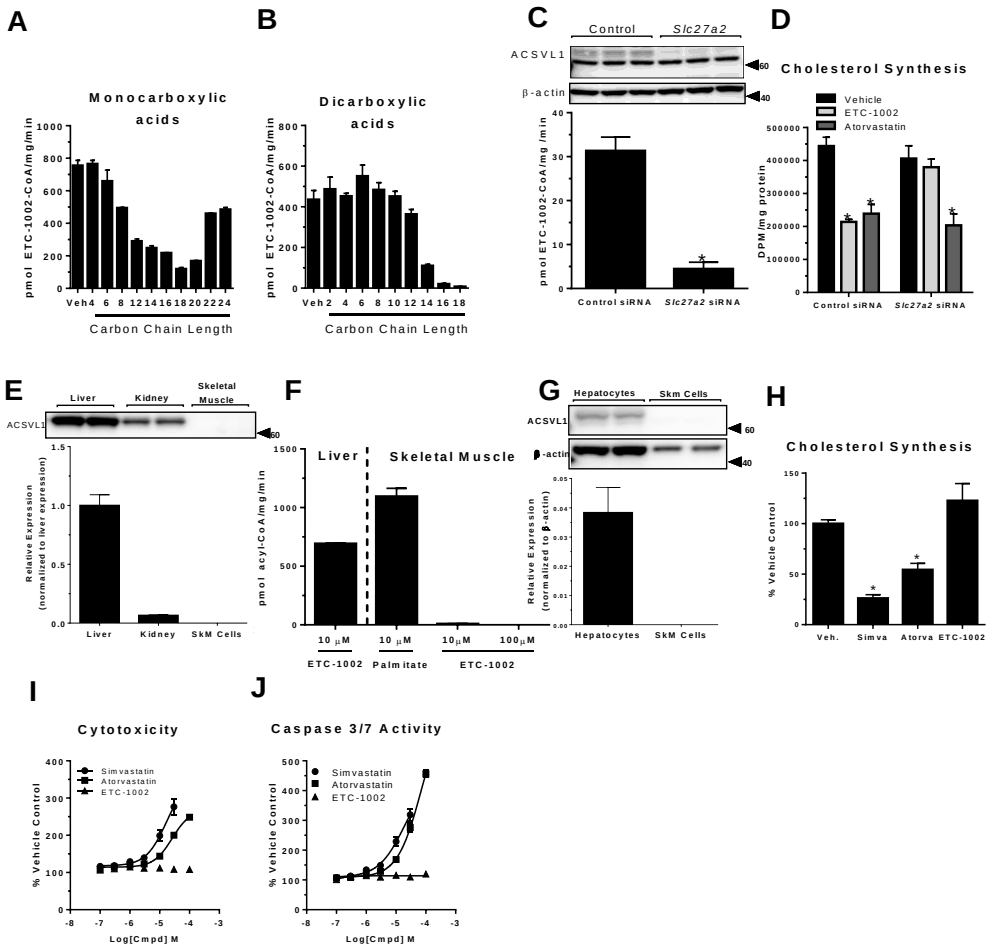


Figure 2. Differentiation of ETC-1002 from the myotoxic effects of statins. [^{14}C]-ETC-1002 (10 μM) conversion to [^{14}C]-ETC-1002-CoA in human liver microsomes was determined in the presence of 30 μM unlabeled (A) monocarboxylic and (B) dicarboxylic saturated competitive fatty acid substrates. RH7777 cells were treated with negative control (Control) or *Slc27a2* (ACSVL1) siRNA; n = 6, for 48 hrs and (C) ACSVL1 expression determined by western blot and [^{14}C]-ETC-1002-CoA synthesis determined; n = 3. (D) Both control and

Slc27a2 siRNA-treated RH7777 cells were treated with vehicle, ETC-1002 (30 μM), or atorvastatin (0.5 μM), and *de novo* cholesterol synthesis measured over 4 hrs; n = 6 transfections. (E) Relative ACSVL1 expression in microsomes prepared from human liver, kidney, and skeletal muscle. (F) ETC-1002-CoA synthetase activity in microsomes prepared from human liver and skeletal muscle; Palmitate (10 μM) used as a positive control for skeletal muscle microsome viability. (G) ACSVL1 expression was measured in primary human hepatocytes (hepatocytes) and primary human skeletal muscle cells (SkM Cells) by western blotting and expressed relative to β-actin. (H) Primary human myotubes were treated with vehicle (Veh.), simvastatin 10 μM (Simva), atorvastatin 10 μM (Atorva), or ETC-1002 100 μM in the presence of [^{14}C]-glucose for 12 hours, and incorporation into non-saponifiable lipids determined; or treated with 0.1 μM to 100 μM simvastatin, atorvastatin, or ETC-1002 for 48 hours and (I) cytotoxicity was measure by (GF-AFC/bis-AAF-R110 cleavage, and (J) Caspase 3,7 activity (DEVD cleavage) determined. Data for myotube cytotoxicity assays are expressed as mean ± SEM, n = 2 performed in triplicate. Data for liver (n = 50 donors pooled) and kidney (n = 50 donors pooled) microsome preparations are representative of two independent experiments performed in duplicate, and expressed as mean ± SD. Data for human skeletal muscle microsomes (n = 4 pooled donors) are the mean ± SEM of two independent experiments performed in triplicate. Multiple comparisons were made using an one-way ANOVA, Bonferroni's multiple comparisons test; * $p < 0.05$

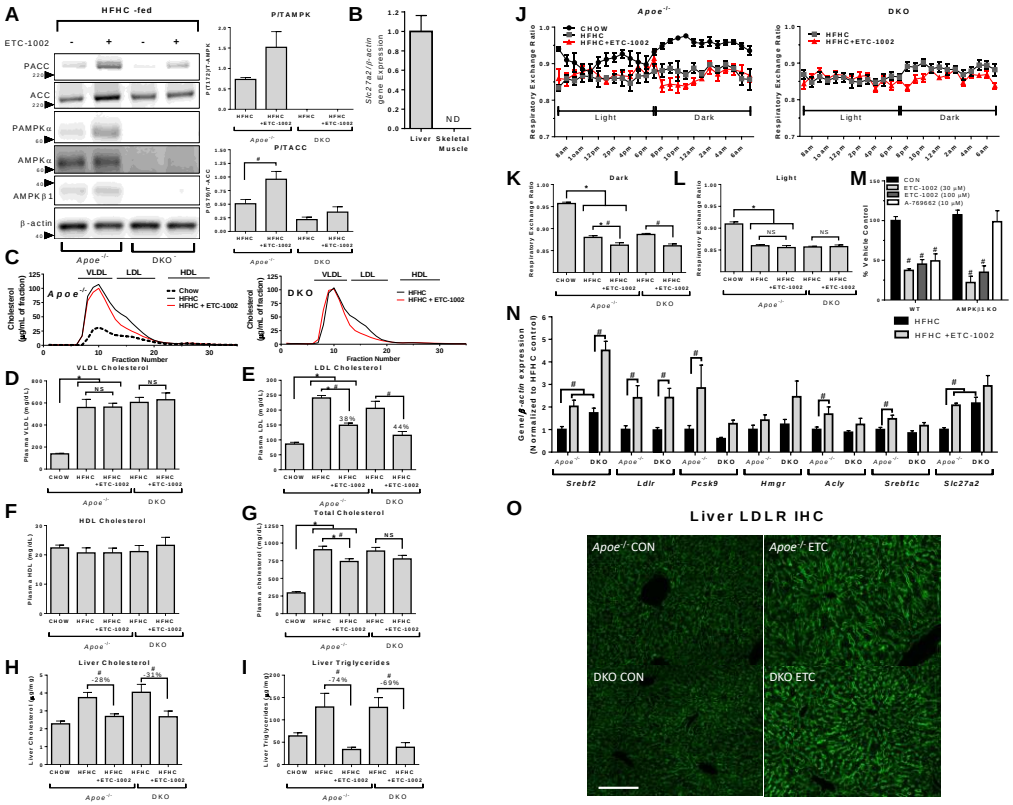


Figure 3. ETC-1002 mediates effects on lipid metabolism independently of AMPK β1. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ (DKO) mice were fed a high fat-high cholesterol diet for 12 weeks with or without ETC-1002 targeted to achieve a 30 mg/kg/day dose and (A) total and phosphorylated (T172) AMPKα and total and phosphorylated (S79) acetyl-CoA carboxylase (ACC) measured in livers from *Apoe*$^{-/-}$ (n = 4) and DKO (n = 3). (B) *Slc27a2* (ACSVL1) expression in liver and tibialis anterior from untreated *Apoe*$^{-/-}$ mice (n = 6). (C) Representative FPLC tracings from chow, HFHC-diet-fed ± ETC-1002 *Apoe*$^{-/-}$ and DKO mice. Plasma (D) VLDL, (E) LDL, (F) HDL (n = 4 for chow-fed mice; n = 6 for remaining

treatment groups), and (G) Total cholesterol (n = 10); liver (H) cholesterol, and (I) triglycerides (n = 4 for chow-fed, and n = 6 for remaining treatment groups) determined at the end of study. (J) Respiratory exchange ratio (RER) was measured over 24 hrs following 10 weeks on diet and mean RER during (K) dark (7pm to 7am) and (L) light (7am to 7pm) cycles calculated. (M) Total lipid synthesis in primary hepatocytes isolated from WT and AMPK β1 KO mice treated for 4 hrs with the indicated concentration of ETC-1002, A-769662, or vehicle (CON) in the presence of ^{3}H-acetate (n = 3-8 independent experiments performed in duplicate. (N) *Srebf2*, *Ldlr*, *Pcsk9*, *Hmgr*, *Acly*, *Srebf1c*, and *Slc27a2* gene expression (n = 4 chow-fed and n = 5 for remaining treatment groups) determined at the end of study. (O) Representative images for IHC staining of LDLR in frozen liver sections from ETC-1002-treated *Apoe*$^{-/-}$ and DKO mice (20X); bar = 100 µM. Data are expressed as mean ± SEM. Multiple comparisons were made using an *one-way (within *Apoe*$^{-/-}$ treatment groups) or $^{\#}$ two-way ANOVA (between *Apoe*$^{-/-}$ and DKO treatment groups); Bonferroni's multiple comparisons test; *and $^{\#}$ $p < 0.05$

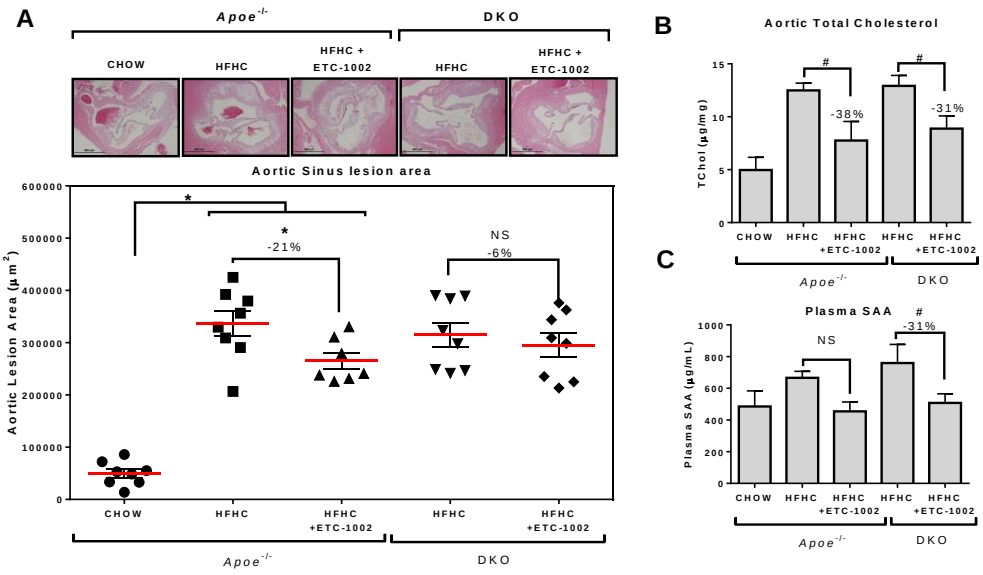


Figure 4. ETC-1002 treatment reduces the progression of atherosclerosis. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ (DKO) mice were fed a high fat-high cholesterol diet for 12 weeks with or without ETC-1002 targeted to achieve a 30 mg/kg/day dose. (A) Sections of the aortic sinus from control and ETC-1002 - treated mice were stained with H & E and atherosclerotic lesion size determined; n = 8 for all treatment groups except ETC-1002 treated *Apoe*$^{-/-}$ (n = 7). (B) Total cholesterol (TChol) was measured in whole aorta (n = 4 for *Apoe*$^{-/-}$; n = 6 for DKO HFHC-fed mice) and (C) plasma serum amyloid A (SAA) measured at the end of study (n = 10). Data are expressed as mean ± SEM. Multiple comparisons were made using an *one-way (within *Apoe*$^{-/-}$ treatment groups) or $^{\#}$ two-way ANOVA (between *Apoe*$^{-/-}$ and DKO treatment groups); Bonferroni's multiple comparisons test; *and $^{\#}$ $p < 0.05$.

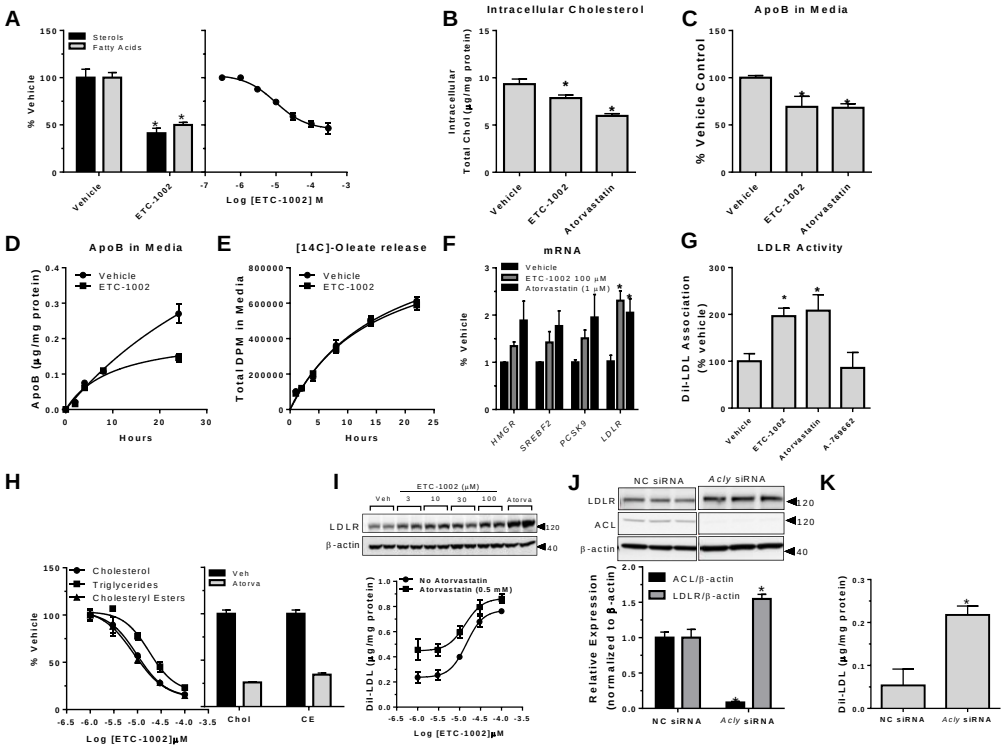


Figure 5. ACL suppression increases LDLR. (A) Primary human hepatocytes (PHH) were treated with vehicle or ETC-1002 (100 µM) for 18 hours in the presence of [^{3}H]-H_2O or pre-treated with the indicated concentrations of ETC-1002 for 1 hour, following a 3 hour [^{14}C]-acetate pulse. Counts incorporated into the non-saponifiable (sterols), and saponifiable (fatty acids) lipid fractions were determined and expressed as % vehicle. (B) Primary human hepatocytes were treated with vehicle, atorvastatin (0.5 µM), or ETC-1002 (100 µM) for 36 hrs and total intracellular cholesterol concentrations were determined in cell lysates. (C) Media was assayed for apoB by ELISA. (D) PHHs were treated with ETC-1002

for the indicated time points and the apoB concentrations were measured in the media. (E) PHH were pre-labeled overnight with [^{14}C]-oleate and the effects of ETC-1002 treatment on oleate-derived counts in the media determined at the indicated time points. PHH hepatocytes were treated with ETC-1002 and atorvastatin, and (F) mRNA levels for *HMGR*, *SREBF2*, *PCSK9*, and *LDLR* were determined by RT-qPCR or (G) LDL receptor activity determined; A-769662 (10μM). (H) RH7777 cells were treated with the indicated concentrations of ETC-1002 or atorvastatin (0.5 μM) and [^{14}C]-lactate incorporation into cholesterol (Chol), cholesteryl esters (CE), and triglycerides determined. (I) LDLR-mediated DiI-LDL uptake in response to the indicated concentrations of ETC-1002 ± atorvastatin (0.5μM). RH7777 cells were subjected to negative control (NC) or *Acly* siRNA-mediated gene silencing (n =3) and analyzed for (J) ACL LDLR protein levels, or (K) LDLR-mediated DiI-LDL uptake. Data for primary human hepatocytes are expressed as mean ± SEM of n =2 donors performed in triplicate (A, B, C, F and G; except A-769662 treatment was 1 donor) or representative of two independent experiments showing similar results (D and E). (H) IC_{50} and (I) EC_{50} determinations were made using non-linear curve fit model. Multiple comparisons were made using (A, J, and K) unpaired Student's t-test, or (B, C, F, and G) an one-way ANOVA, Bonferroni's multiple comparisons test; * $p < 0.05$

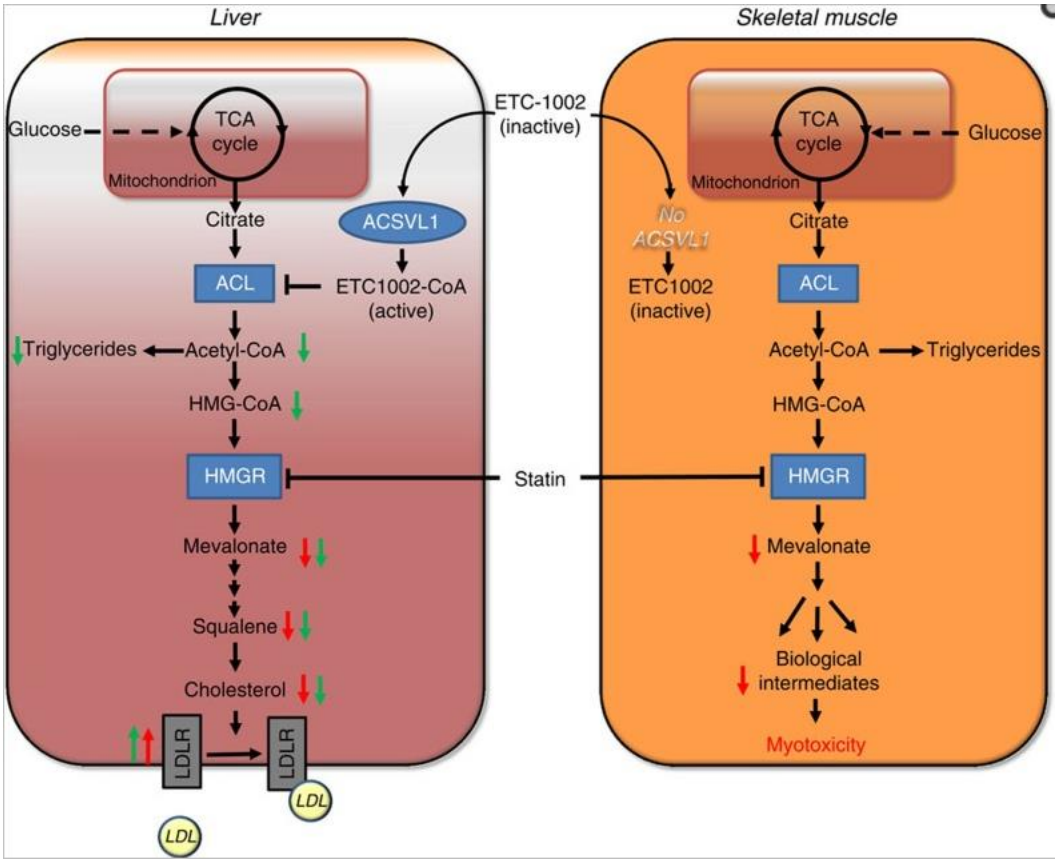
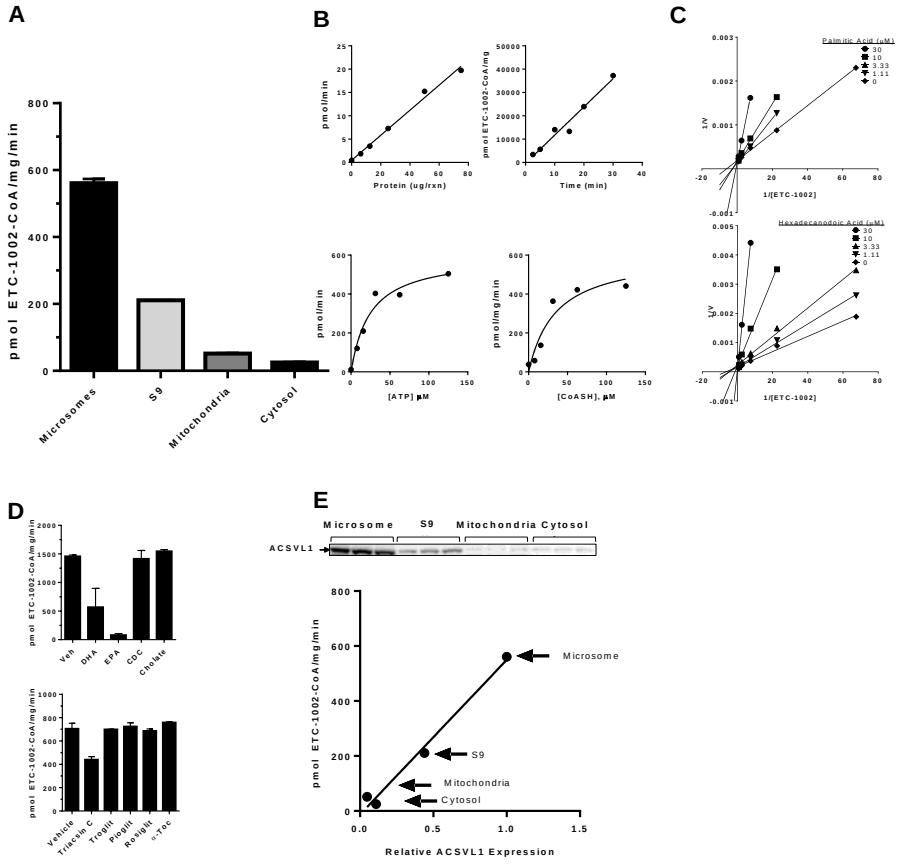


Figure 6. The mechanism of action of ETC-1002. In liver, ETC-1002 (bempedoic acid) is activated to ETC-1002-CoA by very long-chain acyl-CoA synthetase-1 (ACSVL1), and subsequently inhibits ATP-citrate lyase (ACL). Similar to inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) by statins, inhibition of liver ACL by ETC-1002-CoA results in the suppression of cholesterol synthesis and compensatory LDLR upregulation and LDL particle clearance from the blood. Skeletal muscle does not express ACSVL1 and is unable to convert ETC-1002 to its active form. Therefore, ETC-1002 does not suppress the synthesis of cholesterol or the associated biological intermediates that are

required to maintain normal muscle cell function, or promote the associated toxicity. Green and red arrows indicate the effects of ETC-1002 and statins, respectively.

Supplementary Material

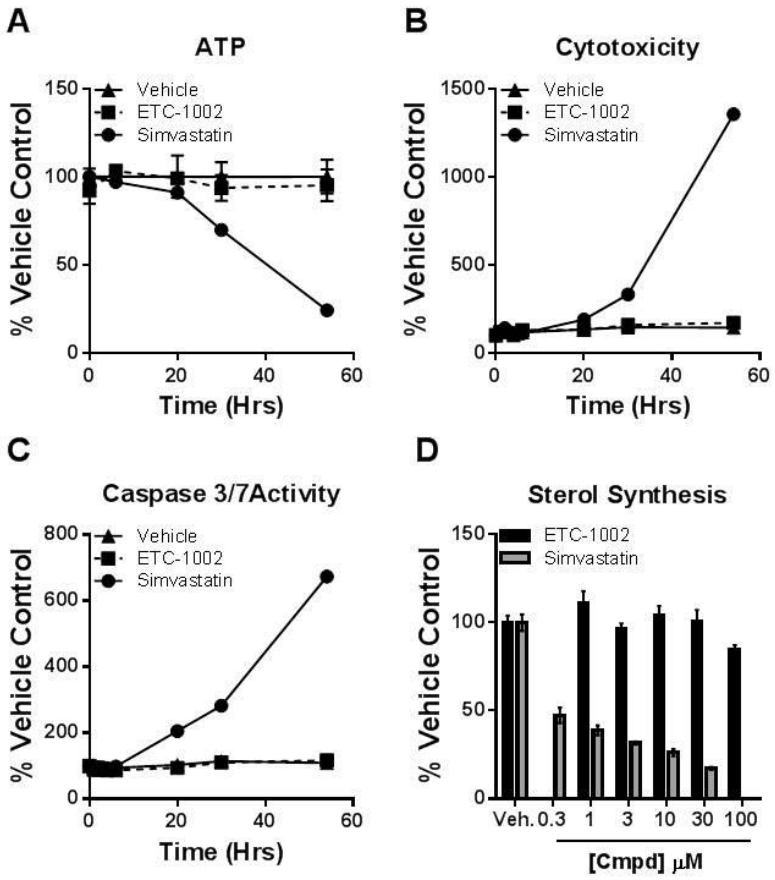
Supplementary Figure 1



Supplementary Figure 1. Characterization of the ETC-1002-CoA synthetase. (A) Equal total protein from subcellular fractions of human liver homogenates were incubated with [^{14}C]-ETC-1002 and duplicate measures of conversion to [^{14}C]-ETC-1002-CoA was determined. (B) ETC-1002-CoA synthetase activity was measured in human microsomes and standard assay kinetics determined. (C) [^{14}C]-ETC-1002 (10 µM) conversion to [^{14}C]-ETC-1002-CoA in human liver

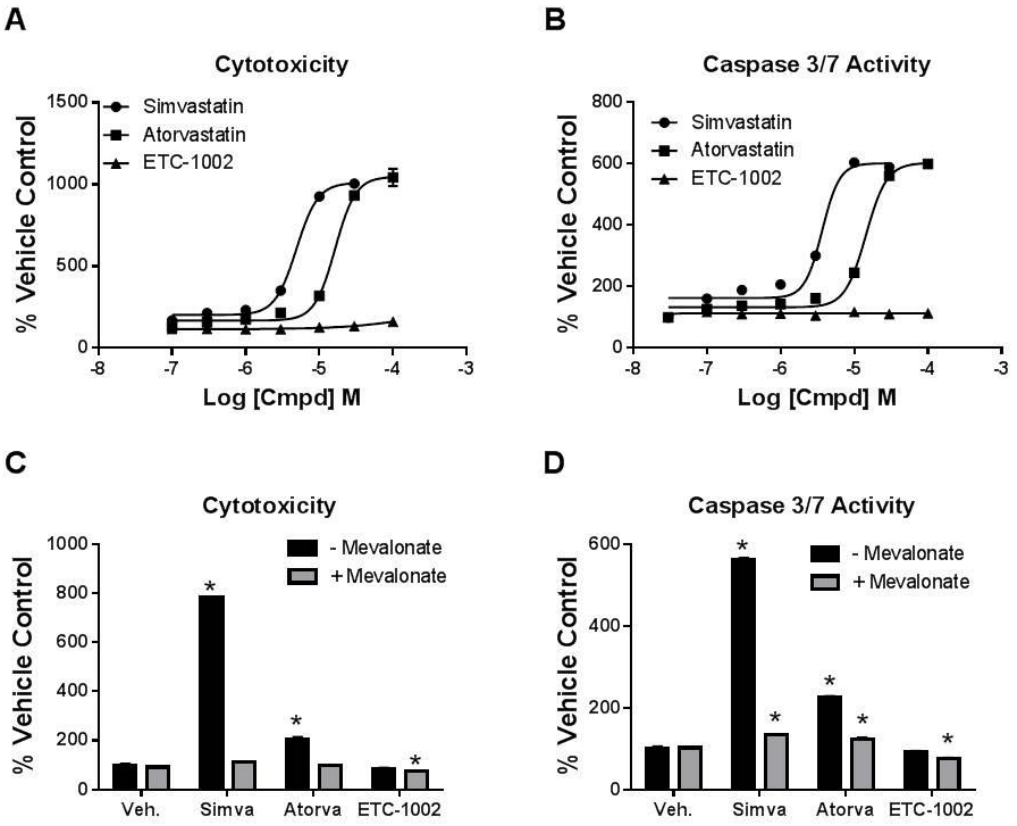
microsomes was determined in the presence of unlabeled substrates. Lineweaver-Burk plots represent competitive inhibition of ETC-1002-CoA synthetase activity by palmitic acid and hexadecanodoic acid against ETC-1002. (D) [^{14}C]-ETC-1002 (10 μM) conversion to [^{14}C]-ETC-1002-CoA in human liver microsomes was determined in the presence of 30 μM unlabeled competitive substrates or 10 μM inhibitors, Troglitazone (Troglit), Pioglitazone (Pioglit), Rosiglitazone (Rosiglit), and α-Tocopherol (α-Toc). (E) Relative ACSVL1 expression was determined in subcellular fractions from human liver homogenates from (A) by western blotting. Relative ACSVL1 expression was plotted against the ETC-1002-CoA synthetase activity for each respective subcellular fraction and linear regression analysis was performed. Data are expressed as mean ± SEM of duplicate (A-D) or triplicate (E) measures, and representative of a least two independent experiments.

Supplementary Figure 2



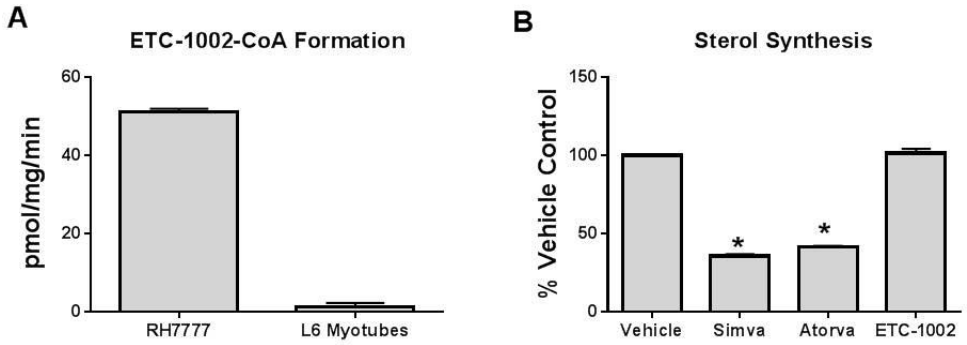
Supplementary Figure 2. Temporal effects of simvastatin and ETC-1002 on L6 cytotoxicity. L6 mytotubes were incubated with vehicle, ETC-1002 (100 µM), or simvastatin (10 µM) for the indicated times and (A) ATP, (B) cytotoxicity, and (C) Caspase 3/7 activity was determined. (D) Rates of sterol synthesis in the presence of the indicated concentrations of ETC-1002 and simvastatin were determined over a 24 hour period. (A through C) were performed in duplicate and (D) in triplicate. Data are presented as percent control of mean ± SEM DPM/well.

Supplementary Figure 3



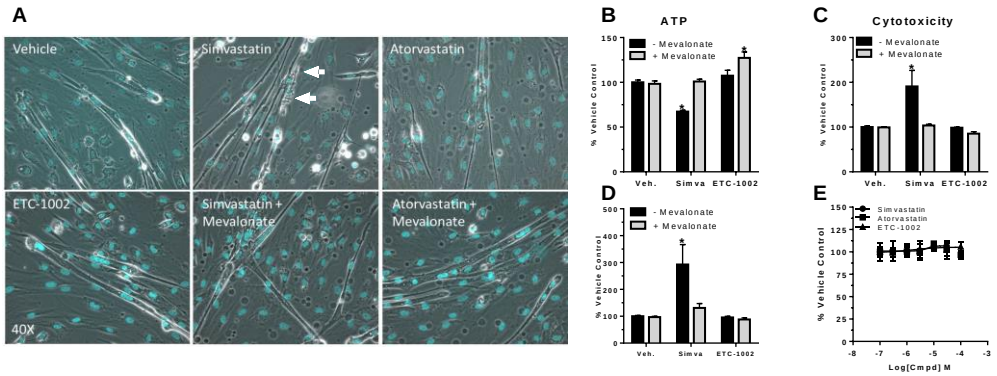
Supplementary Figure 3. Mevalonate rescues statin-induced cytotoxicity in L6 cells. L6 mytotubes were incubated with vehicle or the indicated concentrations of ETC-1002 or simvastatin for 48 hours and (A) cytotoxicity or (B) caspase 3/7 activity determined. L6 myotubes were incubated with vehicle, simvastatin (simva) (10 µM), atorvastatin (atorva) (10 µM), or ETC-1002 (100 µM) ± mevalonate (500 µM) for 48 hours and (C) cytotoxicity and (D) caspase 3/7 activity measured. Data are mean ± SEM of triplicate measures. Multiple comparisons were made using an one-way ANOVA, Bonferroni's multiple comparisons test; * $p < 0.05$.

Supplementary Figure 4



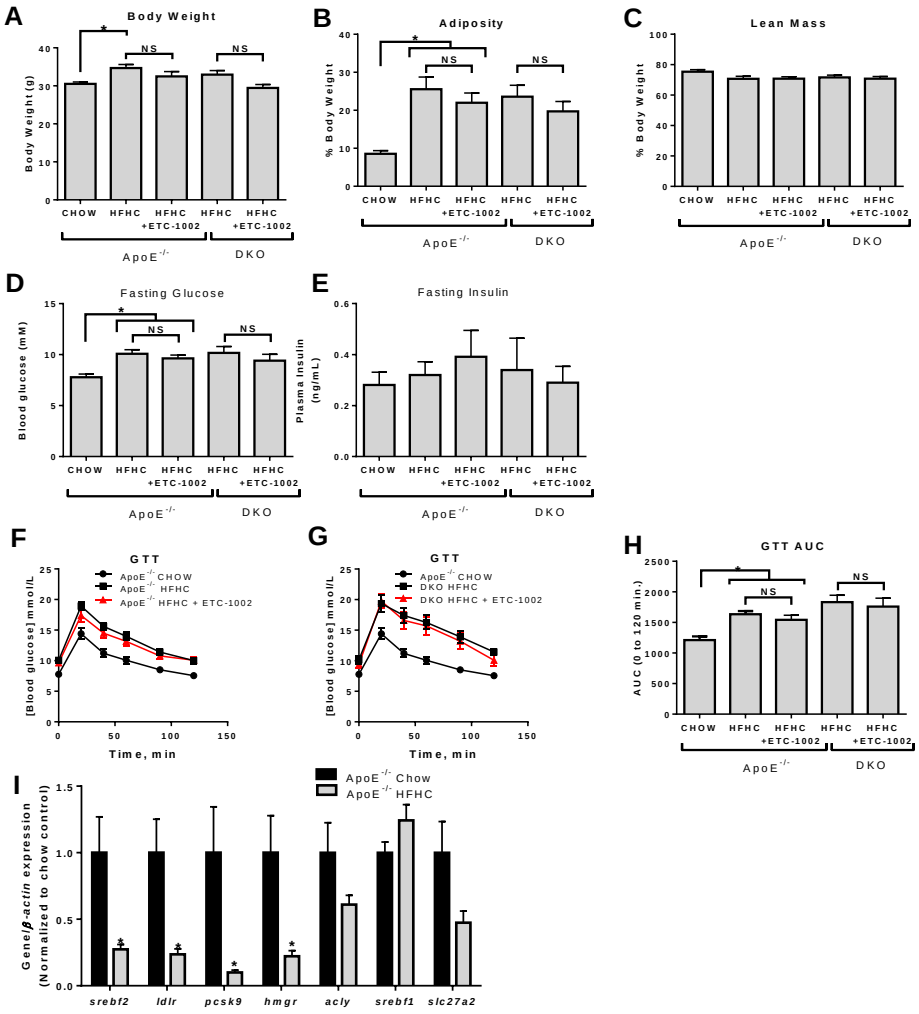
Supplementary Figure 4. ETC-1002 does not inhibit sterol synthesis in L6 myotubes. (A) McArdle (RH7777) cell and L6 myotube lysates were generated and ETC-1002-CoA synthetase activity was measured in the presence of 10 µM [14C]-ETC-1002. Data are expressed as mean ± SEM pmol ETC-1002-CoA generated per mg total protein per min from 3 preparations. (B) Rates of sterol synthesis were measured over 24 hours in the presence of vehicle, simvastatin (Simva) (10 µM), atorvastatin (Atorva) (10 µM), or ETC-1002 (100 µM). Data are the mean ± SEM of DPM/ mg total protein and expressed as percent control, and representative of two independent experiments. Multiple comparisons were made using an one-way ANOVA, Bonferroni's multiple comparisons test; * $p < 0.05$

Supplementary Figure 5



Supplementary Figure 5. Mevalonate rescues statin induced cytotoxicity in primary human myotubes. Primary human myotubes were treated with vehicle, 10 μM simvastatin (+/- 500 μM mevalonate), atorvastatin 10 μM (+/- 500 μM mevalonate), or ETC-1002 (100 μM) for 48 hours and (A) merged images of bright field and fluorescent capture of Hoechst-stained cultures (arrows indicate membrane blebbing), and (B) ATP, (C) cytotoxicity was measure by (GF-AFC/bis-AAF-R110 cleavage, and (D) Caspase 3,7 activity (DEVD cleavage) determined. (E) Primary human myotubes treated with 0.1 μM to 100 μM simvastatin, atorvastatin, or ETC-1002 for 12 hours and viability determined by MTT assay. Data are the mean of two independent experiments performed in triplicate. Multiple comparisons were made using an one-way ANOVA, Bonferroni's multiple comparisons test; * $p < 0.05$

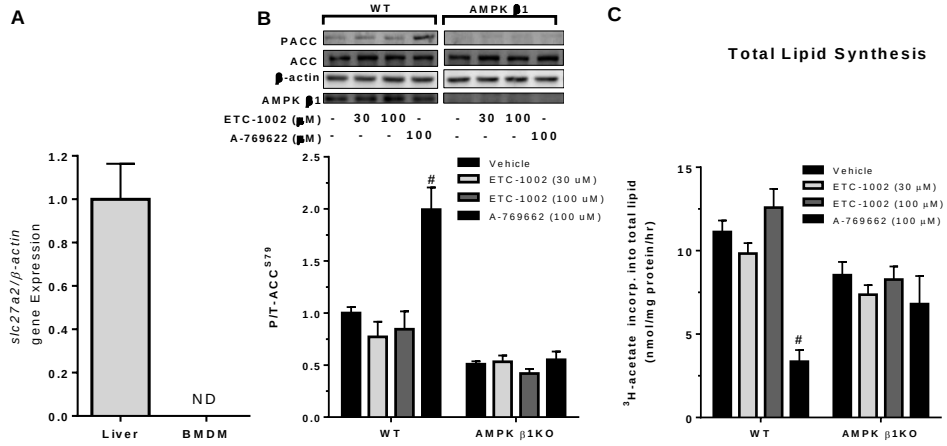
Supplementary Figure 6



Supplementary Figure 6. Metabolic outcomes are unaffected by ETC-1002 in *Apoe*$^{-/-}$ mice. *Apoe*$^{-/-}$ and DKO mice were fed a high fat-high cholesterol (HCHF) diet for 12 weeks with or without ETC-1002 to achieve a 30 mg/kg/day dose. (A) Body mass (B) Adiposity, and (C) lean mass determined at sacrifice (n = 15 for *Apoe*$^{-/-}$, and n = 11 for control DKO, and n = 12 for ETC-1002 treated DKO).

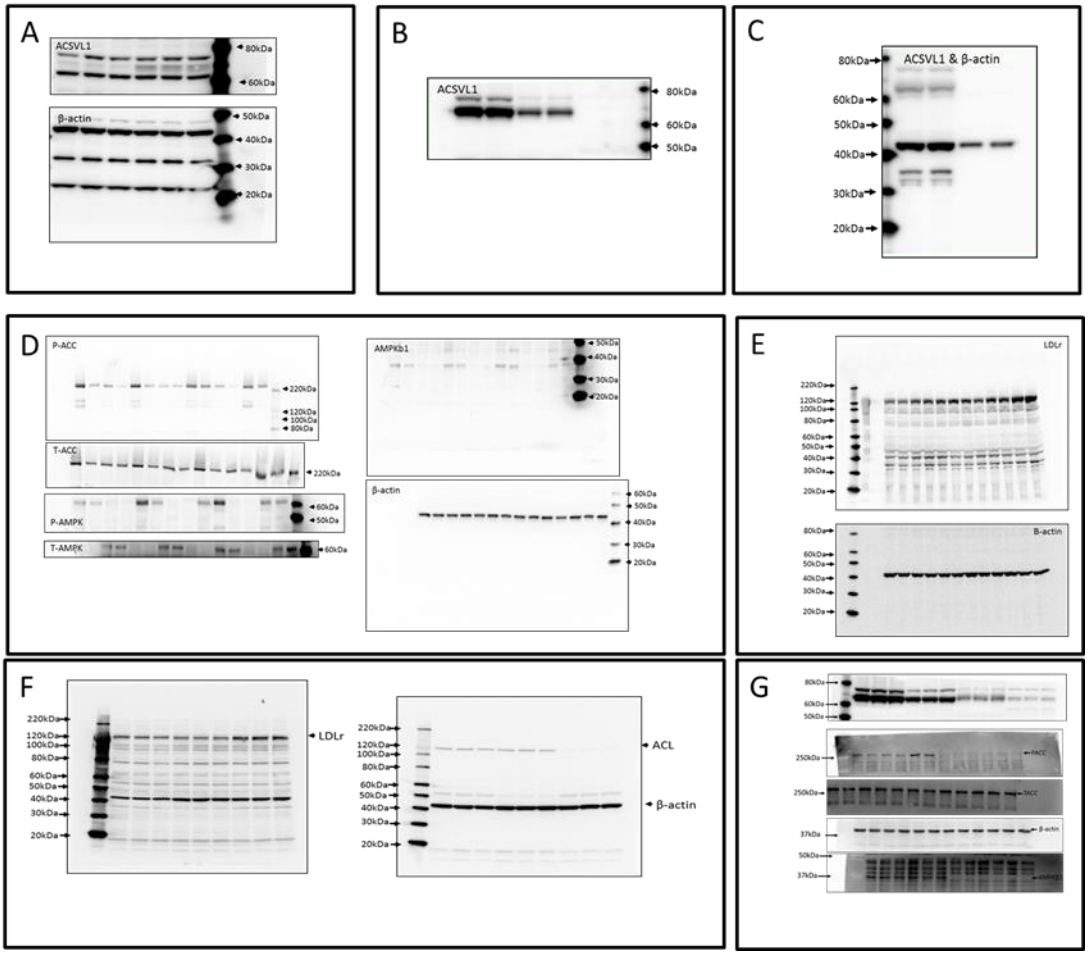
Fasting (D) glucose and (E) insulin were determined at 10 weeks on diet (glucose; n = 15 for *Apoe*$^{-/-}$, and n = 11 for control DKO, and n = 12 for ETC-1002 treated DKO; and insulin n = 6/group). Glucose tolerance test (GTT) was performed in (F) *Apoe*$^{-/-}$ and (G) DKO mice and (H) $AUC_{(0\ -120\ min.)}$ calculated. (I) Effects of HFHC diet on *Srebf2*, *Ldlr*, *pcsk9*, *Hmgr*, Acly, *Srebf1*, and *Slc27a2* gene expression normalized to chow-fed mice was (n= 4 chow-fed and n = 5 for remaining treatment groups) determined at the end of study. Multiple comparisons were made using (I) unpaired Student's t-test or an *one-way (within *Apoe*$^{-/-}$ treatment groups) or $^{\#}$ two-way ANOVA (between *Apoe*$^{-/-}$ and DKO treatment groups); Bonferroni's multiple comparisons test ; * $p < 0.05$.

Supplementary Figure 7



Supplementary Figure 7. ETC-1002 is inactive in bone-marrow derived macrophages. Hematopoietic stem cells were isolated from WT and β1 KO mice by taking the tibia and femur from both legs of one mouse and spinning out the bone marrow for 5 min at 4500 rpm in DMEM containing 4.5 g/L glucose, sodium pyruvate (110mg/L), 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin. The following day, the stem cells were stimulated to differentiate into BMDM under L929. (A) Following 8 days of differentiation, *Slc27a2* and β-actin expression was measured and compared to liver expression. (B) Phosphorylation of ACC was measured in BMDM treated with ETC-1002 (30 and 100 μM), and A-769662 (100 μM). ETC-1002 did not increase ACC phosphorylation. (C) ETC-1002 did not lower lipogenesis in WT or β1 KO. Two-way ANOVA was used to detect statistical differences. n = 3-8 performed in triplicate measures. Data are expressed as mean ± SEM. *P < 0.05, significantly different from control.

Supplementary Figure 8



Supplementary Figure 8. Gel images. Uncropped gels are shown for figures (A) 2C, (B) 2E, (C), 2G, (D), 3A, (E) 5I, (F), 5J and (G) supplementary figures 1E and 7B.

Supplementary Table #1.

ETC-1002 Dose (mg/kg/day)	ETC-1002-CoA (ng/mg) ± SEM		
	Liver	Skeletal Muscle	Fat
10	1.4 ± 0.2	BLQ*	BLQ*
30	4.0 ± 0.7	BLQ*	BLQ*

*BLQ = Below limit of quantitation; Limit of Quantitation = 0.11 Liver, 0.22 Skeletal Muscle, and 0.01 Fat

CHAPTER FOUR

4 Targeting ATP-Citrate Lyase in Hyperlipidemia and Metabolic Disorders

Stephen L. Pinkosky, Pieter H. E. Groot, Narendra D. Lalwani, and

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The identification of ACL as the molecular target of bempedoic acid coupled with the marked LDL-C lowering achieved in randomized clinical trials, has elevated ACL as a potentially important strategy to treat hypercholesterolemia and other related metabolic disorders. In this manuscript, we aimed to summarize the published literature pertaining to ACL biology and integrate these findings with key emerging concepts in metabolic and cardiovascular disease pathophysiology and treatment. Specifically, we highlight that as the primary source of nuclear and cytosol acetyl-CoA, the role of ACL in metabolism extends beyond committing excess energy substrate to the biosynthesis of cholesterol and fatty acids, and may reprogram metabolism by also influencing protein acetylation. This emerging concept suggests that ACL signals a nutrient rich metabolic state that is then integrated with other nutrient availability and energy signals to maintain lipid homeostasis. We discuss the potential implications of this mechanism in the context of health and disease, and draw important parallels between the suppression of cholesterol synthesis by ACL suppression, to the well-established links between statin therapy and cardiovascular risk reduction, through a synthesis of findings from preclinical studies, randomized clinical trials, and Mendelian randomization studies. We also highlight the evidence that suggests that the observed concomitant suppression of fatty acid synthesis is likely to reduce hepatic lipotoxicity and promote insulin sensitization in patients with metabolic syndrome and NAFLD.

S.L.P and G.R.S. wrote the manuscript and P.H.G. and N.D.L. edited the manuscript and provided comments. I was the lead author.

Targeting ATP-Citrate Lyase in Hyperlipidemia and Metabolic Disorders

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Abstract:

Chronic over nutrition and a sedentary lifestyle promote imbalances in metabolism, often manifesting as risk factors for life-threating diseases such as atherosclerotic cardiovascular disease (ASCVD) and non-alcoholic fatty liver disease (NAFLD). Nucleocytosolic acetyl-CoA has emerged as a central signaling node used to coordinate metabolic adaptations in response to a changing nutritional status. ATP-citrate lyase (ACL) is the enzyme primarily responsible for the production of extramitochondrial acetyl-CoA and is thus strategically positioned at the intersection of nutrient catabolism and lipid biosynthesis. Here, we discuss several recent findings from preclinical studies, as well as Mendelian and clinical randomized trials demonstrating the importance of ACL activity in metabolism and supporting its inhibition as a potential therapeutic approach to treating ASCVD, NAFLD and other metabolic disorders.

Modern Challenges in Cardiovascular and Metabolic Health Require New Therapeutic Strategies

A combination of human genetic factors, overnutrition, and a sedentary life style promote derangements in cholesterol and triglyceride metabolism; these can manifest as one or more risk factors associated with increased probability of developing a number of life-threatening metabolic and/or cardiovascular diseases. The importance of maintaining cholesterol homeostasis in humans is strongly supported by both epidemiologic cohort studies and meta-analyses of multiple Mendelian- and statin randomized trials that clearly demonstrate a causal association between elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) (hypercholesterolemia) and atherosclerotic cardiovascular disease (ASCVD) risk[1,2]. While a causal association for circulating triglyceride levels is less clear[3,4], aberrations in liver triglyceride metabolism also manifest as other metabolic ASCVD risk factors including insulin resistance, Type 2 Diabetes (T2D), and non-alcoholic fatty liver disease (NAFLD)[5,6]. Moreover, NAFLD also poses an independent health challenge as it is now the most common cause of chronic liver disease in the Western world and a leading cause of liver-related morbidity and mortality worldwide[7]. Of relevance, neither ASCVD nor NAFLD is adequately addressed by currently available treatment options. There are no FDA-approved therapies for NAFLD, and because many patients are not effectively treated for lipid disorders with the current standard of care, ASCVD remains the

leading cause of death and disability in the western world [8]. As such, therapeutic strategies that target cholesterol and triglyceride metabolism are required to provide patients with more potent LDL-C reduction regimens and therapeutic options to treat NAFLD. ATP-citrate lyase (ACL) is an enzyme uniquely positioned at the intersection of nutrient catabolism, and cholesterol and fatty acid biosynthesis. In this review, we discuss emerging evidence supporting that ACL –derived acetyl-CoA not only serves as carbon precursor for cholesterol and fatty acid biosynthesis, but also a key metabolic checkpoint used by cells to sense nutrient availability and coordinate metabolic adaptions. We raise key remaining questions regarding the potential role of ACL in controlling lipid metabolism, mitochondrial biogenesis, apoptosis, and inflammation by influencing protein acetylation. We also review findings from recent preclinical studies, and Mendelian and clinical randomized trials that support that the strategic position of ACL in metabolism provides a unique therapeutic opportunity to treat hypercholesterolemia, and potentially address the overlapping pathophysiology that exist between ASCVD and NAFLD. Finally, we highlight how over the next few years, the continued clinical investigation of bempedoic acid (BA), will potentially establish whether lowering LDL-C by targeting ACL constitutes a new viable strategy to reduce ASCVD risk and potentially treat other associated metabolic disorders.

ACL Regulation, and Role in Lipid Metabolism

ACL is a ubiquitous enzyme at the nexus of nutrient catabolism and synthesis of cholesterol and fatty acids. In mammals, it is highly expressed in lipogenic tissues including adipose, liver and lactating mammary glands[9]. In the presence of ATP and Coenzyme A (CoA), ACL catalyzes the cleavage of citrate to acetyl-CoA and oxaloacetate (OAA) (Box 1, Figure I).

Fatty acids and cholesterol are the two fundamental building blocks supporting the synthesis of more complex lipids that serve several functions in cell physiology, including structural components of cellular membranes, energy transport and storage, bioactive signaling molecules, and substrates for post-translational modification of signaling proteins (figure 1). The biosynthesis of lipids starts in the mitochondria where acetyl-CoA units derived from the metabolism of non-lipid nutrients are condensed with oxaloacetate (via the tricarboxylic acid (TCA) cycle) to form citrate, and exported to the cytosol by the citrate transport protein (CiC). The subsequent cleavage of citrate back to acetyl-CoA by ACL in the cytosol is a requisite step for the de novo synthesis of cholesterol and fatty acids (Figure 1). Mevalonate, the product of HMG-CoA reductase within the cholesterol biosynthetic pathway, is also a building block for the synthesis of several important biological intermediates and products including isoprenoids, CoQ10, and dolichol

(reviewed in [10,11]) (Figure 1). When nutrient availability exceeds biosynthetic and energy requirements, cells utilize this pathway for energy storage by esterifying lipids into cholesteryl esters and triglycerides[12]. Under certain conditions, such as those associated with metabolic reprogramming in mouse and human tumors [13-17] or in human liver following excessive alcohol consumption [18], ACL can be bypassed by direct activation of cytosolic acetate to acetyl-CoA by acetyl-CoA synthetase2 (ACSS2). However, this pathway does not appear to completely compensate for the absence of ACL[19], nor quantitatively important for the de novo synthesis of lipids in humans under normal physiological conditions[18,20].

While the pathways of de novo cholesterol and fatty acid biosynthesis are both dependent on the supply of cytosolic acetyl-CoA from ACL, they are largely subject to distinct regulatory mechanisms. Moreover, transcriptional regulation of the fatty acid synthesis pathways differs between white adipose and liver [21]. In liver, ACL is co-regulated along with all members of the lipogenic enzyme set, including enzymes required to generate NADPH reducing equivalents [22]. The entire lipogenic enzyme set is essentially controlled by the three transcription factors: sterol regulatory element binding protein 1c (SREBP-1c), carbohydrate-response element binding protein (ChREBP) [23,24], and liver X receptors (LXR) [25,26]. Although the expression of both SREBP-1c and ChREBP have been

shown to be induced by LXR [24,26], in rodent liver, the induction of gene expression is primarily mediated by SREBP-1c in response to elevated glucose and insulin (i.e. the fed state) [22] (Figure 1). By contrast, the cholesterol biosynthesis pathway is controlled by sterol-response element binding protein-2 (SREBP-2) and is activated in response to low intracellular cholesterol levels as observed with reduced dietary supply (reviewed in [27]) (Figure 1).

In addition to transcriptional regulation of ACL, in vitro studies using purified enzyme and rat adipocytes indicate that covalent activation of the enzyme can occur through phosphorylation at Ser454 by cAMP-dependent protein kinase and protein kinase B/Akt [28-31] which desensitizes the enzyme to allosteric modulation in vitro [31]. However, in vivo evidence supporting the physiological relevance of Ser454 on lipid synthesis has not been established. In addition, to phosphorylation of ACL at Ser454, ACL is also phosphorylated at Thr446 and Ser450 by glycogen synthase kinase 3; however, this does not appear to affect enzyme activity[29]. It is noteworthy that ACL phosphorylation has been shown to play a role in modulating nuclear acetylation in cancer cells, macrophages, and T-cells, resulting the modulation of several cellular processes ranging from inflammation to DNA repair [32-35]. This raises the possibility that hormones signaling nutritional status

such as insulin, might affect these processes in multiple cells types. Additional investigation seems warranted.

ACL: A Metabolic Checkpoint for Sensing Excess Nutrients?

During normal transitions between fasting and feeding, cells maintain energy homeostasis by integrating signals of energy and nutrient status at key metabolic nodes coordinating multiple processes. For example, the AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy sensor that responds to energy deficit by mediating regulatory phosphorylations of numerous substrates including acetyl-CoA carboxylase (ACC) and HMG-CoA reductase[36-39] (Figure 2). The net result of these phosphorylation events is a switch from energy consuming processes (*e.g.* cholesterol, fatty acid and protein synthesis) to energy producing processes (*e.g.* fatty acid β-oxidation, glucose uptake) and restored energy balance. Under conditions of prolonged energetic stress such as exercise training or caloric restriction[40], this pathway promotes mitochondrial biogenesis, an effect which is potentiated by activating Sirtuin 1 (SIRT1)-dependent deacetylation of critical transcription factors and co-activators such as p53 and peroxisome proliferator-activated receptor gamma co-activator1α (PGC-

1α)[41,42]. As such, AMPK and SIRT1 cooperate to elicit both acute and chronic metabolic adaptations to reverse cellular energy deficits and maintain metabolic flexibility (Figure 2)[41].

Despite the importance of activating AMPK-SIRT1 under energetic stress, the ability of this pathway to reprogram cellular metabolism under conditions of nutrient excess appears to be minimal, as evidenced by the relatively benign metabolic phenotype of *Ampk* and *Sirt1* genetic loss of function mouse models fed hyper-caloric high-fat diets (HFD)[40,43]. However, recent reports suggest that extra-mitochondrial acetyl-CoA concentrations might impact protein acetylation and exert influence over metabolism by limiting the supply of substrate for acetyltransferases such as GCN5, independently of the AMPK-SIRT deacetylation axis [20,44,45]. This is supported by studies showing that the acetylation status of multiple transcription factors, enzymes, and histones are closely linked to extra-mitochondrial acetyl-CoA concentrations, and that this regulatory mechanism could be critical for maintaining metabolic flexibility during changes in nutritional status (Figure 2) [20,41,44,46]. In contrast to mitochondria, the cytosolic acetyl-CoA pool can exchange with the nuclear pool via the nuclear pore complex [47], and is therefore often regarded as one nucleocytosolic pool. As mentioned, ACL is the primary enzyme that generates cytosolic acetyl-CoA by catalyzing the cleavage

of citrate produced from the mitochondrial metabolism of macronutrients (Box 1, Figure I). Because cytosolic acetyl-CoA is also the final common substrate supporting the conversion of excess mitochondrial-metabolism of nutrients to both cholesterol and fatty acid biosynthesis for storage, ACL potentially provides a logical checkpoint to signal nutrient availability by also promoting metabolic adaptations via substrate-level protein acetylation (Figure 2) [46]. Indeed, this was demonstrated in mammalian cells where nuclear ACL-derived acetyl-CoA is required for GCN5-dependent histone acetylation in response to both growth factor stimulation and glucose availability, and in primary adipocytes, this is required for glucose-induced transcriptional regulation of select genes such as Glut4 which is important for increasing glucose uptake [20].

It is noteworthy that GCN5 also directly acetylates and inhibits non-histone proteins such as the transcription factor, PGC-1α [48,49], which suggests an important link between nucleocytosolic acetyl-CoA levels and mitochondrial biogenesis [41], and potentially ACL. In mice, GCN5-dependent modulation of PGC-1α acetylation and activity has been shown to reciprocally regulate energy expenditure in response to caloric excess or caloric restriction [50]. Because GCN5 is dependent on ACL for acetyl-CoA substrate, ACL activity could also reciprocally control PGC-1α activity by providing acetyl-CoA for its acetylation

when energy/substrate levels are high, or limiting its acetylation when energy/substrate levels are low. When coupled with the AMPK/SIRT1 energy sensing pathway, cells could then integrate changes in ACL activity with cellular energy status to promote appropriate funneling of macronutrients toward energy production (fatty acid β-oxidation) or storage (lipid synthesis), while ensuring sufficient metabolic capacity (e.g. mitochondrial biogenesis) upon changing energy/nutritional status (Figure 2). This raises the intriguing possibility that targeting ACL could potentially offer a point of therapeutic intervention aimed at restoring metabolic homeostasis by short-circuiting chronic signals of calorie/energy excess and enhancing mitochondrial function. However, whether the suppression of ACL results in PGC-1α activation and improved mitochondrial function in the context of metabolic disease, has not been studied and warrants investigation.

ACL Inhibition: Lowering Blood LDL-C to Reduce ASCVD Risk

Given its strategic position in the lipid biosynthesis pathway, ACL has been considered an attractive target for lipid-lowering even before satins and their effects on cholesterol homeostasis were elucidated (Box 2). Cells carefully maintain intracellular free cholesterol concentrations within a narrow range, primarily through a highly sensitive regulatory feed-back mechanism involving the

transcription factor, SREBP-2 (Figures 1 and 3) reviewed in [27]). Indeed, statins exploit this pathway by inhibiting liver HMG-CoA reductase, thereby reducing intracellular cholesterol levels and triggering SREBP-2-mediated LDLR upregulation in human hepatocytes [10,11,22,27]. This can result in a new homeostatic state where cells acquire more cholesterol from circulating LDL particles, thus reducing LDL-C [10] and its potential to cause ASCVD (Figure 3) [1]. Given the dependence of cholesterol biosynthesis on ACL activity, ACL inhibition is anticipated to promote effects on LDLR-mediated LDL particle clearance in a manner similar to statins[51]. This is supported by in vitro studies in human liver cells demonstrating that LDLR upregulation can result from ACL suppression via both siRNA-mediated and pharmacological ACL suppression using (-)-hydroxycitrate [51,52].

Moreover, other approved LDL-C-lowering therapies can also impact LDL metabolism (in a manner similar to statins), by mimicking dietary cholesterol restriction via reducing cholesterol absorption (Figure 3). A meta-regression analysis of 49 clinical cardiovascular outcome trials showed that oral therapeutic statin administration, or, non-statin therapies such as ezetimibe, and bile acid sequestrants, that could mimic some aspect of the SREBP-2 cholesterol-sensing mechanism, provided ~ 23% relative risk reduction of major vascular events as

defined by a composite of acute myocardial infarction (MI) or other acute coronary syndrome, cardiovascular death, stroke, or coronary revascularization, per 1 mmol/L of plasma LDL-C lowering [2]. Moreover, lowering plasma LDL-C levels by increasing LDLR activity via injectable anti- proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody therapy was recently shown to also reduce the number of major vascular events in a cardiovascular disease outcomes trial, suggesting that preserving LDLR activity is mechanistically sufficient to lower plasma LDL-C and associated cardiovascular disease risk [53]. These findings have led to the consensus that lowering LDL-C via LDLR-mediated clearance provides a proportional and predictable reduction in major vascular events[1]. Therefore, ACL inhibition might be expected to produce biologically equivalent effects on LDL metabolism and ASCVD risk reduction, as observed with other interventions such as statin, PCSK9 inhibition, and ezetimibe, which also upregulate LDLR activity (Figure 3).

In the absence of clinical efficacy endpoints, investigators and drug developers have turned to the principles of Mendelian randomization as a means to inform cardiovascular risk/benefit associated with novel drug targets[54]. This approach has been validated retrospectively in humans for ASCVD using LDL-C lowering variants in *HMGR*, and prospectively, to predict the outcomes of the Improved

Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) [55,56] and Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (FOURIER) trials using *HMGR*/*NPC1L1* (target of Vytorin, simvastatin/ezetimibe combination) and *PCSK9* variants, respectively[2,55]. Using similar methods, a study reported the effects of lowering LDL-C blood levels, as mediated from multiple independently inherited single nucleotide polymorphisms (SNPs) in the gene region encoding ACL (gene: *ACLY*) [57]. In addition to lowering LDL-C, these variants were also associated with a shift in plasma biomarkers such as ApoB, HDL-C, hsCRP, and triglycerides in a way that was remarkably similar to LDL-C lowering SNPs in *HMGR* and the effects of statins in randomized clinical trials[57]. These findings strongly suggest that the mechanism of LDL-C lowering via ACL inhibition might be biologically equivalent to that of statins. However, this remains to be fully demonstrated. Importantly, by constructing a genetic score based on the weighted association of these SNPs with lower LDL-C levels, these studies have also shown that exposure to life-long lower LDL-C levels mediated by SNPs in *ACLY* was causally associated with a reduction in ASCVD risk ; furthermore, when the odds ratios were adjusted per 10 mg/dL LDL-C lowering, the relative risk reduction appeared to be similar to that observed with lower plasma LDL-C mediated by polymorphisms in other validated genes including *HMGR*, *NPC1L1*, *PCSK9*, and *LDLR*[57]. Using a 2X2 factorial analysis designed to mimic the effects of combination therapy in a

randomized clinical trial, subjects were randomized first based on their *ACLY* LDL-C score, then by either their *HMGR* LDL-C score or *NPC1L1* LDL-C score. These analyses showed that *ACLY* SNPs provided additive LDL-C lowering and a proportional reduction in ASCVD risk, when combined with either *HMGR* or *NPC1L1* SNPs[57]. This suggests that ACL inhibitors might be able to provide an additive ASCVD benefit when combined with existing LDL-C lowering therapies such as statins and ezetimibe in patients with hypercholesterolemia. However, further studies are warranted, and whether pharmacological inhibition of ACL will corroborate these findings remains to be established.

While it is known that circulating LDL-C levels are largely regulated by either controlling the rate of hepatic production of its triglyceride-rich precursor VLDL particle, or by the rate of LDLR-mediated LDL particle clearance [58], it should be noted that important differences exist between rodents and humans. Studies in rodents show that when carbohydrates are consumed in excess of caloric requirements, elevated insulin and blood glucose stimulate hepatic de novo lipogenesis (DNL) and subsequent esterification of fatty acids to form triglycerides for storage in the liver, or transport via plasma VLDL to white adipose tissue for fat storage. As such, the suppression of hepatic DNL in rodents has been reported to promote marked reductions in plasma triglycerides [59] By contrast, DNL in

healthy humans on a balanced diet appears to be relatively low, contributing only ~5% -10% of the hepatic VLDL triglyceride pool with the majority of fatty acids coming from dietary sources and peripheral lipolysis[60]. Furthermore, when subjects were provided a diet supplemented with excess carbohydrate, glucose was oxidized at the expense of fatty acids, rather than being converted into fatty acids and triglycerides for storage or secretion into the blood via VLDL [61]. Therefore, in contrast to rodents, the suppression of hepatic DNL in healthy humans might not significantly impact plasma triglycerides. However, it is noteworthy that liver DNL is upregulated in individuals with hepatic insulin resistance [62-65] or presenting with NAFLD (see below)[66]. Therefore, inhibition of DNL could promote reductions in plasma triglycerides in these patient populations but this remains to be further explored.

DNL and NAFLD: A Rationale for Using ACL Inhibitors?

Metabolic Syndrome and ACL blockade

Beyond the cardiovascular benefit resulting from cholesterol synthesis inhibition and LDL-C lowering, the concomitant inhibition of fatty acid synthesis resulting from ACL blockade may improve other disease outcomes associated with metabolic syndrome, a cluster of ACSVD and T2D risk factors thought to arise

from imbalances in energy utilization and storage. In the liver, metabolic syndrome manifests as an accumulation of ectopic lipid (steatosis)-- a requisite condition for the onset of a spectrum of liver-related pathologies collectively referred to as NAFLD[5,67-69]. Although the underlying molecular mechanisms leading to hepatic steatosis and its transition to the more dangerous progressive form, non-alcoholic steatohepatitis (NASH), are not well understood, these conditions appear to be closely linked to insulin resistance in adipose tissue and liver in humans. For instance, in adipose tissue, insulin resistance results in increased lipolysis which promotes the influx of fatty acids into the liver for subsequent storage as triglycerides[70]. In liver, insulin resistance can result in a pathological response where elevated blood insulin levels do not effectively suppress gluconeogenesis but potently activate DNL [70-72]. Together, increased hepatic fatty acid influx and DNL can lead to the accumulation of triglycerides and associated lipotoxic metabolites such as long-chain acyl-CoAs, diacylglycerol, lysophosphatidic acid, and ceramides [73], which might further perpetuate insulin resistance[74], promote VLDL production, and increase plasma triglyceride concentrations [75,76]. Because VLDL is an LDL precursor, increased VLDL production can also lead to an elevated numbers of LDL particles in circulation [58,77]; however, in many patients with metabolic syndrome, LDL-C might not be elevated [78]. This might be partially explained by multiple defects in lipid and lipoprotein metabolism that result in “atherogenic dyslipidemia” marked by high plasma triglycerides, low

HDL-C, and small dense, and triglyceride-rich, LDL particles, a profile that might not be effectively treated by available treatment options [78-81]. In addition, the chronic exposure of hepatocytes to lipotoxic metabolites associated with NASH can also promote endoplasmic reticulum (ER) stress and mitochondrial dysfunction as evidenced by increased reactive oxygen species [82-84], which in turn, can eventually lead to chronic pro-inflammatory signaling [85] and propagation of hepatocellular injury and apoptosis[86]. This may indicate a state of pathological transition to NASH, marked by hepatic stellate cell activation, collagen deposition starting in the peri-sinusodial space, and finally, hepatic fibrosis [84,87-89]. Indeed, histological features of NASH, including steatosis, hepatocellular ballooning, and lobular inflammation with or without peri-sinusoidal fibrosis are evident at this stage (Figure 4). If left untreated, NASH can result in cirrhosis and primary liver cancer [90].

ACL Blockade in Human Studies

The importance of DNL in the pathogenesis of human metabolic disease is supported by several studies showing that hepatic fatty acid synthesis and the lipogenic enzymes set are significantly increased in patients with metabolic syndrome associated risk factors including obesity, hypertriglyceridemia, and insulin resistance [62-65]. Specifically in NAFLD patients, studies measuring the incorporation of stable isotopes into plasma and liver lipids, indicated that DNL

might no longer be subject to normal diurnal rhythm regulation in the liver, and contributed to >25% of hepatic and circulating VLDL triglycerides [63]. Moreover, the therapeutic utility of targeting DNL has been recently corroborated in NASH patients where hepatic ACC inhibition rapidly reduced DNL, hepatic steatosis, and markers of fibrosis within 12 weeks of treatment [91,92]. In addition, array-based DNA methylation and mRNA expression profiling of liver samples from morbidly obese patients (presenting early to later stages of NAFLD), identified *ACLY* as 1 of 9 genes to be alternatively methylated and expressed in subjects with NAFLD [93]. These studies showed that consistent with the anticipated repressive effects of DNA methylation on gene expression [94], an approximately 9% reduction in *ACLY* methylation was associated with an increase in *ACLY* mRNA in NASH patients compared to control subjects. This suggests that ACL might be deregulated in NASH patients and an important contributor its pathogenesis. Collectively, these findings suggest that pharmacological suppression of hepatic ACL in patients with insulin resistance and NAFLD might be able to reduce hepatic triglyceride and associated lipotoxic metabolites; this might in turn attenuate VLDL and LDL production, pro-inflammatory signaling, liver injury, and fibrosis progression, although it should be noted future studies are needed to validate this hypothesis (Figure 4).

ACL Blockade in Preclinical Studies

Preclinical studies of metabolic disease and the effects of pharmacologic or genetic blockade on liver DNL has implicated its involvement in the underlying pathophysiology of IR and hepatic steatosis [59,95]. Moreover, malonyl-CoA, the intermediate product of fatty acid synthesis produced by acetyl-CoA carboxylase (ACC), is a potent allosteric inhibitor of carnitine palmitoyltransferease 1 (CPT-1), thus blocking the import of long-chain fatty acids into the mitochondria[96]. As such, at least in mice, pharmacological inhibition of DNL at or upstream of ACC promotes concomitant mitochondrial fatty acid β-oxidation and further reduces cellular triglycerides and improves insulin resistance [59]. With respect to liver ACL, high fructose diets that increase DNL and steatosis also increase liver ACL expression, with similar observations made in mice made insulin resistance via high-fat feeding [97,98]. In another study, ACL expression was shown to be increased in the livers, but not adipose tissue of insulin resistant chow-fed polyphagic Db/db mice [99], suggesting hyper-caloric consumption might specifically upregulate ACL activity in liver. In addition, liver-specific abrogation of ACL using adenovirus-mediated RNA interference, reduced hepatic acetyl-CoA and malonyl-CoA levels, lipogenesis, steatosis, and gluconeogenic genes which led to improved insulin sensitivity and glucose tolerance [99]. Based on the presented preclinical evidence, pharmacological suppression of hepatic ACL could reduce steatosis by promoting concomitant suppression of DNL and activation of long-

chain fatty acid β-oxidation, thus restoring insulin sensitivity and potentially improving NASH.

As discussed above, in addition to potentially improving insulin sensitivity through reductions in hepatic liver lipids, ACL suppression might also promote metabolic adaptions relevant to the pathophysiology of NAFLD by modulating the acetylation status of histone and non-histone proteins [20,41]. For example, reductions in nucleocytosolic acetyl-CoA could be anticipated to block GCN5–dependent PGC-1α inhibition, and promote mitochondrial biogenesis and function. However, this has not been tested. Other histone acetyltransferases (e.g. p300) have been shown to be potentially key regulators of lipogenic gene transcription by catalyzing ChREBP acetylation. In cultured hepatocytes, glucose-activated p300 acetylated ChREBP and increased its transcriptional activity, and in a mouse model of type 2 diabetes and obesity, increased p300 activity was associated with ChREBP hyperacetylation and hepatic steatosis [100,101], although the role of ACL was not specifically studied. Moreover, multiple other transcription factors and proteases important for regulating inflammatory and apoptotic processes such as nuclear factor kappa B (NF-ƙB), Stat1, and caspases, are also controlled by acetylation [45,102,103]. Given the established role of these pathways in the progression of NAFLD [104], it is plausible that ACL suppression could mediate beneficial effects

by also suppressing the acetylation status of these pro- apoptotic and inflammatory regulators. Despite these encouraging findings, the specific role of ACL-dependent acetylation and its putative contribution to steatosis and its transition to NASH pathophysiology has been poorly studied. Consequently, future research examining insulin sensitivity, NAFLD and NASH in liver-specific ACL deficient mice are evidently warranted. Of relevance, ACL might also play an important role in non-hepatic tissues (Box 3), but currently, little is known; hence, thorough evaluation of its role in other tissues may well lead to fruitful areas of investigation.

ACL Inhibition in the Liver via Bempedoic Acid

Significant clinical evidence supporting the therapeutic utility of pharmacological ACL inhibition has recently been generated by investigational use of bempedoic acid (BA). Although not prospectively pursued as an ACL inhibitor in cell-free assays, BA was discovered and optimized in a phenotypic screen where α-substituted dicarboxylic acids were evaluated based on potency for concomitant inhibition of de novo cholesterol and fatty acid synthesis [105]. Subsequent studies confirmed these effects in primary human liver cells and established that BA could inhibit lipid synthesis via ACL inhibition [52,106]. Using siRNA-mediated suppression of very long-chain acyl-CoA synthetase (ACSVL1; gene: *Slc27a2*), BA was shown to be a prodrug that required ACSVL1-dependent intracellular

coenzyme A (CoA) activation to inhibit ACL [52,107]. In vivo studies in rats showed that BA treatment reduced levels of hepatic acetyl-CoA and malonyl-CoA[106], and given that malonyl-CoA is an allosteric inhibitor of CPT-1, it also increased rates of fatty acid β-oxidation [52,105,106]. Consistent with ACL inhibition, BA promoted hypolipidemic effects in a variety of disease models such as dyslipidemic hamsters and obese Zucker rats [105,106], and attenuated atherosclerosis and serum amyloid A (SAA) in high-fat, high-cholesterol fed, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, frequently used as models for hyperlipidemia and associated ASCVD [52,97,106]. In *Apoe*$^{-/-}$ mice, BA treatment reduced liver cholesterol mass, upregulated LDLR expression and lowered plasma LDL-C [52]. In insulin resistant *Ldlr*$^{-/-}$ mice, BA treatment reduced plasma cholesterol, VLDL-C, LDL-C, and triglycerides[97]. Although the effect of BA in reducing plasma LDL-C levels was consistent with previous reports of statins in *Ldlr*$^{-/-}$ mice [108], this effect might also be linked to inhibition of fatty acid synthesis leading to reduced production of the LDL particle precursor VLDL, a pathway known to be increased by insulin resistance [58,77]. Further examination of liver and other metabolic outcomes in *Ldlr*$^{-/-}$ mice showed that BA also reduced diet-induced hepatic inflammatory gene expression (e.g. *Tnf, Ccl3, and Nos2*) and improved glucose tolerance [97]. In order to determine the therapeutic potential of BA in NASH, additional studies are warranted using models that can present informative histological endpoints, including hepatic ballooning and fibrosis.

In clinical studies, BA has been reported to promote dose-dependent LDL-C lowering effects of up to 30% as monotherapy, and up to an additional 24% when added in combination with stable statin therapy, or ~50% when combined with ezetimibe [109-112]. These effects were accompanied by proportional reductions in several plasma biomarkers associated with ASCVD risk such as total cholesterol, non-high density lipoprotein cholesterol (non-HDL-C), plasma apoB and LDL particle number, as well as high-sensitivity C-reactive protein (hsCRP) [109,113]. However, in contrast to rodents [52,106], BA has not demonstrated a consistent effect on plasma triglycerides. As discussed above, this may be potentially due to the low rates of (hepatic) lipogenesis in normoinsulinemic humans compared to rodents, therefore, whether BA reduces plasma triglycerides in insulin resistant patients should be specifically investigated. A post-hoc exploratory analysis in a subset of patients with elevated fasting insulin ($\geq$ 12 μ/IU/ml) showed that at specific doses (40 and 80 mg), BA significantly lowered fasting plasma insulin vs. placebo (- 5.8 ± 1.8, p = 0.005). It is notable that BA also reduced plasma triglycerides at these doses [113]. Thus, further studies on the potential effects of BA on triglycerides and other metabolic endpoints associated with NASH in obese insulin resistant patients seem adequately justified. Furthermore, given that the ASCVL1 is nearly exclusively expressed in the liver, the formation of the active BA CoA conjugate and subsequent ACL inhibition, are also restricted to the liver

[52,114]. Therefore, the absence BA activity in peripheral tissues--including skeletal muscle and adipose-- might be able to provide a mechanistic basis for a putative improved safety profile compared to statins. The initiation of the Cholesterol Lowering via BEmpedoic Acid, an ACL-inhibiting Regimen (CLEAR) Outcomes study will demonstrate if BA-induced inhibition of ACL can reduce ASCVD risk in humans with a favorable safety profile.

Concluding remarks

Cells utilize acetyl-CoA levels to integrate nutrient status with energy levels to ensure the proper funneling of substrate toward energy production or storage. However, upon chronic metabolic insult, this sensing mechanism becomes uncoupled and can lead to discordances among cellular energy status, nutrient catabolism and lipid biosynthesis, which can manifest as risk factors for life threating diseases such as ASCVD, T2D, and NAFLD. ACL is the primary source of nucleocytosolic acetyl-CoA, and is thus a critical enzyme for integrating nutrient status with energy availability, and under conditions of high carbohydrate availability, dictates the synthesis of cholesterol and fatty acids. Moreover, ACL-dependent nucleocytosolic acetyl-CoA production has been implicated in metabolic reprograming in response to changing nutrient availability via histone acetylation. However, many questions regarding the role of nucleocytosolic acetyl-CoA in

health and disease, and whether ACL activity directly impacts mitochondrial function and inflammatory pathways via modulation of histone and non-histone protein acetylation remain unanswered. Preclinical evidence suggests that pharmacological suppression of ACL promotes LDL receptor activity and LDL uptake, as well as a variety of other beneficial effects on lipid and glucose metabolism in models of hyperlipidemia, atherosclerosis and NAFLD. The importance of ACL in humans is strongly supported by Mendelian randomization studies showing that LDL-C lowering SNPs in the ACL gene are associated with a reduced ASCVD risk. Moreover, recent advances have focused on ACL inhibition – particularly via BA - to lower plasma LDL-C levels. BA, currently in late-stages of clinical development, has provided the first evidence in humans that pharmacological suppression of ACL can promote plasma LDL-C reductions, and reduce other biomarkers associated with ASCVD [113]. Furthermore, evidence suggests that ACL inhibition in patients with hepatic insulin resistance, may also promote additional benefits on lipid and lipoprotein metabolism associated with the metabolic syndrome and NAFLD [113]. Although many questions remain, such as whether hepatic ACSS2 can compensate for ACL blockade and impact the therapeutic response in some patients (also see outstanding questions and Box 4), these recent advancements strongly support continued investigation into the molecular mechanisms linking ACL to the onset and progression of ASCVD and associated metabolic disorders. Over the next several years, the investigation of

BA in several clinical studies will reveal the therapeutic utility of ACL inhibition as a strategy for reducing cardiovascular disease risk and improving other diseases of metabolic origin such as NAFLD.

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Box 1. ACL and Synthesis of Fatty Acids and Cholesterol.

Rat ACL was purified and shown to consist of 4 identical subunits with an approximate total molecular weight of 440 kDa [115]. Human ACL is located at chromosome 17q21.2, with a coding region consisting of 1105 amino acids and its sequence is 96.3% homologous with rat ACL [116,117]. In the presence of ATP and Coenzyme A (CoA), ACL catalyzes the cleavage of citrate to acetyl-CoA and oxaloacetate. Although the reaction mechanism was not completely understood for many years, recent findings have better informed the details of substrate binding [118] and its catalytic mechanism. The currently accepted catalytic mechanism is shown in Figure I [119]. This 4 step reaction is a revision to an earlier hypothesis which suggested the involvement of an active site thiol [120], and has thus advanced the understanding of the ACL catalytic mechanism and facilitated drug discovery efforts.

Box 2. Early ACL Inhibitors: Translational Perspectives

Similar to other lipid synthesis enzymes (including HMG-CoA reductase[121]), global knockout studies in rodents have not provided significant insight into the role of ACL in lipid metabolismas homozygous knockout mice die early in embryonic development and heterozygous knockout mice do not produce a notable

phenotype[122]. Therefore, initial insight into understanding the role of ACL in lipid and lipoprotein metabolism has been largely derived from preclinical studies exploring the impact of pharmacological inhibition of ACL.

The first evidence demonstrating a key role of ACL in lipid metabolism was obtained from early studies showing that (-)-hydroxycitrate was a potent inhibitor of purified rat ACL [123], and suppressed cholesterol and fatty acid synthesis, and reduced plasma cholesterol and triglycerides in rat in vivo[124]. However, the development of (-)-hydroxycitrate was primarily focused on weight loss and was soon halted due to lack of efficacy, [125] low bioavailability and multiple off-target effects[126,127]. Efforts to improve upon these properties by generating synthetic analogues of citrate were also unsuccessful [128]. Despite its many limitations, (-)-hydroxycitrate was utilized as a tool to probe ACL biology and was critical in establishing connections between ACL and cholesterol metabolism [51]. Subsequent strategies focused on evaluating analogues of the stable reaction intermediate citryl-CoA, which resulted in the identification of SB-201076 [129]. Although a potent inhibitor of purified ACL (Ki = 1 μM), like previous citrate analogues, the polar nature of SB-201076 limited cell permeability [129]. This was overcome by synthesizing a less polar lactone **prodrug** form (SB-204990), which demonstrated high cell permeability and intracellular hydrolysis to its active form

(SB-201076) [129]. Consistent with ACL inhibition, SB-204990 blocked the incorporation of $^{3}H_2O$ into cholesterol and fatty acids in HepG2 cells and rat liver in vivo, and decreased plasma cholesterol and triglycerides in rats ApoE*3-Leiden transgenic mice [129,130]. Researchers then discovered BMS 303141, which also inhibited human recombinant ACL and lipid synthesis in HepG2 cells (IC_{50} = 8 µM)[131]. In vivo, chronic oral dosing of BMS 303141 lowered plasma cholesterol, triglycerides, and glucose in HFD-fed mice, further supporting the important role of ACL in metabolism [131]. Despite significant enthusiasm surrounding ACL as a putative therapeutic target, interest waned as ACL discovery programs were slow to identify suitable inhibitors with favorable pharmacological properties [128]. Owing to their anticipated lipid-lowering effects, ACL inhibitors were largely viewed as competitors of statins at the time and all ACL discovery programs were discontinued prior to advancing to clinical trials. Although the pharmacologic profile of ACL inhibitors in humans was never demonstrated, findings from preclinical studies do indicate that ACL inhibition can lower fatty acid and cholesterol synthesis in a variety of models, providing a strong rationale for pursuing ACL as a potential enzyme target to treat hyperlipidemia.

Box 3. The Role of ACL in Non-Hepatic Tissues

Although most tissues express basal levels of ACL, its role in non-hepatic tissues remains relatively unclear. For example, some studies show that ACL may be involved in insulin secretion from mouse and human pancreatic islets [132,133]; however, the importance of this mechanism is debated[134]. In human and mouse kidney mesangial cells, ACL was shown to play a role in glucose-dependent histone acetylation and pro-fibrotic gene expression, suggesting that ACL inhibitors might also possess anti-fibrotic activity [135]. In human adipose tissue, glucose stimulates ChREBP-dependent ACL expression and DNL [136,137], suggesting that DNL in adipose tissue may serve as a depot for the clearance and storage of circulating excess glucose and thus protect other tissues from the accumulation of ectopic fat. Accordingly, recent findings showed that obese patients with insulin resistance exhibited decreased expression of lipogenic enzymes in adipose tissue and bariatric weight loss restored DNL in adipose tissue [138]. However, recent studies have indicated that mice lacking adipose tissue *Acly* expression do not present an acute metabolic phenotype, despite exhibiting lower rates of fatty acid synthesis and reduced histone acetylation in adipose tissue [19].

ACL expression has also been documented as elevated in many human cancers including hepatocellular carcinoma, non-small cell lung cancer, breast cancer,

colorectal cancer, and glioblastoma [139-142] and has even been proposed as a biomarker that might aid in predicting cancer progression and prognosis in some patients [143,144]. This is consistent with an important role of enhanced DNL associated with metabolic reprograming of cancer cells that appears to confer growth and survival advantage over normal cells [145-147]. Moreover, investigations into the effects of SNPs in *ACLY* have been shown to be a prognostic marker for survival in patients with non-small cell lung cancer [148]. Based on these observational and genetic studies, as well as on its presumed strategic location in cancer cell metabolism, the impact of ACL suppression on cancer cell survival has been the subject of significant investigation. While the therapeutic potential of ACL inhibition is supported by in vitro and in vivo preclinical studies showing that both pharmacological intervention and RNAi-mediated ACL knockdown can lead to prostate tumor growth arrest [149], it is currently unknown whether this translates into any clinical benefit.

Given that ACL is upstream of HMG-CoA reductase, there may be some unexpected consequences of cholesterol synthesis inhibition in non-hepatic tissues, as evidenced from clinical experience with statin therapy. Indeed, despite their overwhelmingly favorable benefit/risk profile, statins promote undesirable side effects which can limit dose and/or affect compliance. Most common side effects

include muscle cramping, pain, and weakness (myalgia) [150]. Although the underlying pathophysiology of statin-induced myalgia is not completely understood, a leading hypothesis links myotoxicity to downstream consequences of HMG-CoA reductase inhibition in skeletal muscle [151-154]. It is possible that ACL inhibition could have similar effects in skeletal muscle, though this has not been directly tested. Accordingly, recent findings showed that siRNA-mediated ACL knockdown in myotubes reduced mitochondrial function, and ACL activity in mouse skeletal muscle in vivo was associated with increased ATP, suggesting an important role of ACL in maintaining muscle mitochondrial activity [155]. Although the role of ACL in non-hepatic tissues remain relatively unexplored, these initial findings suggest that additional investigations are justified and could lead to important insights.

Box 4. Clinician's Corner

Chronic overnutrition and lack of physical activity can cause derangement in cholesterol and fatty acid metabolism that lead to a variety of diseases including dyslipidemia, ASCVD, T2D, and NAFLD

An elevated level of plasma LDL-C (hypercholesterolemia) is causally associated with ASCVD, and for many patients, is not sufficiently treated by currently available therapies

Therapies that decrease plasma LDL-C by upregulating LDL receptor activity in the liver, reduce ASCVD risk

Targeting liver ACL reduces plasma LDL-C by suppressing cholesterol synthesis and increasing LDL receptor activity and could provide healthcare providers with an additional treatment option for patients with hypercholesterolemia

ACL inhibition could provide additional beneficial effects for patients with other lipid disorders such as NAFLD by reducing lipotoxic metabolite levels and promoting hepatoprotective effects through improved mitochondrial function, and reducing inflammation

The continued clinical investigation of bempedoic acid will further define the beneficial effects of targeting ACL on ASCVD risk and potentially other metabolic disorders such as NAFLD

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Glossary

AMP-activated protein kinase (AMPK): A central regulator of lipid and glucose metabolism that promotes energy producing processes and inhibits energy consuming processes in response to signals of cellular energy deficit

***Apoe*$^{-/-}$ mice:** Mice deficient in the gene encoding apolipoprotein E that develop hyperlipidemia and atherosclerotic lesions

Apolipoprotein B (apoB): A major protein found in LDL and VLDL particles that is involved in the transport and metabolism of lipids

Atherosclerotic cardiovascular disease (ASCVD): A chronic and progressive disease whereby elevated levels of apoB-containing cholesterol-rich lipoprotein particles accumulate in the arterial wall and promote the formation of macrophage-laden plaques that can eventually become inflamed and prone to rupture

ATP-citrate lyase (ACL): An enzyme in the nucleus and cytosol that is primarily responsible for the production of acetyl-CoA required for cholesterol and fatty acid synthesis, and protein acetylation

De novo lipogenesis (DNL): A biosynthetic process by which acetyl-CoA units generated from mitochondrial metabolism of non-lipid macronutrients are converted to fatty acids

Ezetimibe: A small molecule inhibitor of NPC1L1that reduces the absorption cholesterol in the gastrointestinal track that lowers plasma LDL-C by ~20%

Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (FOURIER): The first randomized clinical trial designed to evaluate the efficacy of Evolocumab (PCSK9 inhibitor) for reducing major cardiac events when added to other treatments for dyslipidemia

GCN5: A histone acetyltransferase shown regulate gene transcription and protein function by acetylation

Gluconeogenesis: the synthesis of glucose from non-carbohydrate carbon substrate

Hepatic fibrosis: The accumulation of collagen and other extracellular matrix protein in the liver in response to chronic injury and pro-inflammatory signaling

Hepatic steatosis: The accumulation of fat in the liver

Hepatic stellate cell: A cell type found in the peri-sinusoidal space of the liver that when activated by pro-inflammatory signaling and plays an important role in fibrosis

Hepatocellular ballooning: A term used in histopathology to describe the enlargement of hepatocytes that is indicative of apoptosis in response

High-sensitivity C-reactive protein (hsCRP): An acute phase protein made by the liver in response systemic inflammation that is associated with ASCVD risk

Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT): The first randomized clinical trial designed to evaluate the efficacy of the combination of ezetimibe with simvastatin vs. simvastatin alone, for reducing major cardiac events

Insulin resistance: A condition by which the normal physiological response to insulin become impaired or attenuated

Low-density lipoprotein (LDL): An apoB-containing particle that carries the majority of the circulating cholesterol for delivery to tissues and is sometimes called the "bad" cholesterol

Low-density lipoprotein receptor (LDLR): A protein expressed on the cell surface which binds and mediates the endocytosis of LDL particles

***Ldlr*$^{-/-}$ mice:** Mice deficient in the gene encoding for the low-density lipoprotein receptor that develop hepatic steatosis, hyperlipidemia, and atherosclerotic lesions

Mendelian randomization: A study design whereby variations within a gene of interest are exploited as instrument variables for approximating causal effects of an exposure on a specific disease outcome

Metabolic syndrome: A cluster of factors including obesity, hyperglycemia, hypertriglyceridemia, hypertension, and low high-density lipoprotein that increase one's risk for developing ASCVD, type 2 diabetes, and/or NAFLD

Niemann–Pick C1–like 1 (NPC1L1): A transmembrane protein localized at the apical membrane of enterocytes critical for the absorption of cholesterol in the gastrointestinal track

Non-alcoholic fatty liver disease (NAFLD): A disease spectrum marked by the accumulation of liver fat in the absence of competing liver disease etiologies including significant alcohol consumption and viral infection

Non-alcoholic steatohepatitis (NASH): liver injury and inflammation associated with increased buildup of fat in the liver

Non-high-density lipoprotein cholesterol (HDL-C): The sum of plasma cholesterol associated with LDL and VLDL particles

Peripheral lipolysis: The hydrolysis (or breakdown) of triglycerides to fatty acids in tissues such as adipose

Peri-sinusoidal fibrosis: Fibrosis occurring in the tissue surrounding sinusoids usually as a result of hepatic stellate cell activation and transformation to myofibroblasts

polyphagic Db/db mice: Mice homozygous for a point mutation in the leptin receptor that causes increased food consumption and subsequent obesity and diabetes

Prodrug: An agent that is administered in an inactive form that is subsequently metabolized to an active drug within the body

Sirtuin1 (Sirt1): A member of a class of NAD^+-dependent protein deacetylases that link energy metabolism to transcriptional regulation of several cellular processes

Tricarboxylic acid cycle (TCA): A series of biochemical reaction within mitochondria that produce adenosine triphosphate, nicotinamide adenosine dinucleotide, and biosynthetic precursors through the oxidation of acetyl-CoA derived from lipids, proteins, and carbohydrates

Type 2 diabetes (T2D): A chronic and progressive condition cause by impaired synthesis or response to insulin that results in hyperglycemia

Very low-density lipoprotein (VLDL): An apoB-containing LDL precursor particle secreted by the liver that contains triglycerides and cholesterol for distribution to peripheral tissues

Figures and Legends

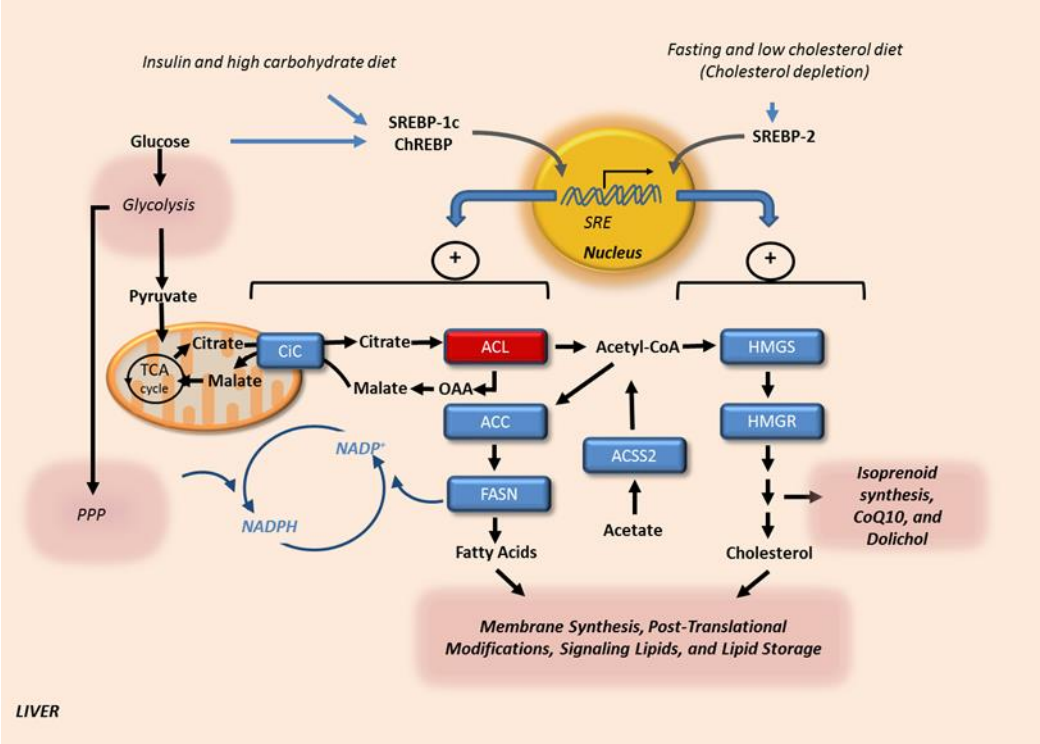

Figure 1. The central role of ACL in mammalian cholesterol and fatty acid synthesis in the liver. During the fed state, nutrients and insulin activate sterol-response element binding protein-1C (SREBP-1c) and carbohydrate-response element binding protein (ChREBP) - dependent lipogenic gene expression. The biosynthesis of fatty acid starts when an increased supply of citrate resulting from increased flux of substrate through the tricarboxylic acid (TCA) cycle, is exported from inside the mitochondria into the cytosol by the citrate transport protein (CiC). By contrast, cholesterol biosynthesis is supported by basal TCA flux and is induced by reduced dietary cholesterol intake. This results in reduces intracellular

cholesterol levels which activates sterol-response element binding protein-2 (SREBP-2)-dependent expression of multiple genes important for maintaining cholesterol homeostasis including cholesterol biosynthesis synthesis enzymes. In the cytosol, the cleavage of citrate by ACL to generate acetyl-CoA is a requisite step for the de novo synthesis of cholesterol and fatty acids. Cytosolic acetyl-CoA becomes committed to the pathway of fatty acid synthesis following conversion to malonyl-CoA by acetyl-CoA carboxylase (ACC), or to the mevalonate pathway via a series of condensation reactions followed by reduction to mevalonic acid by 3-hydroxy-3-methylglutarate-CoA reductase (HMGR), both rate-limiting steps in their respective pathways. Fatty acid synthesis proceeds via a four-step repeating cycle utilizing 7 malonyl-CoA molecules and one acetyl-CoA primer catalyzed by fatty acid synthase (FASN). The FASN reaction cycle is also dependent on the reducing power of NADPH which can come mainly from the pentosephosphate pathway (PPP) shunt. The mevalonate synthesized by HMGR is a building block for the synthesis of several important biological intermediates and products including isoprenoids, CoQ10, dolichol, and cholesterol. Carbohydrate-response element binding protein (ChREBP), 3-hydroxy-3-methylglutarate-CoA synthase (HMGS), liver X receptor (LXR), and nicotinamide adenine dinucleotide phosphate (NADP). Positive signs indicate the transcriptional activation of fatty acid biosynthesis enzymes by SREBP-1c or cholesterol biosynthesis enzymes by SREBP-2.

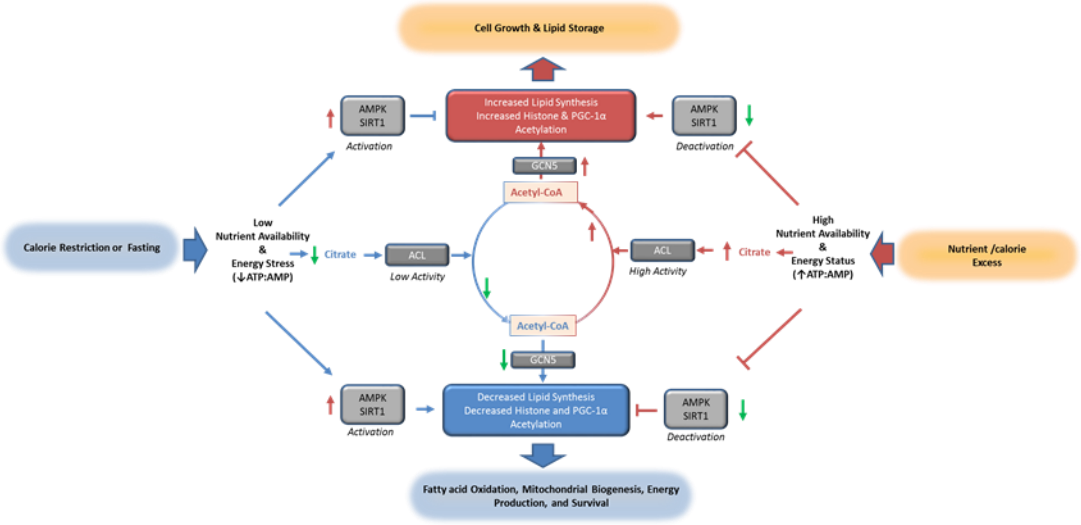

Figure 2. Proposed Model of ACL and AMPK-SIRT1-Mediated Nutrient Sensing in Cells. In this model, mammalian ACL and AMPK-SIRT1 are thought to coordinate metabolic adaptations in response to changing nutritional status. In the fed state (red), nutrient consumption exceeds energy expenditure and carbon substrate, and ATP molecules are abundant. Excess carbon is exported out of the mitochondria in the form of citrate which is cleaved to acetyl-CoA by ACL in the cytosol. The rise in nucleocytosolic acetyl-CoA supplies substrate for cholesterol and fatty acid synthesis, and GCN5-mediated histone and PGC-1α acetylation. The rise in ATP:AMP deactivates AMPK signaling which 1) reverses inhibitory phosphorylations of rate-limiting enzymes for cholesterol and fatty acid synthesis, thus allowing the conversion of acetyl-CoA to lipids for storage or support cell

growth, and 2) prevent SIRT1-dependent protein deacetylation. Protein acetylation further perpetuates the fed signal by regulating gene transcription. By contrast, in the fasted state where nutrients are limited and carbon substrate and ATP levels are low, citrate is retained in mitochondria for energy production and ACL activity is low. This results in reduced nucleocytosolic acetyl-CoA, which prevents flux into cholesterol and fatty acid synthesis, and GCN5-mediated histone and PGC-1α acetylation, resulting in enhanced fatty acid β-oxidation for ATP production. This is further perpetuated by the activation of AMPK resulting from reduced ATP:AMP ratio, which catalyzes inhibitory phosphorylations of the rate limiting enzymes in cholesterol and fatty acid synthesis. Furthermore, the activation of AMPK promotes SIRT1-dependent protein deacetylation to ensure the appropriate transcriptional response is evoked including genes involved in mitochondrial biogenesis (e.g.PGC-1α).

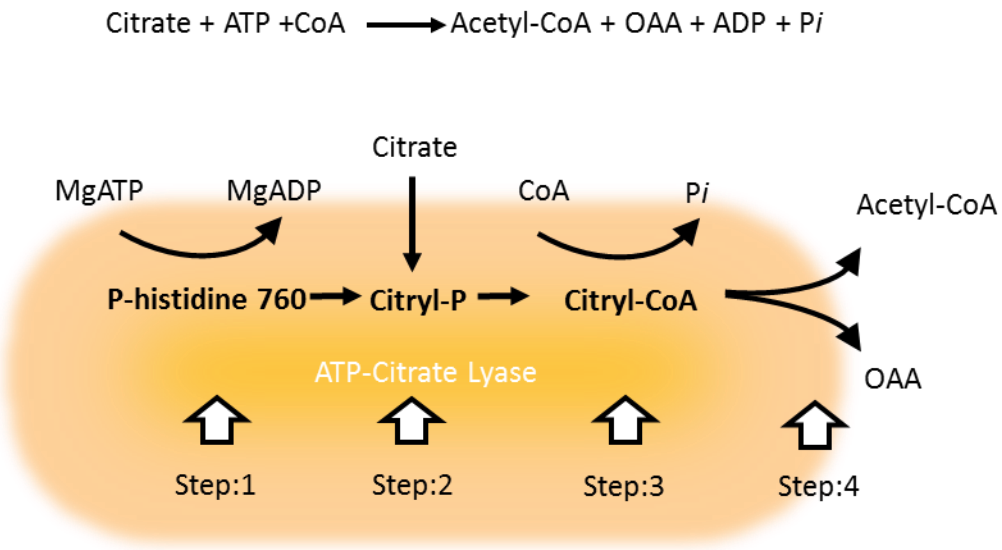

Figure I in Box 1. The ACL Biochemical reaction. The ACL reaction is initiated when 1) the enzyme is phosphorylated at histidine 760, which then catalyzes the formation of 2) an enzyme-bound citryl-phosphate followed by the formation of a 3) citryl-CoA intermediate after a CoA attack. Finally, 4) the citryl-CoA intermediate is cleaved and acetyl-CoA and oxaloacetate are released.

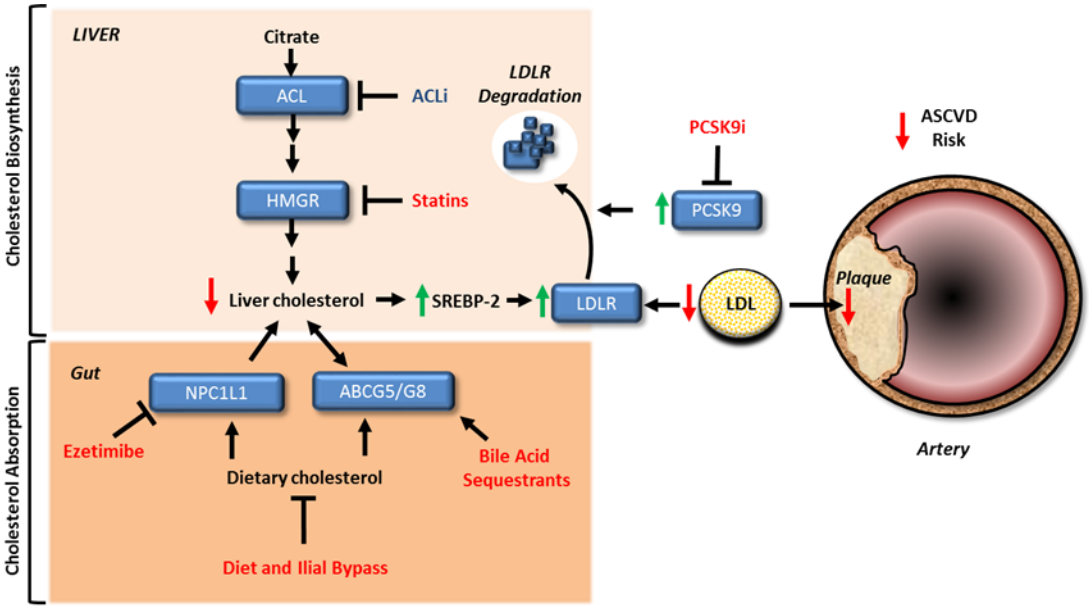

Figure 3. ACL inhibition can promote hepatic LDLR upregulation and decrease ASCVD risk. All therapeutic interventions that that up regulate LDLR activity in the liver have been demonstrated to reduce ASCVD risk in humans. Therapies that reduce hepatic cholesterol by blocking cholesterol absorption in the intestine (e.g. bile acid sequestrants (Welchol), ezetimibe, diet, and ilial bypass) or by inhibiting the de novo synthesis of cholesterol directly in the liver (e.g. ACL inhibitors and statins), all result in reduced hepatic cholesterol levels and compensatory SREBP2-mediated LDLR upregulation. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition prevents LDLR degradation and also increased LDLR activity and reduces ASCVD risk. Interventions in red indicate validation by both human genetics and randomized clinical trials. Interventions in blue indicate validation by human genetics only.

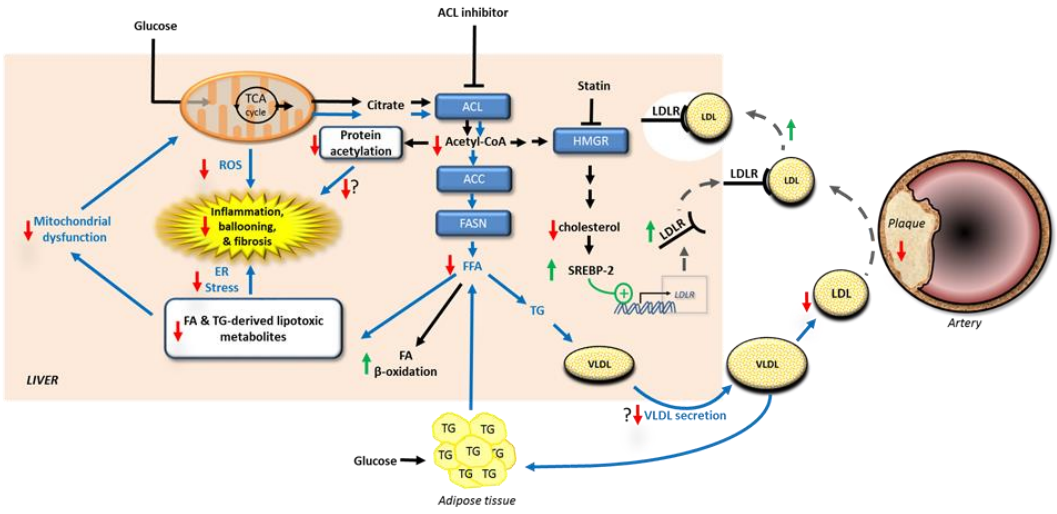

Figure 4. Proposed Model of ACL Inhibition on Human Hepatic Lipid and Lipoprotein Metabolism. The diagram illustrates the Anticipated Effects of ACL inhibition under Normal and Insulin Resistant (IR) States. Under normoinsulinemic conditions, hepatic fatty acid synthesis rates are low and most of the cytosolic acetyl-CoA is funneled toward de novo cholesterol synthesis (black arrows). Similar to statins, ACL inhibition results in reduced intracellular cholesterol and activation of SREBP-2-mediated LDLR upregulation, and increased LDL particle clearance from the circulation. This results in less retention of LDL in the arterial wall, lowering ASCVD risk. With IR, the concentration of hepatic fatty acids rise due to increased influx from enhanced lipolysis in adipose tissue and DNL (blue arrows). This promotes VLDL secretion, and elevated blood LDL. Chronic exposure to increased hepatic triglycerides (TG) can lead to the accumulation of

lipotoxic metabolites promoting mitochondrial dysfunction, ROS production, and ER stress (blue arrows). This can lead to inflammatory signaling, hepatocyte injury, and eventually fibrosis (i.e. NASH). Inhibition of ACL could have the following beneficial effects on lipid metabolism in individuals with metabolic syndrome: 1) suppress hepatic lipogenesis and promote fatty acid β-oxidation resulting in reduced levels of free fatty acids and TG, 2) decreased levels of TG could result in decreased VLDL assembly and secretion, and thus, reduced plasma TG,VLDL-C and LDL-C, 3) reduced levels of TG-derived lipotoxic metabolites and protein acetylations contributing to ER stress, inflammatory signaling, mitochondrial dysfunction and/or apoptosis, and 4) reduced hepatocyte ballooning, inflammation, and fibrosis. Black arrows indicate conditions under normoinsulinemia, blue arrows: effects accelerated by IR; green and red arrows: effects of ACL inhibition.

Outstanding Questions

What are the underlying molecular mechanisms that link the regulation of nucleocytosolic acetyl-CoA pool to the onset of the metabolic syndrome, ASCVD and NASH?

Are hepatic nucleocytosolic levels of acetyl-CoA elevated in patients with ASCVD, NAFLD, and metabolic syndrome?

Does pharmacological ACL inhibition reduce the acetylation of non-histone proteins such as PGC-1α, NFkB, or Stat3?

Does pharmacological ACL inhibition in hypercholesteremic patients reduce the risk of ASCVD?

Does ACL inhibition in patients with metabolic syndrome reduce liver and systemic triglycerides? Does it improve insulin sensitivity and dysglycemia?

Can ACL inhibition improve or reverse liver pathologies associated with NAFLD/NASH, including fibrosis?

Trends Box

ATP-citrate lyase (ACL) is a cytosolic enzyme dysregulated in many metabolic disorders that catalyzes the production of acetyl-CoA from citrate and as such is positioned at the intersection of oxidative phosphorylation and the synthesis of cholesterol and fatty acids

For many years, ACL has been considered a viable target to treat dyslipidemia, but recent insight into its role in lipid and energy metabolism provides additional rationale for its potentially utility to address modern challenges in cardiovascular and metabolic health

ACL-dependent protein acetylation has also emerged as an important signal to coordinate lipid metabolism and gene expression with nutritional status

ACL suppression reduces atherogenic lipoproteins, hepatic fat and inflammation, attenuates experimental atherosclerosis, and improves dysglycemia in multiple disease models

Mendelian randomization studies, which have emerged as a means to genetically validate new drug targets, have recently shown that LDL-C lowering single nucleotide polymorphisms (SNPs) in and around the *ACL* gene are associated with reduced ASCVD risk

Late-stage clinical trials demonstrate that the novel ACL inhibitor, bempedoic acid, reduces elevated levels of LDL-cholesterol, and promotes beneficial effects on other metabolic risk factors associated with ASCVD and NAFLD

CHAPTER FIVE

5 Conclusions and Future Directions

5.1 Introduction

Cells have evolved multiple mechanisms to integrate intracellular lipid and energy signals with metabolic processes to promote whole-body energy homeostasis. The uncoupling of energy and lipid metabolism and the subsequent accumulation of ectopic lipids in various tissues appears to be a hallmark trait of multiple metabolic diseases with increasing prevalence in the developed world such as ASCVD and NAFLD. One component of lipid metabolism that is tightly linked to energy status and is often dysregulated in metabolic disease is de novo lipid synthesis (Diraison, Dusserre et al. 2002; Donnelly, Smith et al. 2005; Tamura and Shimomura 2005; Ference, Ginsberg et al. 2017). However, the specific mechanisms by which cells integrate energy and lipid signals and how the dysregulation of these processes can lead to aberrant lipid biosynthesis are still being investigated. AMPK is an evolutionarily conserved master regulator of metabolism that links lipid and energy metabolism by promoting regulatory phosphorylation of several enzymes and transcription factors in lipid metabolism in direct response to changing energy status (AMP: ATP) (Steinberg and Kemp 2009). AMPK gauges metabolic status by interacting with the intracellular environments via the two regulatory subunits, β and γ. While the mechanism by which the γ subunit senses energy status through competitive binding of AMP, ADP, and ATP has been well characterized, the regulatory role of the β subunit and

its physiological relevance is less well understood. For many years the β subunit was largely regarded as a scaffold to facilitate interactions between the α catalytic and γ regulatory subunits or by mediating glycogen binding (Polekhina, Gupta et al. 2005; McBride, Ghilagaber et al. 2009; Oakhill, Chen et al. 2010); however, the identification of a class of small molecule synthetic activators that allosterically modulate kinase activity via specific interactions with the S108 phosphorylation site within the CBM of β1 subunit, suggested the presence of a previously unidentified natural ligand for this regulatory subunit (Sanders, Ali et al. 2007; Hawley, Fullerton et al. 2012; Xiao, Sanders et al. 2013; Cokorinos, Delmore et al. 2017; Myers, Guan et al. 2017). In the present studies, we aimed to identify this natural ligand and characterize its role in mediating adaptations in lipid metabolism under physiological and pathological conditions, and to also determine whether this mechanism can be exploited for therapeutic intervention.

5.2 LCFA-CoA esters as novel regulators of AMPK

We conducted a targeted screen of multiple metabolites with established roles in lipid metabolism as well as several cofactors and vitamins that share structural similarities with a known synthetic AMPKβ1-selective agonist. We show for the first time, that LCFA-CoAs specifically interact with AMPK β1-containing heterotrimers via broad interactions involving the catalytic AMPKα-subunit and the β1-S108 drug binding site to promote allosteric activation of the kinase. In vitro studies using purified active mammalian, insect, and bacterial

AMPK α1β1γ1 heterotrimers, demonstrated that saturated LCFA-CoA ranging from 12 to 18 carbon chain length, were optimal for increasing kinase activity by approximately 2-3-fold. Palmitoyl-CoA, the product of DNL and substrate for CPT-1-dependent mitochondrial LCFA β-oxidation, was used as the representative ligand, and demonstrated concentration-dependent enzyme activation within physiologically relevant concentrations ($EC_{50} \leq 1\mu M$). Interestingly, the respective free acids of the LCFA-CoA esters were inactive in these assays, suggesting that the metabolically activated CoA ester form of LCFAs specifically regulate kinase activity. Moreover, the magnitude and potency of activation was comparable to that observed with AMP. Consistent with findings from synthetic activators (e.g. A-769662), palmitoyl-CoA did not activate β2-containing AMPK heterotrimers or AMPKα1β1γ1 complexes containing a single S108A point mutation within the β1-subunit. However, in contrast to A-769662, studies using truncated $AMPK\alpha1_{(1-392)}$ and $\alpha2_{(2-279)}$, showed that palmitoyl-CoA also directly activated the α2 catalytic subunit independently of the β subunit, but not α1, suggesting the potential for broader interactions with α2-containing heterotrimers. Also, unlike A-769662, palmitoyl-CoA did not protect against PP2Cα-mediated AMPKα (Thr172) dephosphorlation in vitro. Despite these differences, treatment with palmitate or A-769662 increased ACC (Ser79) phosphorylation status in WT primary mouse hepatocytes, while hepatocytes isolated from AMPKβ1-S108A KI mice were refractory to both treatments. We then demonstrated the physiological relevance

of this sensing mechanism in promoting metabolic adaptation in hepatic lipid metabolism in response to excess dietary fat by showing that an oral gavage of Intralipid® following a fast-carbohydrate refeed staging regimen, rapidly increased hepatic AMPK activity and suppressed RER in WT mice, but not AMPKβ1-S108A KI mice. Moreover, we show that the deletion of AMPKβ1/β2 in brown adipose tissue did not attenuate Intralipid®-dependent suppression of RER, which further implicated the role of liver AMPK.

These studies potentially establish a novel mechanistic link by which dietary fat trigger a feedback mechanism involving direct allosteric interactions with the AMPKβ1 subunit resulting in enhanced mitochondrial β-oxidation and suppressed fatty acid synthesis. These studies show for the first time that AMPK is regulated by physiological signals other than changes in the AMP: ATP ratio, and sense intracellular fatty acyl-CoA levels through the β1-S108 drug binding site and are thus the first natural ligands identified which regulate enzyme activity through this mechanism. It is noteworthy that LCFA-CoAs have also been shown to inhibit ACC (thus also inhibiting fatty acid synthesis and increasing fatty acid oxidation) and therefore, the identification of this novel point of regulation adds to the weight of evidence for the importance of fatty acyl-CoAs as a critical modulator of substrate utilization. Given the demonstrated cytotoxicity of LCFA-CoAs and their associated metabolites, this mechanism could facilitate fuel switching to safeguard the liver from lipotoxicity associated with elevated fatty acids levels, an

established requisite condition for the onset of metabolic disorders such as insulin resistance and NAFLD.

However, several questions remain, such as why this mechanism would be specific for β1-containing AMPK complexes, and what the implications are for this mechanism in other tissues and species. Given that palmitoyl-CoA directly activates the isolated α2 subunit but is also dependent on S108 for activation of the trimer, these finings suggest that it may interact with the kinase more broadly than the synthetic agonists. Moreover, it is surprising that despite the requirement for S108, palmitoyl-CoA did not protect the kinase from PP2Cα-dependent de-phosphorylation as observed with A-769662 and AMP. Therefore, further characterization of this specific interaction by studying binding or x-ray crystallography would be very informative. Moreover, understanding how fatty acid signaling cooperates with energy deficit signals (i.e. increased AMP:ATP) to regulate AMPK activity, and what other downstream effects apart from lipid metabolism are affected should be studied further. Also, how this mechanism might influence metabolism under a variety of physiological conditions that exert energetic stress through either energy deprivation or excess, and whether this mechanism is dysregulated in human lipid disorders, is of particular interest.

5.3 Role of AMPK and ACL in lipoprotein metabolism and atherosclerosis

In chapter 3, we began addressing some of the above questions by investigated the importance of this mechanism in a model of hypercholesterolemia and atherosclerosis. To this end, we crossed *Ampkβ1*$^{-/-}$ mice with *Apoe*$^{-/-}$ mice to generate *Ampkβ1*$^{-/-}$ /*Apoe*$^{-/-}$ double knockout (DKO) mice. Following 12 weeks of HFHC diet feeding, we measure the impact of AMPKβ1 deletion on multiple disease outcomes including glycemic parameters (fasting glucose, insulin, and GTTs), plasma lipoprotein profiles (LDL-C, VLDL-C, HDL-C, and non-HDL-C), body composition, liver lipid levels, and atherosclerosis. As expected *Apoe*$^{-/-}$ mice fed the HFHC diet developed elevated liver lipids, plasma non-HDL-C, fasting blood glucose, body weight, adiposity, and an accelerated progression of atherosclerosis compared to mice fed a normal chow diet. However, we were unable to detect any difference between *Apoe*$^{-/-}$ and DKO mice for any disease model outcomes. This was unexpected given the impact observed in response to Intralipid in the acute in vivo setting. Moreover, despite the observed upregulation of *Srebf2* in livers from DKO mice, no difference in the expression of other key genes in fatty acid or cholesterol metabolism were observed, including *Srebf1c*, *Hmgr*, or *Ldlr*, suggesting that loss of AMPKβ1 did not impact these pathways. The lack of effect observed on LDL metabolism appear consistent with previous reports suggesting that neither AMPK activation nor germline deletion, affect plasma LDL-C (Dong, Zhang et al. 2010; Dong, Zhang et al. 2010; Wang, Zhang

et al. 2011; Cai, Ding et al. 2016; Ding, Zhang et al. 2016; Ma, Wang et al. 2017). Although the reasons why the deletion of AMPKβ1 did not produces a metabolic phenotype in a chronic setting of lipid overload which would be expected to increase cellular concentrations of LCFA-CoAs are unknown, but it suggests that other redundant or compensatory metabolic mechanisms were sufficient to overcome this AMPK-dependent mechanism, and prevent the accumulation of LCFA-CoAs and hepatic triglycerides.

In an attempt to circumvent this, we aimed to mimic LCFA-CoA-dependent activation of AMPK using bempedoic acid, a synthetic long-chain (15 carbon backbone) aliphatic dicarboxylic acid that is in late-stage clinical development for the treatment of hypercholesterolemia. Bempedoic acid also forms a CoA conjugate, but due to several strategic structural modifications such as α, α gemdimethyl substitutions, is protected against subsequent metabolic processes such as mitochondrial β-oxidation, or esterification into triglycerides or cholesteryl esters. Bempedoic acid was previously shown to suppress rodent hepatic de novo cholesterol and fatty acid synthesis, and to activate AMPK in both in vitro and in vivo settings (Pinkosky, Filippov et al. 2013). It is also noteworthy that bempedoic acid was shown to inhibit ACL in liver cells; however, the underlying molecular mechanisms and relative contribution of these potential targets was unknown. We first investigated the mechanisms by which bempedoic acid activated AMPK using the same enzyme preparations as described for the assessment of natural LCFA-

CoA, and confirmed that similar to natural LCFA-CoAs, the CoA conjugate of bempedoic acid indeed mediated β1-dependent AMPK activation while the free acid did not. Through a variety of techniques using human liver preparations, we characterized and identified the specific isoform that mediates the CoA activation of bempedoic as ACSVL and confirmed its expression in human and mouse liver.

We then assessed the effects of bempedoic acid in the DKO mouse, which not only offered the opportunity to interrogate the importance of β1-dependent AMPK activation in a hypercholesterolemic model of atherosclerosis, but also elucidate the relative contribution of AMPK activation and ACL inhibition. Given that bempedoic acid is in late-stage clinical trials, these answers would provide important insight into how engaging either of these first-in-class molecular targets, could translate to humans. To this end, a HFHC diet ± bempedoic acid was fed to both *Apoe*$^{-/-}$ and DKO mice for 12 weeks, and consistent with previous in vivo studies (Pinkosky, Filippov et al. 2013), AMPK activity was increased in the livers from *Apoe*$^{-/-}$ mice. As anticipated by the demonstrated β1 specificity for AMPK activation in vitro, bempedoic acid treatment did not increased liver AMPK activity in DKO mice. While treatment with bempedoic acid did not impact glycemic parameters such as fasting glucose or GTT in either *Apoe*$^{-/-}$ or DKO mice, it did lower plasma LDL-C, decrease hepatic cholesterol and triglycerides, suppressed RER, upregulated hepatic *Ldlr*, and attenuated the progression of atherosclerosis in both *Apoe*$^{-/-}$ and DKO mice. These findings suggest that bempedoic acid mediates

its pharmacodynamic effects independently of AMPK, which implies a dominant role of ACL inhibition.

When taken together, it appears that despite the robust activation of AMPK by LCFA-CoA esters observed in vitro, and the observed acute effects of Intralipid gavage on hepatic AMPK and RER, studies in HFHC-fed DKO mice were unable to demonstrate AMPK-dependent effects. Moreover, using bempeoic acid to mimic the β1-selective activation of AMPK by LCFA-CoAs also did not demonstrate AMPK-dependent pharmacodynamic effects. Although the reasons for the lack of translation to a chronic disease model is not known, it might be due to the presence of other compensatory mechanisms that mask the loss of AMPK function. As previously discussed in the context of lipoprotein metabolism, several important differences between rodents and humans have been identified which must be taken into account when considering the translation of findings between species. During the course of these studies, it was shown that while in rodent liver, AMPK is predominantly expressed as α1β1γ1 or α2β1γ1 heterotrimeric complexes with little β2 expression observed, in human liver, the β2 isoform is predominantly expressed (Wu, Puppala et al. 2013). These findings likely deemphasize the role of β1-selective activation in the liver of humans, but raise potentially important questions regarding the effects of LCFA-CoAs in other tissues that highly express the AMPKβ1 such as macrophages. Due to the lack of ACSVL1 expression in macrophages, bempedoic acid could not be used to address this question due to the

absence of CoA thioesterification in this cell type. Future studies could be conducted with natural LCFA that are activated in macrophages to better assess the importance of this mechanism. Of interest, evidence suggests that palmitoleate does suppress inflammation in macrophages through an AMPK-dependent mechanism (Chan, Pillon et al. 2015). Therefore, additional investigations of this potential activity in other cell types including macrophages, could shed light on the physiological role of different isoform combinations in different species, and the ligands that regulate their activity.

The lack of AMPKβ1 expression in human liver combined with the demonstrated AMPK independence and liver-specificity of bempedoic acid, prompted us to focus on elucidating the molecular mechanisms linking the suppression of ACL activity to lipid synthesis inhibition, LDL-C lowering and associated attenuated atherosclerosis. Given the pre-established effects of cholesterol synthesis suppression via statins on plasma LDL-C, we pursued studies aimed to confirm similar connections for blockade by ACL inhibition. Using siRNA-mediated suppression of ACL, we demonstrated that like inhibition of HMG-CoA reductase, reduced ACL expression promoted the upregulation of LDL receptor protein and activity. These effects were reproduced using pharmacological inhibition in primary human hepatocytes where increases in *LDLR* mRNA, protein, and activity were all increased by bempedoic acid treatment. Consistent with no observable LDL phenotype in DKO mice and predominant expression of AMPKβ2

in human liver, β1-selective AMPK activation with A-769662, did not increase LDL receptor activity in primary human hepatocytes. When taken together, these findings suggest that AMPK is not a major regulator of LDL metabolism in these models, and that bempedoic acid mediates its LDL-C lowering effects by inhibiting ACL. These findings raise important questions about ACL as a new target to potentially treat dyslipidemias, ASCVD, and other metabolic disorders in humans. As many aspects of ACL biology are relatively unexplored, several questions remain regarding how this strategy is similar or differentiated from existing therapies and whether it is safe. Moreover, owing to its strategic position at the intersection of carbohydrate catabolism and cholesterol and fatty acid synthesis, it is of interest to explore whether targeting ACL could benefit other lipid disorders of metabolic origin.

5.4 Targeting ATP-Citrate Lyase in Hyperlipidemia and Metabolic Disorders

To begin addressing some of these questions, we attempted to integrate information known about ACL biology and put it in the context of modern challenges in cardiovascular and metabolic health. Specifically we aimed to determine if sufficient evidence supports the involvement of ACL in the pathogenesis of ASCVD and/or NAFLD, and whether evidence exists to justify its inhibition as a potentially viable strategy to treat either of these diseases. Given the demonstrated impact of ACL suppression on plasma LDL-C observed in both

preclinical and clinical investigations of bempedoic acid, we first aimed to understand how pharmacological ACL inhibition might impact ASCVD. When considering the position of ACL upstream of HMG-CoA-reductase in the cholesterol biosynthesis pathway, the confirmation of the anticipated downstream effects on cholesterol metabolism following ACL inhibition, namely cholesterol synthesis suppression and LDL receptor upregulation, essentially demonstrates a mechanism for LDL-C lowering that is similar to statins. This is important because the details of this pathway have been well characterized and proven to not only lower elevated LDL-C, but also promote a proportional reduction in ASCVD risk (Silverman, Ference et al. 2016). Indeed for many years the association between LDL-C and ASCVD risk (i.e. the LDL hypothesis) has been considered to be so reliable that it is an accepted surrogate marker for ASCVD and sufficient for the approval of many LDL-C lowering therapies. Over the years, this "LDL hypothesis" has been further strengthened by epidemiologic cohort studies and meta-analyses of several large statin cardiovascular outcomes trials (Ference, Ginsberg et al. 2017). Moreover, other mechanism of LDL-C lowering that increase LDL receptor activity such as blocking cholesterol absorption in the gut via ezetimibe, or blocking PCSK9-dependent LDL receptor degradation with PCSK9 blocking antibodies, have further confirmed this hypothesis (Sabatine, Giugliano et al. 2017). It is important to point out that upregulation of the LDL receptor reduces LDL-C by increasing whole-particle clearance which results in a

corresponding and proportional reduction in plasma LDL-C, LDL particle number, and apoB. Consistently, clinical studies investigating the efficacy of ACL inhibition by bempedoic acid have demonstrated proportional reductions in LDL-C, LDL particle number, and apoB (Ballantyne, Davidson et al. 2013). However, therapeutic approaches that lower LDL-C by changing lipoprotein particle composition (i.e. cholesteryl ester transfer protein; CETP inhibition) may not lead to proportional ASCVD risk reduction (Schwartz, Olsson et al. 2012). ACL inhibition does not appear to be subject to this as bempedoic acid promotes proportional reductions in LDL-C and apoB on all background therapies assessed, most notably, statins (Ballantyne CM 2015; Thompson, Rubino et al. 2015).

Despite the overwhelming evidence suggesting that ACL inhibition will promote a reduction in ASCVD risk proportional to the reduction in LDL-C, this must eventually be shown in a large cardiovascular outcomes trials. However, investigating the potential benefit of a drug on ASCVD efficacy endpoints is a massive undertaking involving the enrollment of thousands of subjects in a placebo controlled randomized clinical trial which must run for several years to accumulate sufficient data for reliable interpretation. Therefore, prior to such resource intensive endeavors, investigators are utilizing the principles of Mendelian randomization as a means to mimic these trials with the aim of "genetically validating" novel LDL-C lowering molecular targets. Indeed, these techniques have been validated retrospectively by interrogating human LDL-C lowering

HMGR variants to mimic statins trials, and prospectively by predicting outcomes of several recent drug trials (Ference, Majeed et al. 2015; Ference, Robinson et al. 2016; Ference, Kastelein et al. 2017). Importantly, Mendelian randomization studies designed to mimic a cardiovascular outcomes trial with an ACL inhibitor showed that lowering LDL-C via this mechanism should promote a concomitant reduction in ASCVD risk, and that this mechanism should not be susceptible to attenuation as observed with CETP inhibition (Ference 2017). When taken together, findings from our in vitro and in vivo preclinical studies implicating ACL-dependent LDL receptor upregulation, support the LDL-C lowering efficacy observed in clinical trials, and when combined with findings from statin cardiovascular outcomes trials, epidemiologic cohort studies, and Mendelian randomization trials, the data strongly suggest that ACL inhibition should reduce ASCVD risk.

Owing to its unique position in metabolism, ACL is linked to several other cellular processes that may demonstrate importance in other metabolic diseases such as NAFLD. Most obvious is the potential impact of the suppression of DNL under various pathological conditions. In normal healthy humans, DNL rates are quite low (Postic and Girard 2008); however, in certain metabolic disorders such as insulin resistance, it can dramatically increase (Diraison, Dusserre et al. 2002; Schwarz, Linfoot et al. 2003; Donnelly, Smith et al. 2005; Tamura and Shimomura 2005; Lambert, Ramos-Roman et al. 2014) and promote steatosis,

hypertriglyceridemia, the accumulation of hepatic lipotoxic metabolites, and further propagation of insulin resistance (Nagle, Klett et al. 2009; Farese, Zechner et al. 2012; Gruben, Shiri-Sverdlov et al. 2014; Mota, Banini et al. 2016). Moreover, the contribution of elevated DNL to the pathogenesis of NASH has been established (Lambert, Ramos-Roman et al. 2014), and its pharmacologic blockade in humans has shown promising results (Kim, Addy et al. 2017; Lawitz 2017; Stiede, Miao et al. 2017). However, important questions remain regarding the impact of this strategy on circulating triglycerides levels (Kim, Addy et al. 2017). Therefore, it is of significant interest to investigate whether ACL inhibition in humans could provide similar beneficial effects for NASH, and how this will impact circulating triglycerides. While the therapeutic utility of ACL blockade for NASH is supported by findings from various genetic preclinical models (Wang, Jiang et al. 2009) the specific assessment of pharmacological blockade on important disease outcomes such as NAFD activity score (NAS) and hepatic fibrosis, have not been investigated.

The role of ACL in controlling nucleocytosolic acetyl-CoA levels and protein acetylation status suggests that the function of ACL might extend beyond providing substrate for lipid synthesis, and may represent an important signaling node in energy metabolism. This suggests a logical link between nutrient availability and protein acetylation because ACL activity is highest when nutrient and energy levels are also high. By contrast, when energy levels are low, carbon is

retained in the mitochondria for oxidation and energy production, and ACL activity is low. As such, the product of ACL, acetyl-CoA reflects the energy status of the cell, and transduces this signal by promoting protein acetylation and modulation of gene expression (Wellen, Hatzivassiliou et al. 2009; Cai and Tu 2011). This signal would be further integrated with energy status through the actions of AMPK, GCN5, and Sirt1, which fine tune these metabolic adaptions by facilitating protein phosphorylation and acetylation/deacetylation reactions in response to changes in energy and redox status. In support, ACL activity has been shown to be tightly linked to histone and non-histone protein acetylation status, and promote metabolic adaptations by modulating the expression of genes involved in energy, lipid and carbohydrate metabolism (Wellen, Hatzivassiliou et al. 2009; Wellen and Thompson 2012; Shi and Tu 2014). It is possible that this signaling network could become uncoupled upon chronic caloric excess and reduced energy expenditure, resulting in inappropriate metabolic adaptations leading to a discordance between energy and lipid metabolism, and the accumulation of ectopic lipids. Therefore, in the context of chronic over-nutrition and reduced energy expenditure, it could be anticipated that key proteins are hyper acetylated, which result in aberrant metabolism, and that ACL blockade might facilitate the reversal of this by signaling reduced nutrient availability. Although this concept has mainly been studied in the context of cancer cell metabolism, many of the mechanisms might have implications for regulating mitochondrial biogenesis, and several survival,

apoptotic, and inflammatory processes important in the pathogenesis of NASH (Kramer, Baus et al. 2006; Aagaard-Tillery, Grove et al. 2008; Bricambert, Miranda et al. 2010; Jeninga, Schoonjans et al. 2010; Rothgiesser, Erener et al. 2010; Yi, Pan et al. 2011; Machado and Diehl 2016).

Findings from our investigations of ACL has several implications for the regulation of metabolism under normal conditions and its dysregulation under pathological conditions. As such, many questions remain and should be the subject of future investigations. For example, does ACL blockade reduce ASCVD risk, and/or prevent the progression of NAFLD in humans? How does the liver acetylome change between normal subjects and those with metabolic syndrome, steatosis, and/or NASH? What are the key proteins, transcription factors, and downstream effectors responsible for the maladaptation of metabolism, and does ACL inhibition reverse these effects? Moreover, the finding that the CoA conjugate of bempedoic acid inhibits ACL raises the intriguing possibility that like HMG-COA reductase and ACC, ACL is regulated by LCFA-CoAs. This would add to the complexity of ACL's role in sensing the energy status of cell and should be investigated further. These questions, and many others, will be critically important to address as we begin unraveling the molecular pathology of metabolic diseases, and novel approaches to treat it.

5.5 Summary

In summary, we have identified LCFA-CoAs as the first β1-S108-dependent physiological regulators of AMPK. Although several questions remain regarding the impact of this regulatory mechanism in chronic disease models, these findings have expanded our understanding of how cells acutely integrate lipid and energy signals to maintain lipid homeostasis in rodents. By assessing the effects of HFHC-feeding and by pharmacologically mimicking the activity of LCFA-CoAs using bempdoic acid, we assessed the impact of this previously uncharacterized mechanism on multiple disease outcomes in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ / *Ampkβ1*$^{-/-}$ double knockout mice. Bempedoic acid demonstrated multiple beneficial effects on lipid metabolism and attenuated atherosclerosis; however, there were no differences between genotypes in response to the HFHC diet or bempedoic acid treatments. Additional mechanistic studies revealed ACL inhibition is the primary mechanism of action mediating the pharmacodynamic effects of bempedoic acid and provided evidence for the therapeutic utility of this strategy to treat hypercholesterolemia and ASCVD in humans. Through our studies of AMPK and ACL, we identify novel connections between lipid biosynthesis and energy metabolism and strengthen the importance of their proper integration in health and disease. Moreover, we propose that the unique position of ACL in metabolism represents a metabolic checkpoint with potential relevance for treating other diseases with metabolic origins including NAFLD.

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