ROLE OF ACETYL COA CARBOXYLASE IN LIVER LIPID METABOLISM AND INSULIN SIGNALING

GENETIC DISRUPTION OF ACETYL COA CARBOXYLASE PHOSPHORYLATION BY AMP-ACTIVATED PROTEIN KINASE INCREASES LIVER LIPID ACCUMULATION AND INSULIN RESISTANCE

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ABSTRACT

In obesity, nonalcoholic fatty liver disease (NAFLD) has been associated with the development of hepatic insulin resistance; however, the potential causality and molecular mechanisms underlying this relationship are unclear. Acetyl coA carboxylase (ACC), which exists as two separate isoforms (ACC1 and ACC2), is an important metabolic enzyme which controls the production of the metabolic intermediate malonyl coA, and hence, fat metabolism. AMP-activated protein kinase (AMPK) has been shown to inhibit ACC activity by phosphoryating ACC1 at Ser79 and ACC2 at Ser221. The objectives of this were to determine the physiological importance of AMPK phosphorylation of ACC as it relates to the development of NAFLD and insulin resistance.

To address the above objectives, we examined the metabolic phenotype of C57Bl6 mice with a targeted ACC1 Ser79 to Ala and ACC2 Ser221 to Ala double knock-in mutation (ACC DKI), which would inhibit AMPK phosphorylation of ACC and compared them to wild-type (WT) mice. Basic body characteristics, assessment of insulin sensitivity, and assessment of liver steatosis were used.

ACC DKI body mass and energy expenditure were not different compared to WT. Liver ACC activity and malonyl coA were higher in ACC DKI mice. The livers of ACC DKI mice displayed greater triacylglycerol accumulation and aggregation of neutrophils. Fasting blood glucose and insulin were both elevated in ACC DKI mice. Glucose and insulin tolerance were impaired and hyperinsulinemic-euglycaemic clamps revealed that ACC DKIs had liver insulin resistance. Insulin-stimulated glucose disposal rate was also reduced.

In summary, we have shown that the phosphorylation of ACC1 Ser79 and ACC2 Ser221 is critical for maintaining ACC activity and malonyl coA levels in the liver. The dysregulation of this pathway results in liver fat accumulation and the development of insulin resistance. These studies demonstrate that AMPK phosphorylation of ACC is essential for maintaining metabolic homeostasis.

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LIST OF ABBREVIATIONS

ACC	Acetyl CoA Carboxylase
ACC DKI	ACC1 Ser79 to Ala and ACC2 Ser221 to Ala Double Knock-In
ACC1	Acetyl CoA Carboxylase 1
ACC1 KI	ACC1 Knock-In
ACC2	Acetyl CoA Carboxylase 2
ACC2-/-	ACC2 knockout mice
ACC2 KI	ACC2 Knock-In
AICAR	AMPK Activator 5` Aminoimidazole-4-Carboxamide-Riboside
ALT	Alanine Aminotransaminase
AMPK	5` Adenosine Monophosphate-Activated Protein Kinase
AST	Aspartate Aminotransaminase
AUC	Area Under the Curve
BMI	Body Mass Index
CLAMS	Comprehensive Lab Animal Monitoring System
CPT-1	Carnitine Palmitoyl-Transferase-1
CREB	cAMP Response Element Binding Protein
CRTC2	CREB Regulated Transcription Coactivator 2
CT	Computed Tomography
CVD	Cardiovascular Disease
DGAT1	Diglyceride Acyltransferase 1
FOXO1	Forkhead Box Protein O1

G6Pase	Glucose-6-Phosphate
GDR	Glucose Disposal Rate
GINF	Glucose Infusion Rate
GTT	Glucose Tolerance Test
H&E	Hematoxylin and Eosin
HGO	Hepatic Glucose Output
HGPbasal	Basal Hepatic Glucose Production
IGT	Impaired Glucose Tolerance
IKK	IκB kinase
IL-1β	Interleukin-1Beta
IL-6	Interleukin-6
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
ITT	Insulin Tolerance Test
JNK1	c-Jun N-Terminal Kinase-1
LACC1KO	Liver-Specific ACC1 Knockout
LKB1	Liver Kinase B1
MCD	Malonyl CoA Decarboxylase
MetS	Metabolic Syndrome
NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
NEFA	Non-Esterfied Free Fatty Acids

PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing 1% Tween-20
PEPCK	Phosphoenolpyruvate Carboxykinase
PGC-1α	Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PI3K	Phosphatidylinositol-3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
RER	Respiratory Exchange Ratio
SEM	Standard Error Mean
Ser	Serine
T2D	Type 2 Diabetes
TA	Tibialis Anterior
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
TNF-α	Tumour Necrosis Factor Alpha
VLDL	Very Low Density Lipoprotein
VCO2	Volume of Carbon Dioxide Production
VO2	Volume of Oxygen Consumption
WT	Wild-type

1.0 INTRODUCTION

1.1 General Introduction

Modern society is experiencing an obesity epidemic. Over 400 million people in the world are obese (BMI>30) and 1.6 billion adults are overweight (BMI> 25). The excessive accumulation of fat in obese individuals leads to an array of metabolic complications and risk factors for cardiovascular disease and type 2 diabetes (T2D) categorized as the Metabolic Syndrome (MetS). Risk factors include abdominal obesity, dyslipidemia, glucose intolerance, and hypertension (Ritchie and Connell 2007). Interestingly, although abdominal fat accumulation is a risk factor for the development of insulin resistance, individuals with MetS are not necessarily obese (Kotronen and Yki-Jarvinen 2008). Kotronen and Yki-Jarvinam (2008) suggest that a fatty liver is the primary driver of insulin resistance, independent of obesity; and both obese and lean individuals may present with nonalcoholic fatty liver disease (NAFLD). Worldwide, the clinicalprevalence of NAFLD is between 6-14% (Clark 2006); however, Canadians report a prevalence rate closer to 25% (Palasciano, Moschetta et al. 2007). Ferreira et al. (2010) recently revealed significant co-morbidity between type 2 diabetes (T2D) and NAFLD but the molecular mechanism(s) underlying the concurrent manifestation of these disorders remain unknown. Two prevailing hypotheses exist. The first is that lipid accumulation in hepatocytes leads to hepatic insulin resistance and subsequent progression to T2D (Marchesini, Brizi et al. 1999; Browning and Horton 2004; Biddinger, Hernandez-Ono et al. 2008; Sachithanandan, Fam et al. 2010), while the second theory suggests that liver insulin resistance and T2D leads to NAFLD (Sanyal,

Campbell-Sargent et al. 2001; Buettner, Ottinger et al. 2004; Bugianesi, Gentilcore et al. 2005; Lonardo, Lombardini et al. 2006; Monetti, Levin et al. 2007; Cusi 2009; Gastaldelli, Harrison et al. 2009; Szendroedi, Chmelik et al. 2009). The goal of this project is to understand the molecular underpinnings of the relationship between T2D and NAFLD, which should aid in the development of therapeutic and preventative strategies for the pathogenesis of NAFLD and insulin resistance.

1.2 Insulin Signaling in the Liver

Insulin maintains euglycemia by promoting adipose, muscle, and liver glucose uptake (Klover and Mooney 2004). High glucose levels increase blood insulin, which in turn inhibits catabolic pathways for lipid (lipolysis), protein (breakdown), and carbohydrate (glycogenolysis) storage within cells (Saltiel and Kahn 2001). It is important to distinguish the role of insulin signaling in the liver from that of the muscle and adipose tissue: muscle and adipose tissue glucose uptake occurs via insulin-stimulated glucose transporters, while liver glucose uptake occurs via passive glucose transport. The primary role of insulin in the liver is to promote glycogen and fatty acid synthesis, and to uniquely control gluconeogenesis, which is the production of glucose from non-carbohydrate substances like lactate or amino acids (Pilkis and Granner 1992; Klover and Mooney 2004). Insulin regulation of glucose metabolism in the liver occurs in both the fasted and fed state. During fasting, when blood glucose levels are low (hepatocyte insulin signaling is reduced and glucagon is increased), the liver maintains blood glucose levels through glycongenolysis and gluconeogenesis. Glucagon binding to

hepatocytes increases glycogenolysis via activation of protein kinase A (PKA) and subsequently, PKA phosphorylation of glycogen phosphorylase kinase and glycogen phosphorylase. After depletion of glycogen stores, transcriptional regulation of hepatocyte gluconeogenesis begins (Klover and Mooney 2004). For instance, transcription factor cyclic AMP-inducible factor CREB (cAMP response element binding protein) interacts with CREB regulated transcription coactivator 2 (CRTC2) in response to a negative change in energy balance (Herzig, Long et al. 2001). Hormonal regulation of CRTC2 during fasting (elevated levels of glucagon) leads to CRTC2 dephosphorylation and subsequent translocation to the nucleus to upregulate CREB and other gluconeogenic enzymes (Koo, Flechner et al. 2005; Liu, Dentin et al. 2008). Further, CREB induces coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression (Herzig, Long et al. 2001), which will then interact with forkhead box protein O1 (FOXO1) to upregulate the transcription of gluconeogenic enzymes, like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Puigserver, Rhee et al. 2003), resulting in an increase in fasting blood glucose.

In the fed state, when blood glucose and insulin levels are elevated and glucagon low; gluconeogenesis and glycogenolysis are inhibited and glycogen synthesis is increased. This occurs because insulin binding to the α subunit of its receptor leads to tyrosine phosphorylation of the β subunit and subsequent requirement of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), with IRS-2 being the more dominant substrate in the liver (Michael, Kulkarni et al. 2000; Klover and Mooney 2004). IRS-2 then

phosphorylates and activates phosphatidylinositol-3 kinase (PI3K) via its p85 subunit to instigate activation and translocation of pyruvate dehydrogenase kinase (PDK)-1 and Akt to the plasma membrane. Phosphorylation and activation of Akt (Yang, An et al. 2003; Klover and Mooney 2004) increases glycogen synthesis by accelerating the activity of glycogen synthase (Lin, Yang et al. 2000; Yang, An et al. 2003; Klover and Mooney 2004). Akt also phosphorylates FOXO1 and inhibits binding of coactivator PGC-1α resulting in the down-regulation of gluconeogenic enzymes, like PEPCK and G6Pase (Yang, An et al. 2003; Klover and Mooney 2004). In summary, hepatocytes depend on intact insulin signaling during fed and fasting states to enable proper regulation of hepatic glucose output and circulating blood glucose.

1.3 Insulin Resistance

Insulin resistance is defined as a precursor to T2D, impaired glucose tolerance (IGT), and cardiovascular disease (CVD) (Reaven 2005). It is commonly associated with obesity as approximately 90% of individuals with type 2 diabetes are, in fact, obese. T2D is the most common manifestation of the Metabolic Syndrome in obese individuals, accounting for 85% of cases (WHO).

In insulin resistance, the body does not respond to increases in insulin after a meal and is thus unable to inhibit hepatic glucose production and bring down blood glucose levels to normal, leading to long-term hyperglycemia (high blood glucose levels) and hyperinsulinemia (high blood insulin levels) (Saltiel and Kahn 2001; Cheng, Dube et al. 2002). Despite extensive research, the exact cause(s) of insulin resistance are not fully

understood, but many believe lipids play a central role. Insulin resistant individuals commonly develop high blood free fatty acid concentrations and advanced accumulation of lipids in a number of body tissues including the liver (Saltiel and Kahn 2001). The regulatory protein, c-Jun N-terminal kinase-1 (JNK1) is one of many stress-activated kinases that are upregulated in obesity (Hirosumi, Tuncman et al. 2002; Tuncman, Hirosumi et al. 2006). Hepatic JNK1 knockout mice are glucose intolerant, insulin resistant, and show fat accumulation in hepatocytes (Sabio, Cavanagh-Kyros et al. 2009). Other proteins upregulated by lipids that also inhibit insulin signaling include IκB kinase (IKK), protein kinase C (PKC) and protein phosphatase 2A (PP2A) (Angulo 2002; Clouston and Powell 2004). Given the essential role the liver plays in regulating glucose homeostasis, the focus of this thesis is to understand the mechanisms regulating insulin sensitivity in this organ.

1.4 Nonalcoholic Fatty Liver Disease (NAFLD)

Referred to as the liver presentation of metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) is typified by a build-up of triacylglycerols in hepatocytes and is often linked to insulin resistance (Seppala-Lindroos, Vehkavaara et al. 2002; Kim, Lee et al. 2004). NAFLD is the most common form of liver dysfunction in the United States and it, along with obesity, type 2 diabetes, and metabolic syndrome, are widespread and increasingly prevalent (Bellentani and Marino 2009). As many as 30-40% of obese individuals have NAFLD (McCulloaugh 2006) that is commonly detected by high levels of serum aspartate transaminase (AST) and alanine transaminase (ALT) enzymes in the

circulation (Bjornsson and Angulo 2007). Because of the varying extent of lipid accumulation in the liver (Bjornsson and Angulo 2007), liver abnormalities range from steatosis, which is the least serious, to nonalcoholic steatohepatitis (NASH) and fibrosis or cirrhosis, which is most serious (Brunt 2001). The stages of progression appear based on the duration of NAFLD. Given that fatty liver causes liver dysfunction, research is focused on better understanding the molecular mechanisms involved in NAFLD as currently there are no effective treatments for this very prevalent disease (Malaguarnera, Di Rosa et al. 2009).

Some studies suggest that insulin resistance leads to the complications of NAFLD (Sanyal, Campbell-Sargent et al. 2001; Buettner, Ottinger et al. 2004; Bugianesi, Gentilcore et al. 2005; Lonardo, Lombardini et al. 2006; Monetti, Levin et al. 2007; Minehira, Young et al. 2008; Cusi 2009; Gastaldelli, Harrison et al. 2009; Szendroedi, Chmelik et al. 2009), while others suggest that fat accumulation and liver complications contribute to the development of insulin resistance (den Boer, Voshol et al. 2004; Savage, Petersen et al. 2007; Anderson and Borlak 2008; Biddinger, Hernandez-Ono et al. 2008; Sachithanandan, Fam et al. 2010). Kraegen and colleagues assessed high-fat diet induced insulin resistance in liver tissue of adult male Wistar rats. Hepatic glucose output suppression by insulin was significantly reduced within just three days of high-fat feeding, suggesting a role of lipids in the development of hepatic insulin resistance (Kraegen, Clark et al. 1991). Similarly, both lipid-infusion and high-fat feeding resulted in hepatic insulin resistance, as assessed by hyperinsulinemic-euglycemic clamp in rats (Li, Yang et al. 2006). Additionally, weight loss in an adult population has been

associated with improved NAFLD and insulin resistance (Petersen, Dufour et al. 2005). Overall, these findings indicate that liver lipids are likely interfering with insulin signaling to lead to hepatic insulin resistance, but genetic evidence supporting this hypothesis does not currently exist.

Although there is data to support the theory that a fatty liver leads to insulin resistance, other studies suggest the opposite may be true. For instance, two divergent mouse models both with hepatic steatosis did not develop hepatic insulin resistance (Monetti, Levin et al. 2007; Minehira, Young et al. 2008). A number of human studies also support the finding that a fatty liver does not necessarily induce insulin resistance (Bevilacqua, Bonadonna et al. 1987; Brunt 2001; Sanyal, Campbell-Sargent et al. 2001; Bugianesi, Gentilcore et al. 2005; Gastaldelli, Harrison et al. 2009; Szendroedi, Chmelik et al. 2009). Furthermore, deletion of IRS-1 (Araki, Lipes et al. 1994; Tamemoto, Kadowaki et al. 1994) and IRS-2 (Kubota, Tobe et al. 2000) leads to insulin resistance independent of obesity or fatty liver. These findings indicate that lipid accumulation does not necessarily lead to hepatic insulin resistance and that insulin resistance can occur independently from liver lipid accumulation.

1.5 Acetyl CoA Carboxylase (ACC)

Acetyl coA carboxylase (ACC) is an enzyme containing a biotin carboxyl carrier protein, a biotin carboxylase domain and a carboxyl transferase domain (Hardie 1989). There are two forms of ACC: ACC1 and ACC2. ACC1 is more highly expressed in the liver and in lipogenic tissues like adipose tissue, while ACC2 is more common in

oxidative tissues like heart and skeletal muscle (Bradbury 2006). Both enzymes catalyze the decarboxylation of acetyl coA to malonyl coA, classified as the first committed step of fatty acid biosynthesis (Saha, Laybutt et al. 1999). Malonyl coA subunits are synthesized to make fatty acids and further converted to triacylglycerol via a complex of fatty acid synthases (Saha, Laybutt et al. 1999). ACC1 and ACC2 differ slightly in structure; they have the same enzymatic domain, but ACC2 has an extra NH_2-terminal that allows for its unique attachment to the mitochondrial membrane (Abu-Elheiga, Brinkley et al. 2000). Studies by Wakil et al. (1983) have suggested that given ACC2's cellular localization at the mitochondrial membrane, this enzyme is the primary regulator of fatty acid oxidation. In view of this finding, the malonyl coA pool generated by ACC2 would be inhibiting carnitine palmitoyl-transferase-1 (CPT-1), the rate-limiting enzyme controlling fatty acyl-CoA entry into the mitochondria. However, as will be described in the section below regarding animal models of ACC, this theory is currently an area of great controversy.

1.6 Characterization of ACC in Animal Models

The first genetic model examining the importance of ACC enzymes *in vivo* was generated by Wakil and colleagues who found that compared to wild-type littermates, ACC2 knockout mice ($ACC2^{-/-}$) had continuous fatty acid oxidation, less adipose tissue storage, reduced liver lipid and triacylglycerol content, and were more insulin sensitive despite a 30% increase in food intake (Abu-Elheiga, Almarza-Ortega et al. 1997; Abu-Elheiga, Matzuk et al. 2001). The same effect was seen in mice fed a high-fat diet and a

high-carbohydrate diet (Abu-Elheiga, Matzuk et al. 2001; Choi, Savage et al. 2007). Importantly, tissue-specific quantities of malonyl coA in $ACC2^{-/-}$ mice were not different in the liver but were much lower in muscle and heart compared to wild-types. This suggests that ACC2 is the predominant source of malonyl coA in muscle but not liver, which is probably due to the lower prevalence of the ACC1 isoform in skeletal muscle (Abu-Elheiga, Matzuk et al. 2001). The authors postulated that reduced steatosis in the livers of $ACC2^{-/-}$ mice is likely the result of increased skeletal muscle fatty acid oxidation. The group, therefore, proposes that if the same is true in humans, inhibiting ACC2 may help people lose weight without changing food intake levels (Abu-Elheiga, Matzuk et al. 2001).

However, since these exciting initial findings, Olson, Pulinilkunnil et al. (2010) recently reported that genetic deletion of ACC2 from skeletal muscle had little effect on body weight, glucose metabolism, fat mass, or food intake, and no difference in muscle malonyl coA and fatty acid oxidation levels, suggesting that ACC1 production of malonyl coA is probably compensating for the lack of malonyl coA from the ACC2 malonyl coA pool. Similarly, Hoehn, Turner et al. (2010) further demonstrated that increased fatty acid oxidation through either AMPK or ACC2 inhibition is insufficient to induce weight loss unless it is coupled with increased energy expenditure. It is unclear why there is such discrepancy between these two studies compared to those previously reported by Wakil and colleagues but they do highlight that further evidence supporting a role for ACC2 in metabolism is required. Therefore, at present, knockout mouse models have not been able

to give a clear consensus about the role of ACC2 in insulin metabolism and lipid accumulation

Although ACC1 whole-body knockout mice are embryonic lethal (Abu-Elheiga, Oh et al. 2003), Mao et al. (2006) developed a liver-specific ACC1 knockout (LACC1KO) mouse model that had no health problems when eating a normal diet, but showed lower liver ACC activity, malonyl coA levels, and lipid accumulation (Mao, DeMayo et al. 2006). LACC1KO mice also had reduced hepatic synthesis and storage of triacylglycerols when fed a fat-free diet, despite elevated lipogenic gene expression (Mao, DeMayo et al. 2006). This indicates that liver ACC1, as expected, is primarily involved in the synthesis or anabolic side of fat metabolism. Because there was no difference in blood glucose and fasting ketone bodies, the authors concluded that reducing cytosolic malonyl coA and fatty acid synthesis does not affect glucose homeostasis (Mao, DeMayo et al. 2006). Additional tests of glucose and insulin regulation would have helped strengthen their claim with a more complete analysis of glucose tolerance and insulin sensitivity. In contrast, Harada et al. (2007) also developed liver-specific ACC1 knock-out mice but, in contrast to Mao et al. (2006), found there was no difference in liver lipogenesis or malonyl coA levels. This led to the conclusion that malonyl coA accumulation from the ACC2 pool in the liver was sufficient for the regulation of fatty acid synthesis and oxidation (Harada, Oda et al. 2007). These conflicting sets of data suggest that further research into the role of ACC in regulating liver lipid metabolism is required.

Savage et al. (2006) were the first to consider the role of both ACC1 and ACC2 in the liver by inhibiting each with antisense oligonucleotides in rat hepatocytes. They found

that inhibiting both ACC1 and ACC2 individually increased fatty acid oxidation, but that inhibition of the two enzymes together resulted in the greatest increase in fatty acid oxidation. ACC1 suppression alone also resulted in inhibition of lipogenesis in hepatocytes (Savage, Choi et al. 2006). Savage and colleagues, additionally, considered the effects of ACC1 and ACC2 suppression *in vivo* in rats with diet-induced NAFLD and insulin resistance. They found that inhibition of both ACC1 and ACC2 was needed to significantly reduce hepatic malonyl coA and hepatic lipids, and to improve hepatic insulin signaling. Savage and colleagues reported that reducing ACC1 and ACC2 expression effectively increased the rate of fatty acid oxidation when rats are in a fed state. This study gives great evidence to support the role of ACC in NAFLD and insulin resistance even if the authors used an incomplete knock-out of ACC1 and ACC2 by intraperitoneally injecting antisense oligonucleotide inhibitors, which did not result in a complete reduction of ACC enzyme levels (~20% was still present). Also, while the data involving genetic deletion of ACC1 and ACC2 isoforms has revealed valuable information in regards to the potential role of these enzymes in controlling metabolism, genetic deletion studies have several caveats in that there is a strong likelihood for the adaptation of compensation mechanisms, making it difficult to develop a true picture of the enzyme role. In addition, the genetic deletion of proteins is also very unnatural as under most physiological conditions, ACC1 and ACC2 expression are not altered dramatically; thus, making it difficult to assess the importance of these enzymes in metabolic homeostasis.

1.7 AMP-Activated Protein Kinase (AMPK)

5` adenosine monophosphate-activated protein kinase (AMPK) is an important regulator of both lipid and glucose metabolism in the liver and is thus considered an important therapeutic target for the treatment of NAFLD and insulin resistance (Hardie 2008; Steinberg and Kemp 2009). It exists as an αβγ heterotrimer structure consisting of an α catalytic subunit and two regulatory subunits (β and γ). In the liver, both α1 and α2 catalytic subunits are expressed (Viollet, Guigas et al. 2009) and expression of these isoforms is almost completely dependent on the β1 subunit (Dzamko, van Denderen et al. 2010). Activation of AMPK in the liver is mediated via phosphorylation of the Thr-172 residue on its α catalytic subunit, by the upstream protein kinase, liver kinase B1 (LKB1) (Shaw, Lamia et al. 2005). LKB1 phosphorylates AMPK in response to low ATP levels that are characteristically evident during fasting or exercise, and blocks AMPK dephosphorylation by protein phosphatase 2C (Sanders, Grondin et al. 2007; Riek, Scholz et al. 2008). Once activated, AMPK phosphorylates a number of proteins at their serine/threonine residues in multiple metabolic pathways that are critical for controlling glucose, protein and lipid metabolism (Steinberg and Kemp 2009). The activation of AMPK in the liver is believed to be required for the insulin sensitizing effects of metformin and adiponectin; however, due to the large of number of possible substrates, the mechanisms by which AMPK regulates insulin sensitivity and lipid metabolism in the liver are not currently known.

1.8 Regulation of ACC Activity by Post-Translational Mediations

ACC activity, like many metabolic enzymes, is regulated by allosteric and covalent (by phosphorylation) mechanisms. Allosteric regulation of ACC is mediated by citrate, which causes ACC to polymerize, increasing its enzymatic activity (Berglund, Kang et al. 2010). In contrast, covalent regulation of ACC inactivates its enzymatic activity. ACC has a number of phosphorylation sites; some of which are silent and others, which are essential for its inactivation (Halestrap and Denton 1974). ACC was first shown to be phosphorylated by AMPK at Ser79, Ser1200, Ser1215 and by PKA at Ser77 and Ser1200 (Halestrap and Denton 1974; Hagopian and Munday 1997). Subsequently, Davies and colleagues reported that only Ser77 and Ser79 were residues that would result in covalent ACC inhibition by phosphorylation (Davies, Sim et al. 1990). However, upon further investigation, it was found that a mutation in Ser79 was sufficient to abolish the ability of AMPK to phosphorylate and inactivate ACC (Kim, Yoon et al. 1998). Further studies by Ha and colleagues compared AMPK and PKA phosphorylation at Ser77, Ser79, and Ser1200 by mutating each residue individually and showed that a Ser79 mutation was the only mutation that prevented AMPK phosphorylation of ACC (Ha, Daniel et al. 1994). Ser79 is found on the ACC1 isoform, and is conserved on the ACC2 isoform as Ser221 (Abu-Elheiga, Almarza-Ortega et al. 1997). Although the exact importance of PKA is not known, it is generally accepted that AMPK inactivates ACC1 and ACC2 by phosphorylation of the Ser79 residue and Ser221 residue, respectively (Munday, Campbell et al. 1988; Davies, Carling et al. 1992; Hardie, Scott et al. 2003). Surprisingly, despite this *in vitro* evidence from over 20 years ago, direct evidence

linking ACC1 Ser79 and ACC2 Ser221 phosphorylation with the control of fatty acid metabolism *in vivo* does not currently exist.

Considering the important relationship between fatty acid synthesis and fatty acid oxidation in regulating the partitioning of fatty acids, and ultimately NAFLD, we believe it is essential to further study the role of ACC1 and ACC2. In particular, investigating ACC regulation, specifically via phosphorylation, and subsequent effects on fatty acid synthesis and oxidation is an important research focus because this is the mechanism that is believed to primarily regulate enzyme activity. To do this, a model that encompasses both isoforms of ACC would be particularly useful given the overlap in function and potential compensatory role of the two ACC enzyme variants. Looking at a mouse model with knock-in mutations that might prevent phosphorylation of the two crucial phosphorylation sites on ACC1 (Ser79) and ACC2 (Ser221) would allow for direct assessment of the physiological mechanism and importance of AMPK signaling of ACC. It could, therefore, help shed more light on the relationship between AMPK, ACC, NAFLD, and liver insulin sensitivity.

2.0 HYPOTHESIS, OBJECTIVES, AND RATIONALE

2.1 Hypothesis

The Steinberg laboratory has recently generated ACC1 Ser79 to Ala and ACC2 Ser221 to Ala double knock-in (ACC DKI) mice. We hypothesize that ACC DKI mice will have restricted phosphorylation at these critical sites resulting in elevated ACC activity and higher levels of malonyl coA that will lead to lower fatty acid oxidation and increased fatty acid synthesis compared to the wild-type (WT) controls. We hypothesize that this will lead to lipid accumulation in the liver and the development of insulin resistance.

2.1 Objectives

2.1.1 Investigate the effects of knock-in mutations at ACC1 Ser79 and ACC2 Ser221 on liver lipid metabolism in mice.

2.1.2 Investigate the effects of knock-in mutations at ACC1 Ser79 and ACC2 Ser221 on insulin and glucose metabolism in mice.

2.2 Rationale

ACC1, is the most predominant ACC isoform found in the liver but ACC2 is also present. Unpublished data from the Steinberg laboratory has found that ACC1 knock-in (ACC1 KI) mice have modest increases in liver lipid levels and develop a very mild form of liver insulin resistance. There is no sign of insulin resistance in any other tissues. ACC2 knock-in (ACC2 KI) mice have normal liver lipid levels and insulin sensitivity.

However, they develop mild insulin resistance in skeletal muscle without showing any adverse effect on whole-body glucose homeostasis. These findings are in agreement with the recent studies in ACC1 liver-specific knock-out (Harada, Oda et al. 2007) and ACC2 whole-body knockout mice (Hoehn, Turner et al. 2010; Olson, Pulinilkunnil et al. 2010), suggesting that there is functional redundancy between the two ACC isoforms. Using the unique ACC DKI mouse model, we intend to investigate the relationship between ACC1 and ACC2 phosphorylation, NAFLD and insulin resistance. The goal of this project is to measure the effects of a knock-in mutation at two phosphorylation site residues of ACC on NAFLD, glucose tolerance, and hepatic insulin sensitivity in C57Bl/6J mice.

3.0 MATERIALS AND METHODS

3.1 Study Design

All animal procedures have been approved by the McMaster University Research Ethics Board (AUP #09-05-15). Male mice were housed in the Central Animal Facility at McMaster University in an SPF-level room, which had a 12-hour light/dark cycle. Mice were fed a normal chow diet (17% kcal fat, Teklad 22/5, Harlan Laboratories; Indianapolis, IN) with ad libitum access to food and water. Beginning at 6-weeks of age, body mass was measured weekly until the end of experiments. Energy expenditure and feeding behaviour were monitored. Figure 1 depicts the tests performed during the animals' lifespans until 18-weeks of age when mice were sacrificed and tissues were collected for subsequent analyses. The details of these methods are described in detail below.

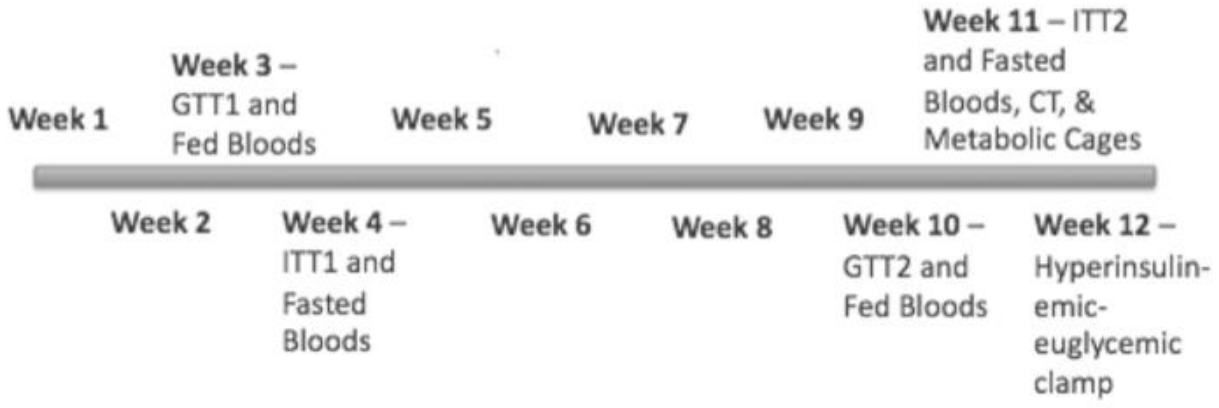


Figure 1. Timeline of Methods Conducted in Mice

3.2 CT Scans

Computed Tomography (CT) scans were conducted at 17 weeks of age at the Animal Imaging Facility at McMaster University. An inhalant anaesthetic, isoflourane (Pharmaceutical Partners of Canada Inc.; Richmond Hill, ON), was used to stabilize and prevent movement of the mouse during the scan. Acquisitions were performed on a GammaMedica Ideas X-SPECT system (North Ridge California). CT acquisitions comprised 1024 projections (1184 x 1120 pixels in size) over 360o with a 70 Kvp and 205 mA cone beam CT system. In order to reconstruct the image, a modified Feldkamp algorithm was used (EXXIM software, Pleasanton, CA), which produced 512x512x512 voxel images (80 micron voxel). Analysis of the CT scan images was conducted using Amira Visage 5.2.2 (San Diego, CA) Imaging Software to measure total body fat composition based on density of the tissues in the voxel image.

3.3 Animal Monitoring in Metabolic Cages

The Oxymax Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) was used to monitor mice for 72-hours via indirect calorimetry. Eight mice per group were placed in metabolic cages at 17 weeks of age. Once the animal monitoring system was equilibrated and calibrated, mice were placed in their individual system to be acclimatized to the CLAMS cages for approximately 12-hours before the experiment began. The CLAMS system measured and monitored 3-day food and water intake, ambient activity, and metabolic gas exchange.

Food intake was averaged over 3-days and was measured by change in weight according to a scale located in the center to the system. Spontaneous activity was

measured using an *X* and *Z* axis with infrared beams set to count beam breaks. Volume of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were also measured every 20 minutes and averaged to assess daily VO_2 and VCO_2. The respiratory exchange ratio (RER) was calculated with the following formula:

$$\text{RER} = \frac{VCO_2}{VCO_2}$$

An RER of 1.0 would indicate 100% oxidation of carbohydrates and an RER of 0.7 would indicate 100% oxidation of fatty acids. Furthermore, total energy expenditure is calculated as the sum of the carbohydrate and fat oxidation calculations. Finally, substrate oxidation (carbohydrate and fat) was calculated using the VO_2 and VCO_2 (mL/hr) as follows:

$$\text{Carbohydrate Oxidation (kcal/hr)} = \frac{((4.585 \times VCO_2) - (3.226 \times VO_2) \times 4}{1000}$$

$$\text{Fat oxidation (kcal/hr)} = \frac{((1.695 \times VO_2) - (1.701 \times VCO_2) * 9}{1000}$$

3.4 Fasting Blood Glucose

Fasting blood glucose was measured when mice were 17-weeks old. Mice were anaesthetized using inhalant isoflourane (Pharmaceutical Partners of Canada Inc.; Richmond Hill, ON). Fasting bloods were collected from the facial vein after a 12-hour overnight fast and fed bloods were obtained following a night of ad libitum access to food. Fasting blood glucose concentrations were recorded using the Accu-Chek Aviva blood glucometer (Roche Diagnostics). Serum was separated from whole blood by

spinning at 7,500 *rpm* for 7 min at 4°C and used to measure insulin with the Rat/Mouse Insulin ELISA kit from Millipore (St. Charles, MO).

3.5 Glucose and Insulin Intraperitoneal Tolerance Tests

We evaluated glucose and insulin tolerance in adult WT and ACC DKI animals using previously validated methods (Watt, Dzamko et al. 2006). Glucose tolerance was measured at 16-weeks of age and insulin tolerance was measured at 17-weeks of age. In the glucose and insulin intraperitoneal tolerance tests, mice were fasted for 6-hours and given either 1.0 gram glucose/kilogram of body mass or 0.7 Units insulin/kilogram of body mass in saline, depending on the test. Blood glucose was measured at 20, 40, 60, 90, and 120 minutes using the Accu-Chek Aviva blood glucometer (Roche Diagnostics). The intraperitoneal tolerance test data was presented with average group blood glucose levels at each time point and area under the curve was calculated and compared between genotypes.

3.6 Serum Biochemistry

Serum was separated from whole blood by spinning at 7,500 *rpm* for 7 min at 4 °C and used to measure non-esterfied free fatty acids (NEFA) and liver function markers alanine transaminase (ALT) and aspartate transaminase (AST) (Watt, Dzamko et al. 2006). NEFAs were measured using the Wako NEFA-HR(2) Microtiter Procedure assay, serum ALT using the MaxDiscovery Alanine Transaminase (ALT) Color Endpoint Assay Kit (Biooscientific, Austin, TX) (expressed in International Units/Liter) and serum AST

using the MaxDiscovery Aspartate Transaminase (AST) Color Endpoint Assay Kit (Biooscientific, Austin, TX). The ALT/AST (DeRitis ratio) is often used in humans to discern between different pathologies (Williams and Hoofnagle 1988). For example, the ALT/AST ratio is elevated in morbidly obese individuals with fatty liver (Adler and Schaffner 1979; Nanji, French et al. 1986).

3.7 Hyperinsulinemic-Euglycaemic Clamp

The hyperinsulinemic-euglycaemic clamp was used so we could assess tissue specific insulin sensitivity including endogenous hepatic glucose production (Kim 2009). Mice underwent surgery to cannulate of the jugular vein in order to enable direct intravenous infusion of glucose during the clamp, which was performed by a post-doctoral fellow, Dr. Morgan Fullerton. On the day of the clamp, mice were fasted for 6-hours before beginning the clamp and placed in restrainers to which they were previously acclimatized. First, to measure basal glucose disposal, D-[^{3}H]-Glucose was continuously infused (7.5 μCi/hr) for a 1-hour time period into the catheter entering the jugular vein. Blood glucose from the tail vein was measured pre- and post-basal glucose infusion. An aliquot of blood was also collected from the tail vein for basal blood measurements at the end of the basal glucose infusion and calculated with the equation below:

$$\text{Basal hepatic glucose production } (HGP_{basal}) = \frac{\text{basal } [^3H] \text{ glucose infusion rate}}{\text{specific activity of glucose}}$$

Next, a constant rate insulin infusion (10mU/kg/min) with 7.5μCi/hr D-[^{3}H]glucose was started, and blood glucose was measured at 10-minute intervals. Variable rates of 50% dextrose solution were infused based on the blood glucose measures until the desired

stable blood glucose was titrated and reached: 5.5-7.5 mmol/L for at least 30 minutes. At this point, the mouse was deemed clamped and has achieved euglycemia (Jamieson, Chong et al. 2005; Steinberg, Macaulay et al. 2006). Blood was then collected from the tail vein. Blood samples were left on ice for 30 minutes to allow for lysis and was deprotonized with $Ba(OH)_2$ and $ZnSO_4$. Glucose infusion rate, glucose disposal rate, hepatic glucose output and percent suppression of glucose were calculated:

$$\text{Glucose infusion rate (GINF)} = \text{Clamp rate} * \frac{454}{\text{weight (mg)}}$$

$$\text{Glucose disposal rate (GDR)} = \frac{\text{clamp } [^3H] \text{ glucose infusate specific activity}}{\text{glucose in plasma specific activity}}$$

$$\text{Hepatic glucose output (HGO)} = \text{GINF} - \text{GDR}$$

$$\% \text{ Suppression} = \frac{HGP_{basal} - HGO}{HGP_{basal}}$$

3.8 Tissue-Specific 2-DG Uptake

Following the hyperinsulinemic-euglycemic clamp, a 100μL bolus of 2-deoxy-D-[^{14}C]glucose (2-[^{14}C]-DG) was infused into the catheter, and blood was collected at 10, 20, and 30 minutes from the tail vein. Blood was left on ice for 30 minutes to clot and samples were lysed and deprotonized with $Ba(OH)_2$ and $ZnSO_4$. Upon uptake into a tissue, 2-[^{14}C]-DG is phosphorylated to become 2-[^{14}C]-DG-P. When phosphorylated, the glucose is unable to exit the tissue because it cannot be metabolized. This is, therefore, a good means of measuring insulin-stimulated glucose uptake. Mice were then sacrificed by a ketamine-xylazine injection into the catheter and tissues collected. Tissues weighed and

removed included the liver, heart, quadriceps, tibialis anterior (TA), gastrocnemius, brown adipose tissue, and white adipose tissue and snap frozen in liquid nitrogen. The tissues were stored at minus 80 degrees Celcius for later analysis (Steinberg et al., 2006b). Glucose uptake into liver (30-50 mg), tibialis anterior (30-50 mg), gastrocnemius (30-50 mg), and epididymal adipose tissue (about 100 mg) were measured. First, the tissue was homogenized in 500µL (300µL for fat samples) cell-lysis buffer (0.05M HEPES, 0.15M NaCl, 0.1M NaF, 0.01M $Na_4O_7P_2$, 0.0005M EDTA, 0.25M Sucrose, 1mM DTT, 1% Triton-X, 1mM $NaVO_4$, 1% Protease Inhibitor Cocktail) with Precellys® 24 Homogenizer (Bertin Technologies; Paris, France). The heterogenous lysate was then rotated for 30 minutes at minus 4 °C, centrifuged for 20 minutes at 13000 rpm at 4 °C, and the supernatant was collected. To measure 2-[^{14}C]-DG, each sample required two scintillation tubes: The first tube measured the total glucose count in 50µL of lysate and 4mL of Ultima Gold. The second was used to measure 2-[^{14}C]-DG-P by treating 1000uL of supernatant retrieved from a precipitation reaction with $BaOH_2$, H_2O, and $ZnSO_4$ and counting the sample in 4mL of Ultima Gold. Finally, glucose uptake was calculated based on 2-[^{14}C]-DG count and the area under the curve of the [^{14}C] activity 2-[^{14}C]-DG blood counts.

3.9 Western Blotting

Liver and TA proteins were extracted from 30-50 mg of tissue samples. The tissue was first homogenized in 500µL cell-lysis buffer (0.05M HEPES, 0.15M NaCl, 0.1M NaF, 0.01M $Na_4O_7P_2$, 0.0005M EDTA, 0.25M Sucrose, 1mM DTT, 1% Triton-X, 1mM

$NaVO_4$, 1% Protease Inhibitor Cocktail) with Precellys® 24 Homogenizer (Bertin Technologies; Paris, France). The heterogenous lysate was then rotated for 30 minutes at minus 4 °C, centrifuged for 20 minutes at 13000 rpm at 4 °C, and supernatant collected. The liver and TA proteins were then analyzed by Western blotting to quantify the expression of phosphorylated and total Akt (pAkt, totalAkt) (60 kDa) (antibodies were purchased from Cell Signaling). 30 μg of protein was separated using gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes (Biorad, PVDF Membrane). After one-hour of blocking at room temperature in phosphate buffered saline containing 1% Tween-20 (PBST) and 5% milk powder, the membrane was washed thoroughly with 3-quick washes to remove the contents of the blocking buffer. The protein of interest was then probed for overnight with antibody dilutions in PBST (pAkt – 1:1000; totalAkt – 1:1000). After overnight incubation, the membrane was washed three times 5-minutes with PBST and then incubated for 1-hour with a horseradish peroxidase conjugated secondary antibody (1:10000 dilution of the antibody purchased from Cell Signaling). The membranes were again washed three times 5-minutes with PBST, and chemiluminescent detection was used to visualize protein bands and a picture was taken. ImageJ, a free NIH imaging program (http://rsb.info.nih.gov/ij/index.html), was used to quantify bands of protein on the membranes. pAkt was expressed as a ratio to totalAkt.

3.10 RNA Analysis

RNA expression of the mitochondrial marker, CPT-1 was assessed using quantitative PCR. Briefly, liver samples (20-30 mg) were homogenized in 1mL TRIzol®

Reagent (Invitrogen) with a mechanical homogenizor. After the RNA was extracted, a nanophotometer (Implen Inc.; Westlake Village, CA) was used to measure the RNA concentration. 1μg of extracted RNA was reverse transcribed with SuperScript® III (Invitrogen) using the manufacturer instructions. Real-time quantitative RT-PCR was then performed using TaqMan® Gene Expression Assays (Applied Biosystems; Foster City, CA) and the Rotor-Gene 6000 (Crobett Research; Mortlake, Australia). CPT1 is involved in fatty acid metabolism and regulates fatty acyl entry into the mitochondria for oxidation. Two housekeeping genes, TATA binding protein and 36B4, were used to assure consistency of the results. The ddCT method (Livak and Schmittgen, 2001) was used to measure gene expression of CPT1 compared to the two housekeeping genes.

3.11 Histology

The initial manifestation of NAFLD is steatosis, referring to liver accumulation of triacylglycerols without any presentation of inflammation or damage. This can progress to liver NASH, which is comprised of steatosis and inflammation. Destruction of liver cells from inflammatory cytokines is known as hepatocellular necrosis, the final, most serious stage of NAFLD involving liver tissue fibrosis—cirrhosis (Marchesini, Brizi et al. 1999).

A small piece of liver was dissected and formalin-fixed in 10% buffered formalin. Paraffin-embedding and slicing of 5-μm thick sections were completed by trained technicians in the histopathological lab in the Department of Medicine at McMaster University. Sections were stained with hematoxylin and eosin (H&E) to assess the overall degree of steatosis compared between groups.

The degree of steatosis was graded based on the percent of hepatocytes with steatosis. Further, steatosis was graded on a 0-3 scale, with 3 graded as highest, according to the NASH scoring system in a minimum of 5 sections (Bondini, Kleiner et al. 2007). Specifically, 0 indicates no inflammation (<5%); 1, mild, few (5-33%); 2, moderate-marked (34-66%); 3, severe (>66%) (Brunt 2001).

3.12 Lipid Analysis

Liver tissue (30-50 mg) was homogenized in phosphate buffered saline (PBS) and lipids were extracted using the Bligh & Dyer lipid extraction protocol (Bligh and Dyer 1959). Lipids were resuspended in chloroform and run on a thin-layer chromatography (TLC) plate in order to separate triacylglycerols (TAGs). Bands of TAGs were scraped from the plate, treated with KOH in 95% EtOH. Finally, samples were analyzed using a glycerol assay (Sigma) to assess the level of TAGs in the liver samples. The amount of glycerol in μg was expressed as a ratio to the grams of total tissue.

3.13 Statistical Analysis

Data was analyzed using GraphPad Prism 4.0 (GraphPad Software Inc.; La Jolla, CA). ACC DKIs were compared to WTs using a two-tailed *t* test. Only body weights and glucose and insulin interperitoneal tolerance tests data for ACC DKI and WT mice was assessed by a two-way repeated measures ANOVA. A *P* value less than 0.05 ($p>0.05$) was considered significant. Data is presented as mean ± SEM (standard error mean).

4.0 RESULTS

4.1 Examination of the ACC DKI Mouse Model

4.1.1 Confirmation of Genotype

The genotype of the ACC DKI mice was confirmed by using a phosphorylation specific antibody that detects phosphorylation of ACC1 Ser79 and ACC2 Ser221. By utilizing a 5 % SDS-PAGE gel we were able to resolve the phosphorylation of ACC2 (~265 kDA) and ACC1 (250 kDA) and found that, consistent with mass spectrometry data obtained by Dr. Rohan Steele (a postdoctoral fellow from the Steinberg laboratory at St. Vincent's Institute, data not shown), both ACC1 Ser79 and ACC2 Ser221 phosphorylation were eliminated in the livers of ACC DKI mice (Figure 2). Thus, the ACC DKI mouse model appears to be an appropriate tool for evaluating the importance of ACC1 Ser79 and ACC2 Ser221 phosphorylation in regulating fatty acid metabolism and insulin signaling in the liver.

4.1.2 ACC Activity Assays

Liver ACC1 and ACC2 activity assays comparing the ACC DKI mouse model to WT controls were performed by Dr. Sandra Galic, a postdoctoral fellow in the Steinberg laboratory at the St. Vincent's Institute in Melbourne, Australia but are included in this thesis as they are essential for validating the use of the ACC DKI model. Livers were collected from WT and ACC DKI mice after an overnight fast, which increases the activity of AMPK and increases ACC phosphorylation (Davies, Carling et al. 1992; Witters and Kemp 1992). ACC1 and ACC2 enzyme activity was measured *in vitro* in

liver immunoprecipates using antibodies specific to each isoform that were generously provided to us by Grahame Hardie (Dundee, UK). ACC activity was measured according to a previously described protocol (Wojtaszewski, Nielsen et al. 2000). ACC1 activity was approximately 3-fold higher with and without citrate in livers from ACC DKI compared to WT mice (Figure 3A). Similar observations were also seen for ACC2 activity, although it should be noted that the activity of ACC2 is an order of magnitude lower than that observed for ACC1 in the liver (Figure 3B). These data indicate that the phosphorylation of ACC1 Ser79 and ACC2 Ser221 is essential for regulating enzyme activity with and without allosteric activation by citrate.

4.1.3 Malonyl CoA

Malonyl coA levels were measured by Dr. Thomas Pulinilkunnil in Professor Jason Dyck's laboratory at the University of Alberta but are included in this thesis as further validation of the ACC DKI mouse model using previously described liquid chromatography/tandem MS analysis (Savage, Choi et al. 2006). We found that, consistent with changes in liver ACC activity, liver malonyl coA levels were two times higher in ACC DKIs compared to WT mice (Figure 4). These findings demonstrate that ACC phosphorylation is a critical regulator of malonyl coA levels and that without it you get constitutive activation of ACC1 and ACC2, that in turn promotes the conversion of acetyl coA to malonyl coA.

4.1.4 Growth Curves and Adiposity

We examined body mass starting at 6 weeks of age and found that there was no genotype difference at any time point (Figure 5A). Consistent with similar body mass, there was no difference in the total adiposity between ACC DKIs and WTs when mice were 17 weeks of age (Figure 6A-C).

4.1.5 Animal Monitoring System Data

Oxygen consumption and calculated whole body energy expenditure (Figure 5B-C) were similar between WT and ACC DKI but surprisingly, ACC DKI mice consumed ~10% less food (Figure 5D). These data demonstrate that ACC phosphorylation is not critical for regulating whole-body energy expenditure but may play a role in regulating appetite.

Given that ACC DKI mice had constitutively active ACC1 and ACC2 and higher levels of malonyl coA, we anticipated that their whole-body rates of fatty acid oxidation would be suppressed. Surprisingly, we found that RER over time and estimated rates of substrate utilization were not different between genotypes (Figure 7A-B). These data suggest that other mechanisms besides the phosphorylation of ACC1 and ACC2 are important for regulating whole-body RER. The relative liver expression of CPT-1 tended to be higher in ACC DKIs compared to WTs ($p=0.054$) (Figure 7C).

4.2 ACC DKI Mice Present with Insulin Resistance

4.2.1 Fasted Blood Measures

Despite a similar body mass and degree of adiposity, we found that ACC DKIs had an ~20% increase in fasting blood glucose levels compared to WT controls (Figure 8A). This effect was even more dramatic when examining fasting insulin levels, which were more than 2-fold higher in ACC DKI mice compared to WTs (Figure 8B). In addition, serum NEFA levels also tended to be higher in ACC DKIs compared to WTs in the fed state (p=0.087) (Figure 8C). These findings indicate the development of whole-body insulin resistance in the ACC DKI mice.

4.2.2 Glucose and Insulin Intraperitoneal Tolerance Tests

Blood glucose levels were higher in ACC DKI mice compared to WTs at 0, 20, 40, 60, and 90 minutes during the GTT, with a significant difference at the 20-minutes time-point (Figure 9A). Overall, the ACC DKIs had significantly higher blood glucose levels during the GTT as shown by a 17% greater GTT area under the curve (AUC) in ACC DKIs compared to WTs (Figure 9B).

Blood glucose levels were higher in ACC DKI mice compared to WTs at 20, 40, and 60 minutes during the ITT (Figure 9C). Overall, the ACC DKIs had significantly higher blood glucose levels during the ITT as shown by a 21% higher ITT area under the curve (AUC) in ACC DKIs compared to WTs (Figure 9D). This, ultimately, suggests a defect in whole-body insulin sensitivity.

4.2.3 Liver Insulin Resistance

During hyperinsulinemic-euglycaemic clamps, the ACC DKIs had a 24% lower GINF rate compared to WTs (Figure 10A). The reduction in the GINF of ACC DKI mice was associated with an ~150% increase in hepatic glucose output (Figure 10B), due to an impaired ability of insulin to suppress hepatic glucose production (Figure 10C). Consistent with liver insulin resistance in DKI mice, the ratio of phosphorylated Akt to total Akt tended to be much lower compared to WTs (Figure 10D). These data indicate that ACC DKI mice have liver insulin resistance.

4.2.4 Glucose Disposal Rate and Tissue-Specific 2-Deoxyglucose Uptake

The ACC DKIs also had an ~18% lower GDR compared to WTs (Figure 11A), which suggests that ACC DKIs have difficulty taking up glucose into target tissues (skeletal muscle and adipose) in response to insulin. However, this impaired rate of glucose disposal did not appear to be due to skeletal muscle insulin resistance as 2-deoxyglucose uptake into mixed gastrocnemius or TA muscle (Figure 11B) and Akt phosphorylation (Figure 11C) were not different between genotypes. Instead, we found that 2-deoxyglucose uptake was reduced in the adipose tissue of ACC DKI mice compared to WTs (Figure 11B). Future studies examining the role of ACC in regulating adipose tissue insulin sensitivity and metabolism are warranted.

4.3 ACC DKI Mice Show Neutrophil Aggregation and Fat Accumulation in the Liver

4.3.1 Histology

Liver weights of the ACC DKIs were not different from the WTs (WT=1043.57±85.0 mg, DKI=1022.86±86.2 g, p=0.66, N=8). H&E sections of ACC DKI (Figure 12A) and WT (Figure 12B) livers showed no prominent difference in steatosis. And had no visible lipid accumulation. However, an accumulation of neutrophils was evident in the ACC DKIs.

4.3.2 Lipid Analysis

Biochemical analysis of lipid levels in the livers of ACC DKIs and WTs showed that ACC DKIs had 3-fold higher levels of TAGs compared to WT controls (Figure 13).

4.3.3 Serum Concentrations

Serum ALT levels tended to be higher in ACC DKIs compared to WTs (p=0.12) (Figure 14A). Serum AST levels were not different between ACC DKIs and WTs (Figure 14B). Consistent with the tendency for increased ALT levels, there was a trend for the ALT to AST ratio to be elevated in ACC DKI mice (p=0.26) (Figure 14C).

5.0 DISCUSSION

NAFLD has been associated with the development of hepatic insulin resistance in obesity; however, the molecular mechanisms underlying this relationship are unclear. ACC, which exists as two separate isoforms, ACC1 and ACC2, is an important metabolic enzyme, which controls the production of the metabolic intermediate malonyl coA. AMPK has been shown to be important for regulating lipid metabolism and inhibits ACC activity through phosphoryation of ACC1 at Ser79 and ACC2 at Ser221. However, the importance of these phosphorylation sites in controlling lipid metabolism in vivo has not been investigated. This thesis shows that compared to wild-type (WT) controls, mice with whole-body knock-in mutations at ACC1 Ser79 and ACC2 Ser221 (ACC DKI), accumulate more liver lipids and have hepatic insulin resistance. The results of this are in agreement with the important role AMPK plays in regulating liver insulin sensitivity and further demonstrate that a primary mechanism by which this may occur is through control of lipid metabolism.

5.1 ACC and Malonyl CoA Regulation

We found that ACC1 and ACC2 activity were both much higher in the livers of ACC DKI mice. These data indicate that under physiological conditions, ACC1 Ser79 and ACC2 Ser221 are the primary phosphorylation sites regulating ACC activity *in vivo*. Thus, while there are multiple phosphorylation sites on both ACC isoforms, our results show that they have minimal physiological importance when it comes to regulating ACC activity. In addition, because malonyl coA levels have been associated with citrate, the

literature has previously suggested that allosteric activation of ACC by citrate may be the reason for increased levels of malonyl coA, instead of a covalent change in the phosphorylation state (Saha, Laybutt et al. 1999; Bavenholm, Pigon et al. 2000). But, researchers have also shown that phosphorylation of ACC reduces the sensitivity of ACC to citrate (Munday, Campbell et al. 1988). The findings of our study build on this and indicate that allosteric regulation of ACC by citrate is unable to overcome covalent control through phosphorylation of ACC Ser79 and Ser221.

Malonyl coA decarboxylase (MCD) is involved in the breakdown of malonyl coA and, along with ACC, regulates malonyl coA levels in the cytosol. We find that consistent with an increase in ACC activity malonyl coA levels are increased by ~2-fold in ACC DKI mice. MCD has been associated with reversing muscle, liver and whole-animal insulin resistance (An, Muoio et al. 2004), but its exact mechanism and importance is not clear in the literature. One study found that AMPK decreases malonyl CoA levels by upregulating MCD and that this occurs via an indirect mechanism (Assifi, Suchankova et al. 2005), while another study conducted in ACC2 knock out animals showed that the deletion of ACC2 decreases the expression of MCD (Olson, Pulinilkunnil et al. 2010), indicating that both AMPK and ACC influence levels of MCD. In the ACC DKI model, the two targeted serine phosphorylation site residues are mutated to inhibit its phosphorylation making ACC constitutively active resulting in higher levels of malonyl coA. This means that MCD cannot compensate for the lack of ACC phosphorylation suggesting that MCD may not have a crucial role in regulating fat metabolism and insulin sensitivity in the liver.

5.2 Body Mass and Energy Expenditure

Body mass and adiposity are not different in the ACC DKIs compared to WTs. The lack of difference in body mass and adiposity is unexpected because, based on the genotype of the ACC DKI mice, it was anticipated that they would have greater fatty acid synthesis and lower fatty acid oxidation, which would likely contribute to greater body mass and adiposity. One reason for a lack of difference could be due to the reduced food intake in the ACC DKIs. Another factor to consider is body fat distribution. Although there is no difference in overall adiposity in the body, assessment of regional fat distribution would help rule out whether the location of fat storage is impacting the ACC DKI mouse model phenotype. In addition, there was no difference in the volume of oxygen consumed and the total energy expenditure between genotypes. Overall, there seems to be an imbalance between the energy consumed versus the energy produced in mice with knock-in mutations at ACC residues Ser79 and Ser221, compared to WTs.

It is important to note that, of the main tissues involved in regulating insulin sensitivity – the liver, muscle, and adipose tissue – skeletal muscle appears to account for the greatest amount of oxygen used (Zurlo F 1990). At rest, the muscle accounts for about 20-30% of resting energy expenditure (Geigy 1970; Rolfe and Brown 1997). In our ACC DKI mice, skeletal muscle sensitivity to insulin is not different compared to WTs, as evidence by no difference in glucose uptake in skeletal muscle, and no difference in Akt phosphorylation levels. Because the muscle plays a more dominant role in energy use, and because it appears to be metabolically healthy in the ACC DKIs, this may be the

reason why there is no evident genotype difference in VO_2 and energy expenditure. The genetic disruption of ACC1 Ser79 and ACC2 Ser221, therefore, is not important to regulating oxygen consumption and energy use. Instead, there may be another mechanism in skeletal muscle that mediates fatty acid oxidation, like mitochondrial biogenesis (Winder, Holmes et al. 2000; Bergeron, Ren et al. 2001) or expression of uncoupling proteins (Suwa, Nakano et al. 2003; Narkar, Downes et al. 2008). Alternatively, others have suggested that malonyl coA is not an important regulator of fatty acid oxidation in skeletal muscle (Odland, Heigenhauser et al. 1996; Bezaire, Heigenhauser et al. 2004). Direct measurement of malonyl CoA levels and fatty acid oxidation in the skeletal muscle of ACC DKI mice would help confirm these findings.

5.3 Respiratory Exchange Ratio and Malonyl CoA Regulation

To obtain energy, chow-fed mice utilize a greater proportion of carbohydrates compared to lipids, as evidenced by an RER close to 1.0 in both ACC DKIs and WTs, with no difference in genotype. However, in the liver, the ACC DKIs show higher expression of malonyl coA compared to WTs. With this finding, one would hypothesize that the ACC DKIs would have lower fatty acid oxidation, and hence, a higher RER compared to WTs but this in not evident in this study. Still, one must note that all abdominal organs require 25% of the whole-body VO_2 at rest (McGilvery RW 1979). This means that the liver alone has a small role in whole-body oxygen consumption and so it is not the ideal value to use in relation to whole-body RER as measured in the metabolic cages. Malonyl coA levels were only measured in the liver making it difficult

to make any conclusion regarding levels of malonyl coA and RER. Future work should measure malonyl coA in other tissues of the ACC DKI mice in order to better assess why the genetic disruption of ACC residues Ser79 and Ser221 does not result in a difference in RER between ACC DKI and WT mice.

Olson, Pulinilkunnil et al. (2010) previously reported a reduced RER in their ACC2 knock-out mouse model compared to WTs. However, the ACC2 knock-out mice unexpectedly did not show a difference in levels of muscle malonyl coA or fat oxidation as assessed by liquid chromatography and ex vivo analysis, respectively. The group proposes that cardiac metabolism or the central nervous system could be mediating a difference in systemic fuel use (Olson, Pulinilkunnil et al. 2010). Furthermore, previous data from the Steinberg laboratory shows no difference in skeletal muscle fatty acid oxidation in ACC2 KI mice. In ACC1 KI mice, however, they do find lower rates of fatty acid oxidation in the liver. Overall, ACC1 KI, ACC2 KI, along with ACC DKI mice did not show genotype differences in RER. In order to better assess the role of ACC1 Ser79 and ACC2 Ser221 on whole body and individual tissue RER, it may be helpful to assess fatty acid oxidation and substrate oxidation in isolated hepatocytes and other tissues, directly. In addition, to better understand the RER and substrate oxidation results, it would be beneficial to directly measure malonyl coA levels, RER, and energy expenditure *in vivo* following injection with the pharmacological AMPK activator 5` aminoimidazole-4-carboxamide-riboside (AICAR), which would be expected to increase ACC phosphorylation and fatty acid oxidation in WT mice. This would directly assess the

importance of ACC's two phosphorylation sites targeted in the ACC DKI mouse model. The same experiment using AICAR could also be completed *ex vivo* in hepatocytes.

CPT1 is critical for regulating fatty acyl CoA mitochondrial flux (Drynan, Quant et al. 1996). Overexpression of CPT-1 in hepatocytes increases fatty acid oxidation and reduces liver triacylglycerol levels (Stefanovic-Racic, Perdomo et al. 2008). In skeletal muscle, the overexpression of CPT-1 also increases rates of fatty acid oxidation and prevents the development of skeletal muscle insulin resistance (Bruce, Hoy et al. 2009). The ACC DKIs tended ($p=0.054$) to have a 50% higher expression of CPT-1 mRNA in the liver. It may be that the transcription of CPT-1 increases in the ACC DKI mice so as to compensate for the inability to oxidize fatty acids as a result of malonyl coA accumulation. It would be interesting to assess substrate utilization with age to see whether there is an age-related change in CPT-1's ability to compensate for the reduced fatty acid oxidation in the ACC DKI mouse model. The increased CPT-1 may be the reason why changes in whole body fatty acid oxidation are not evident in the ACC DKI mouse model compared to WTs. Finally, the fact that the animal monitoring system only measures lipid and carbohydrate oxidation makes it difficult to make precise conclusions. Use of proteins as a fuel source is not measured and is needed to better assess any potential differences in substrate oxidation between genotypes.

5.4 Regulation of Food Intake

One interesting finding is that the ACC DKIs had lower food intake compared to WTs, independent of mouse weight, adiposity, and energy expenditure. A potential

reason for the reduced food intake involves the hypothalamus. For instance, (Hu, Dai et al. 2005) reported that lower hypothalamic malonyl coA levels resulted in higher food intake. We show that the ACC DKI livers have higher levels of malonyl coA compared to WTs. Although not measured, it is likely that we would also observe higher levels of malonyl coA in the hypothalamus of ACC DKIs. In view of Hu et al. (2005) this would mean that higher malonyl coA levels could be leading to a hypothalamic-regulated lowering of food intake, which is evident in the ACC DKIs. A dysregulation in this system involving lipid metabolism and the brain is believed to contribute to insulin resistance and is, therefore, a promising area of research to assess the role of ACC Ser79 and Ser221 on hypothalamic regulation of appetite and insulin signaling.

5.5 Skeletal Muscle Insulin Sensitivity

Skeletal muscle has been shown to predominately express the ACC2 isoform, which is located at the mitochondrial membrane and is associated with fatty acid oxidation. The ACC DKI mice show no impairment in glucose uptake and insulin signaling in skeletal muscle. To date, there are inconsistent findings in the literature with respect to fat oxidation in relation to ACC2 and insulin sensitivity. Certain studies show that increasing levels of fatty acid oxidation protects from high-fat diet-induced insulin resistance (Bruce, Hoy et al. 2009), while others show that excess fatty acid oxidation promotes insulin resistance (Koves, Ussher et al. 2008). Hoehn, Turner et al. (2010) reported that deletion of ACC2 in mice led to a reduction in skeletal muscle malonyl coA levels and an increase in fatty acid oxidation but no changes in energy expenditure or

adiposity. In contrast, Choi, Savage et al. (2007) showed an increase in fatty acid oxidation and an improvement in insulin sensitivity in ACC2 knock-out mice. However, Olson, Pulinilkunnil et al. (2010) showed no difference in malonyl coA levels, rates of fatty acid oxidation, or insulin sensitivity in ACC2 knock-out mice. In our findings, knock-in mutations of ACC1 at Ser79 and ACC2 Ser221 did not impair skeletal muscle insulin sensitivity. Since the predominant ACC isoform in the muscle is ACC2, this study suggests that the ACC2 Ser221 phosphorylation site is not essential to maintaining insulin sensitivity in skeletal muscle.

5.6 Fatty Liver and Hepatic Insulin Resistance

To date, significant research has been conducted to examine the role of AMPK signaling in the development of NAFLD and type 2 diabetes. A number of reviews suggest that lipid accumulation in the liver causes liver and muscle insulin resistance (den Boer, Voshol et al. 2004; Savage, Petersen et al. 2007; Anderson and Borlak 2008). The ACC DKIs present with both hepatic insulin resistance and fat accumulation in the liver. Specifically, the ACC DKIs have hyperglycemia, hyperinsulinemia, impaired glucose and insulin tolerance, hepatic insulin resistance, and triacylglycerol accumulation in the liver. Because the mouse model targets both fatty acid synthesis and oxidation, it is an ideal animal model to assess the importance of lipid metabolism on insulin signaling. In the ACC DKIs, we see increased TAGs in the livers of ACC DKIs and this is associated with the development of hepatic insulin resistance. The ACC DKIs also show a trend for higher NEFA levels in the ACC DKIs compared to WTs. The circulating NEFA levels in

the ACC DKIs are likely impairing glucose metabolism. The data collected in this thesis, therefore, supports the relationship in which lipid accumulation (or NAFLD) leads to impaired insulin signaling, the precursor to type 2 diabetes.

This thesis has reported higher liver lipid levels in ACC DKIs compared to WTs. In addition, analysis of liver histology revealed an aggregation of neutrophils in ACC DKI liver suggesting that the accumulation of lipids in the liver may be as a result of inflammation (Lefkowitch, Haythe et al. 2002). While the measures of liver damage are not significantly different between ACC DKIs and WTs, there is potential for a difference to develop with time, particularly considering the presence of neutrophil aggregation in liver histology slides and the accumulation of TAGs in the livers as assessed by the glycerol assay. Because the level of inflammation does not always correlate with severity of steatosis (Reddy and Rao 2006), assessing the degree of liver inflammation in ACC DKI mice compared to WTs will help to understand the source and mechanism of neutrophil aggregation. A potential theory on the source of neutrophil aggregation is based on findings from a study about the association of liver fibrosis with necroinflammation in chronic hepatitis C patients (Asselah, Boyer et al. 2003; Moucari, Asselah et al. 2008). Necroinflammation has also been correlated with visceral adiposity index. In fact, this index is believed to be a good indicator of adipose-related liver damage and progression of hepatic disease (Salvatore, Buzzetti et al. 2010). The degree of the liver damage and presence of fibrotic tissue, if any, could be assessed in ACC DKI mice using trichrome staining. Therefore, future work should focus on further analysis of liver inflammatory markers and degree of liver fibrosis, in order to characterize the source

of neutrophil aggregation and elevated lipid levels in the livers in the ACC DKI mouse model.

5.7 Adipose Tissue Insulin Sensitivity

The ACC DKI mice show impaired insulin-stimulated adipose tissue glucose uptake. The literature today has established an important link between obesity, insulin resistance, and inflammation. Adipose tissue has been associated with a state of chronic low-grade inflammation in which there is an accumulation of macrophages in the adipose tissue (Wellen and Hotamisligil 2003). The chronic inflammation that surfaces typically involves macrophage expression of pro- inflammatory cytokines like tumour necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Pedersen 2006). Furthermore, AMPK has been associated with this inflammation, where TNF- α, for example, results in inhibition of AMPK activity and induces skeletal muscle insulin resistance (Steinberg, Michell et al. 2006). Further examination of the role of ACC phosphorylation in adipose tissue and potential interactions with inflammatory markers are warranted.

5.8 Future Directions

This thesis has found that mice with knock-in mutations at ACC1 Ser79 and ACC2 Ser221 develop hepatic insulin resistance and lipid accumulation in the liver. In order to better characterize and understand the ACC DKI mouse model as it relates to obesity, it would be interesting to examine responses to a hypercaloric and/or high-fat diet

that induces obesity. In addition, by activating this pathway, naturally and pharmacologically, the role of the AMPK-ACC signaling pathway will be highlighted and will enable a more complete assessment into whether activation leadoff ACC phosphorylation is required for improvements in insulin sensitivity.

5.8.1 Exercise Intervention

Physical activity is a key determinant in the prevention and treatment of obesity and type 2 diabetes (Davis, Forges et al. 2009). Engaging in regular exercise is a lifestyle intervention that can prevent the development of insulin resistance and type 2 diabetes in a high-risk population of overweight individuals (Tuomilehto, Lindstrom et al. 2001). At the molecular level, AMPK has been identified as a driving force and key regulator of the beneficial effects of exercise in obesity and diabetes (Ruderman, Saha et al. 1999). Specifically in the liver, phosphorylation of AMPK and ACC is higher following long-term exercise; however, the exact mechanism and metabolic effects are not understood (Takekoshi, Fukuhara et al. 2006). In addition, a study investigating the effects of exercise in rats fed a high-fat diet found that exercise prevented hepatic steatosis (Gauthier, Couturier et al. 2003). Another groups that cessation of exercise resulted in a worsening of NAFLD properties (Rector, Thyfault et al. 2008). Many *in vivo* studies have shown that exercise impacts liver lipid composition and, due to its activation with exercise, AMPK likely plays a role in this liver phenotype (Camacho, Donahue et al. 2006). The effects of exercise on AMPK activation and liver lipid composition in combination with the effect of AMPK phosphorylation of ACC on liver metabolism

observed in this thesis suggest that an investigation into the effects of exercise on high-fat diet-fed ACC DKI mice might be particularly interesting. Feeding ACC DKI mice a high-fat diet in conjunction with an exercise intervention would be an ideal method to better understand the importance of fat metabolism in the development of insulin resistance and NAFLD using a natural means of increasing ACC phosphorylation.

5.8.2 Metformin Intervention

Metformin is the most widely used anti-diabetic drug with currently over 100 million users worldwide. It has been shown to reduce NAFLD in animal (Lin, Yang et al. 2000) and human studies (Bugianesi, Gentilcore et al. 2005); however, whether this is important for its insulin sensitizing effects is not currently known. In one study, metformin specifically led to lower blood glucose levels via LKB1, the upstream kinase of AMPK (Shaw, Lamia et al. 2005). Research shows that metformin's main effect on AMPK activation occurs in the liver (Zhou, Myers et al. 2001), and the LKB1-AMPK pathway is essential for metformin's gluconeogenic improvements like regulating CRTC2 expression of PGC-1α (Shaw, Lamia et al. 2005). Because research indicates a role for metformin in the AMPK signaling pathway, particularly in the liver, understanding the role of AMPK regulation of ACC activity in the liver in response to metformin is important for determining how metformin improves insulin sensitivity. Testing whether metformin continues to improve insulin sensitivity in high-fat diet-fed ACC DKI mice will allow for a better understanding of whether improvements in NAFLD are important for the insulin sensititizing effects of this commonly used drug.

5.9 Limitations

One limitation in this study may be the duration of tests. The analysis of the ACC DKI mouse model over an extended timeline may have been beneficial as it could have given mice more time for their insulin and liver pathophysiology to progress. Similarly, it would be interesting to look at the progression of changes in insulin sensitivity and fatty liver. Developing a more specific timeline of the development of hepatic insulin resistance and liver lipid accumulation would aid in assessing whether insulin resistance precedes lipid accumulation or vice versa. Also, assessment of food intake in relation to hypothalamic malonyl coA levels could help to better characterize whether there is an effect of hypothalamic malonyl coA on appetite regulation. Furthermore, because ACC can also be phosphorylated by PKA, assessing the degree of PKA phosphorylation in the ACC DKIs compared to WTs would be beneficial to asses the relative importance of AMPK and ACC phosphorylation sites. Similarly, in our study, we cannot rule out any post-translational or allosteric changes in regulation of ACC1 and ACC2. Assessment of MCD levels would also aid in determining whether compensation occurs in order to regulate malonyl coA levels. In addition, a more in depth analysis of liver profile in ACC DKI mice compared to WT mice could help to better characterize the phenotype (i.e. inflammatory markers) of the liver and degree of damage in ACC DKI mice. Finally, one must be cautious in applying the results of this study to a clinical population as assessing whether the same effects occur in a human population still needs to be made.

5.10 Conclusion

The growing prevalence of obesity is a critically important health issue. Obesity is the leading cause of NAFLD. Importantly, fatty liver disease is also a causal factor in the development of hepatic insulin resistance and contributes to both fasting and postprandial hyperglycaemia and the development of type 2 diabetes. However, the mechanisms causing NAFLD are still unclear. ACC1 and ACC2 are believed to be the rate-limiting enzymes regulating fatty acid synthesis and fatty acid oxidation and phosphorylation at S79 and S221, respectively inhibits ACC activity. Characterization of the ACC1 Ser79 and ACC2 Ser221 double knock-in (ACC DKI) mouse model has just begun, and this model will help us understand whether the phosphorylation of ACC by AMPK is necessary to improve glucose and fatty acid metabolism in the liver. In summary, the findings of this thesis show that despite reduced food intake in ACC DKIs fed a chow diet, there was no difference in total body weight, adiposity, energy expenditure, and RER. Importantly, genetic disruption of ACC1 Ser79 and ACC2 Ser221 resulted in an accumulation of triacylglycerols and neutrophils in the liver of ACC DKI mice that was associated with the development of liver insulin resistance. We also observed impaired glucose uptake in epididymal adipose tissue of ACC DKI mice. Although the exact importance of AMPK phosphorylation of ACC in regulating glucose and fatty acid metabolism in liver, skeletal muscle and adipose tissue remains to be defined, this work has set the stage for a number of future directions involving assessment of liver function and use of direct activators of the AMPK-ACC signaling pathway to determine the importance of this pathway for metabolic homeostasis.

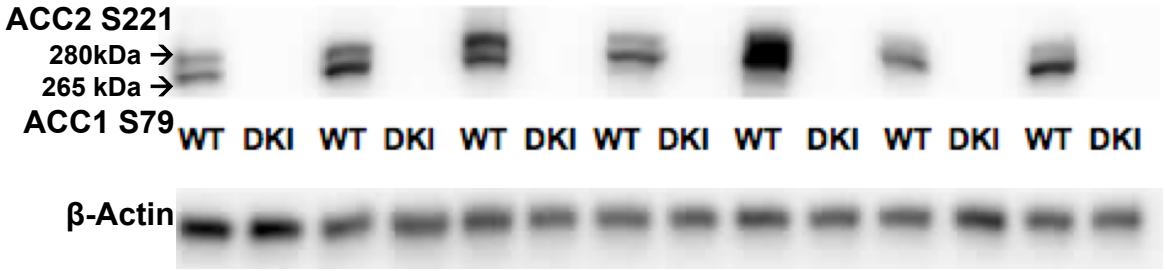
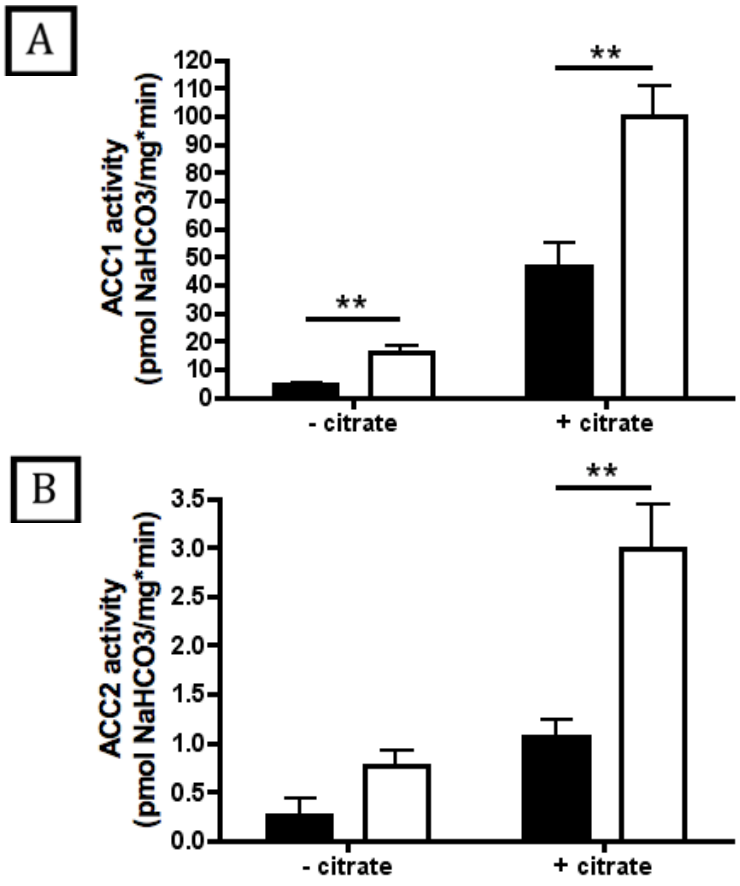
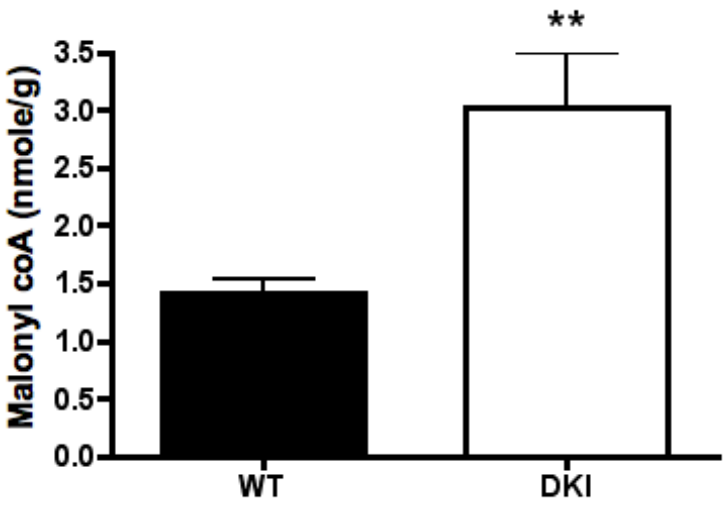


Figure 2. Confirmation of Genotype. Immunoblot showing phosphorylated ACC and loading control expression in the liver of ACC DKI versus WT mice. Each lane represents a different mouse sample and the labels between represent a ACC DKI or WT mouse sample.



(Galic, unpublished)

Figure 3. Liver ACC1 and ACC2 Activity. (**A**) ACC1 enzyme activity in fasted hepatocytes with and without citrate. (**B**) ACC2 enzyme activity in fasted hepatocytes with and without citrate. Shown as WT (black bar) and ACC DKI (white bar) (N=5 per group). All values are expressed in pmol $NaHCO_3$/mg*min as the mean ±SEM and data was analyzed by Student's t-tests (**A-B**). ** Indicates significance versus the control with $P<0.005$.



(Pulinilkunnil, unpublished)

Figure 4. Liver Malonyl CoA. Hepatic malonyl coA levels in a fed state (N=8 per group). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests. ** Indicates significance versus the control with $P<0.005$.

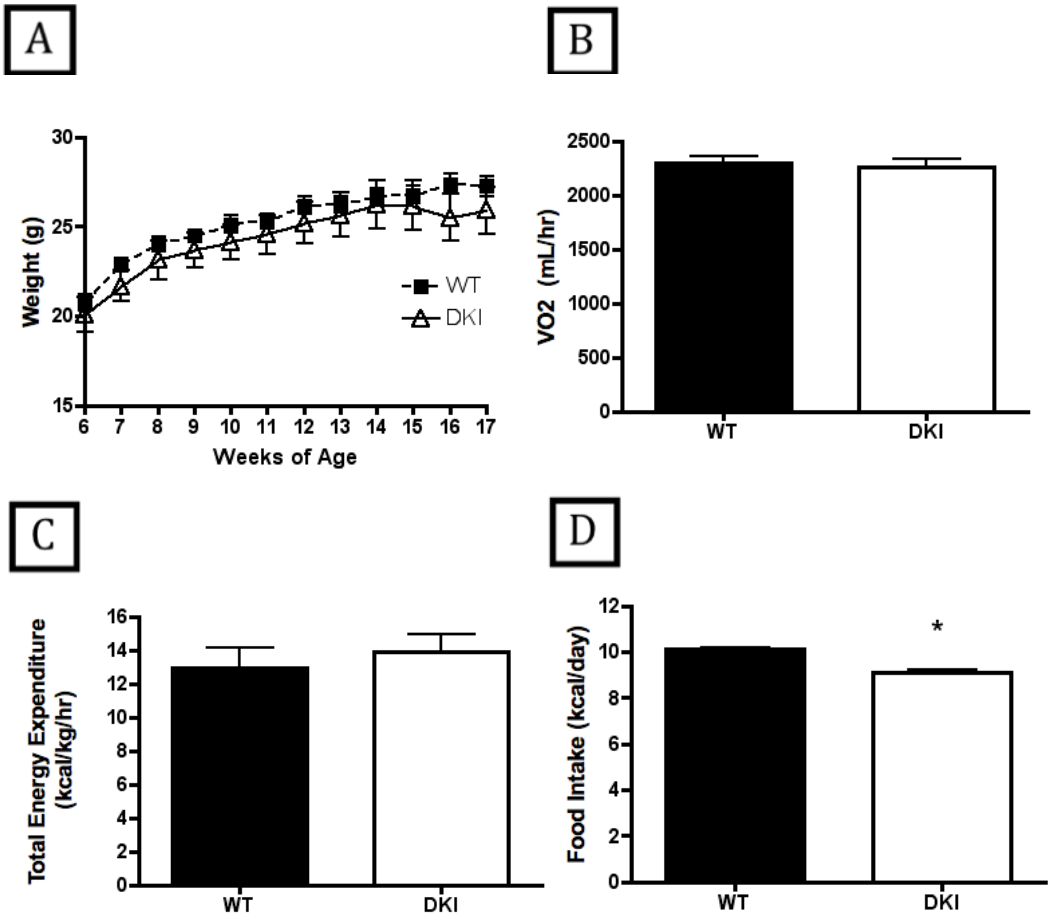


Figure 5. Body Weight, VO_2, Total Energy Expenditure, and Food Intake. (**A**) Growth curve of WT and ACC DKI mice. WT mice are represented by the black square and ACC DKI mice are represented by the white triangle (N=10 per group). (**B**) Average volume of oxygen consumed during the day in WT and ACC DKI mice expressed in mL/hr (N=8 per group). (**C**) Total energy expenditure in WT and ACC DKI mice (N=8 per group). (**D**) Daily average food intake in WT and ACC DKI mice expressed as (kcal/day). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by repeated measures two-way ANOVA (**A**) and Student's t-tests (**B-D**). * Indicates significance versus the control with $P<0.05$.

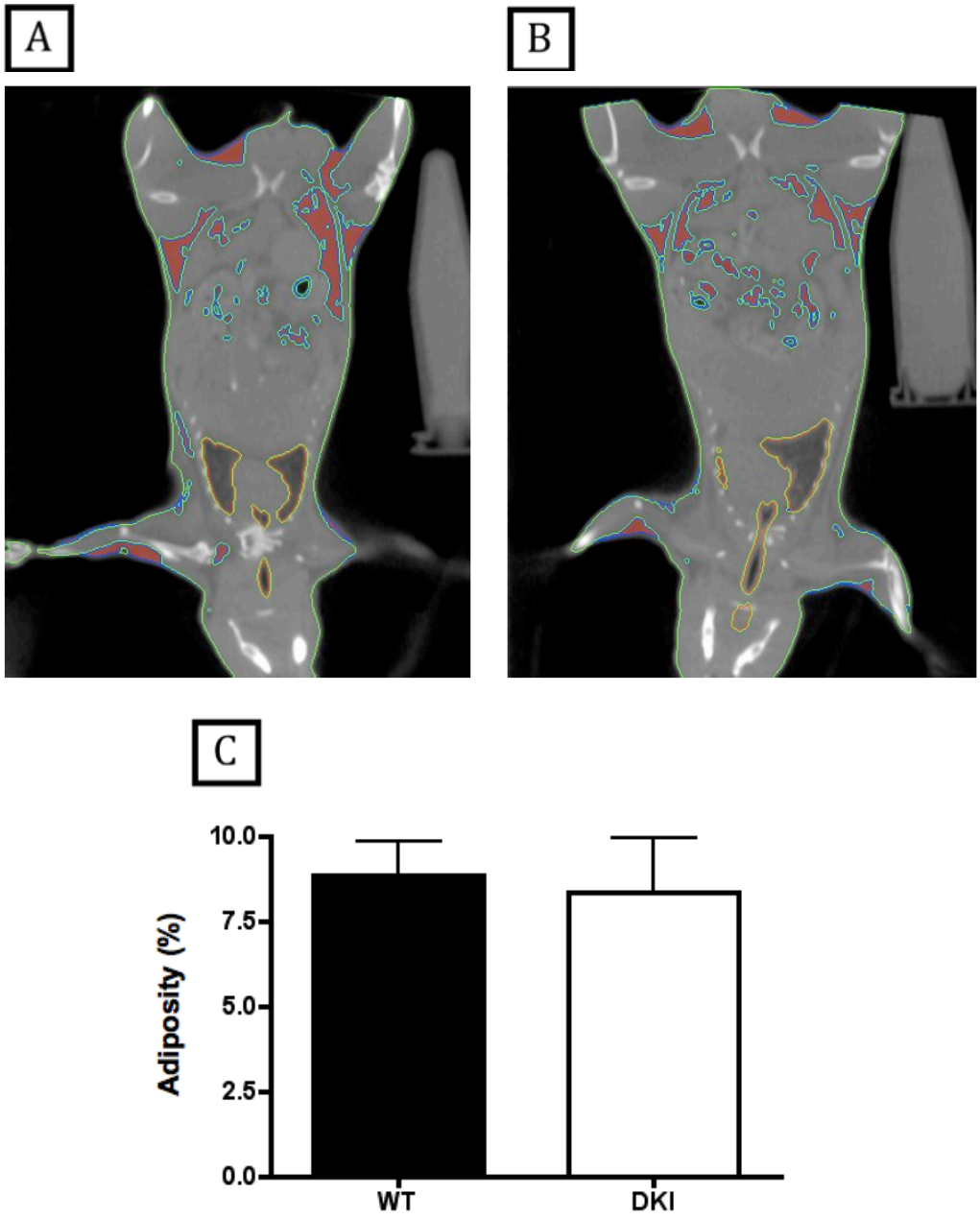


Figure 6. Body Adiposity. (**A**) Representative scan of the body composition of a WT mouse measured by CT with red area highlighting adipose tissue (N=8 per group). (**B**) Representative scan of the body composition of a ACC DKI mouse measured by CT with red area highlighting adipose tissue (N=8 per group). (**C**) Total body fat percent. Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests.

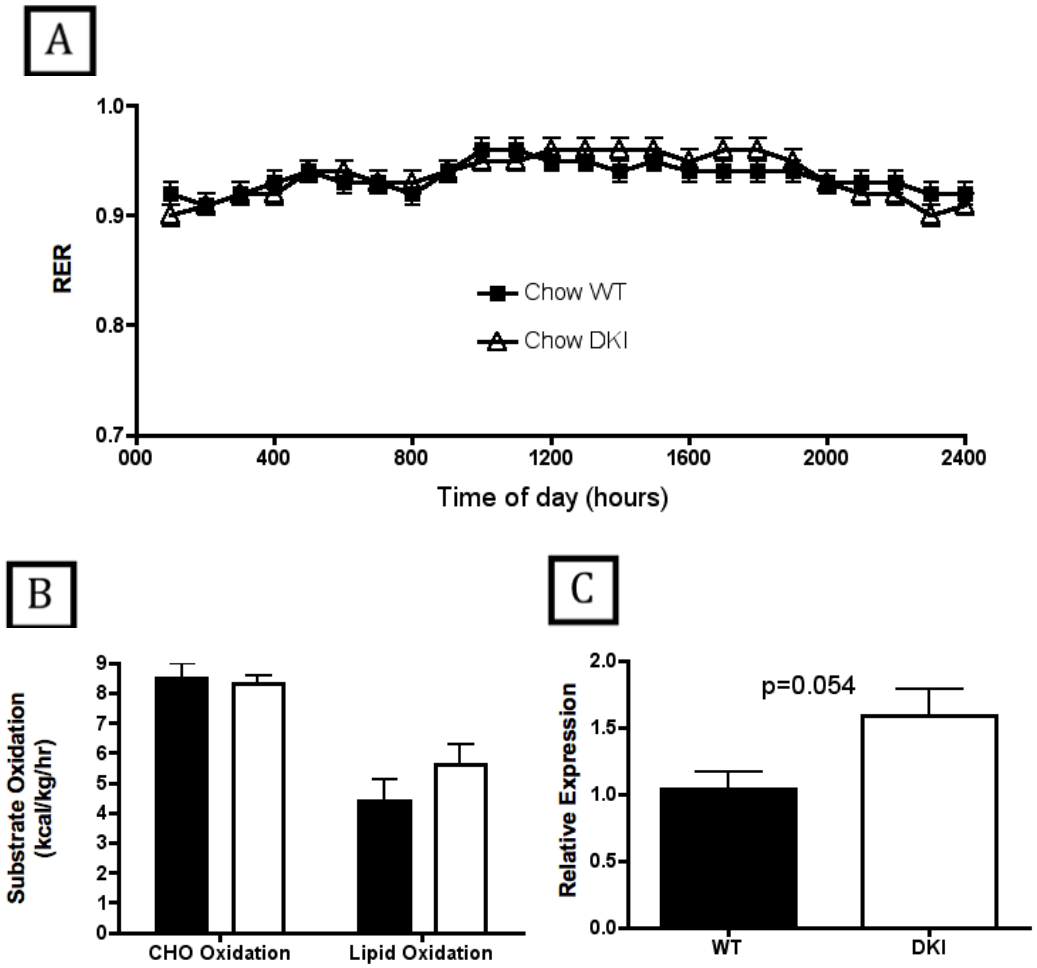


Figure 7. RER, Substrate Oxidation, and CPT-1 Expression. (**A**) RER data comparing WT and ACC DKI average daily RER at 17 weeks of age (N=8 per group). (**B**) Substrate oxidation (carbohydrate (CHO) and lipid) in WT and ACC DKI mice expressed as (kcal/kg/hr) (N=8 per group). (**C**) Hepatic expression of mitochondrial gene CPT1 in WT and ACC DKI mice measured by real-time quantitative PCR (N=8 per group). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests (**A-C**).

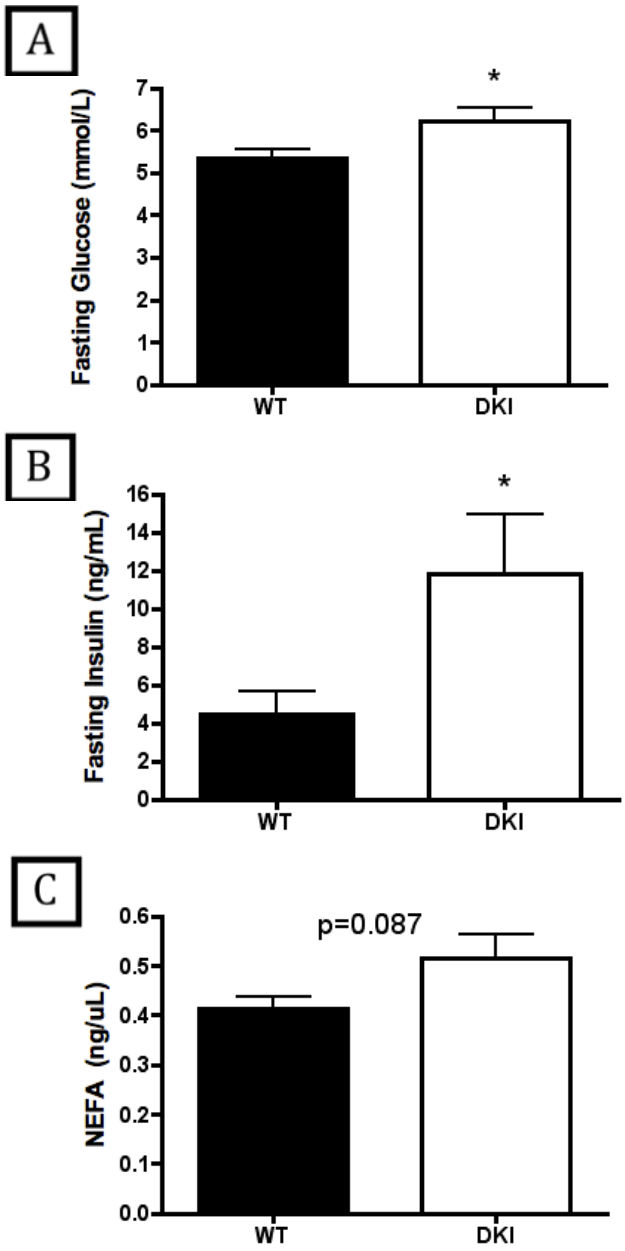


Figure 8. Fasting Blood Glucose, Insulin, and NEFA Levels. (**A**) WT and ACC DKI comparison of 12-hour fasting blood glucose at 17 weeks of age (N=10 per group). (**B**) WT and ACC DKI comparison of 12-hour fasting serum insulin levels at 17 weeks of age (N=8 per group). (**C**) WT and ACC DKI comparison of 12-hour fasting serum NEFA levels at 17 weeks of age (N=8 per group). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests (**A-C**). * Indicates significance versus the control with $P<0.05$.

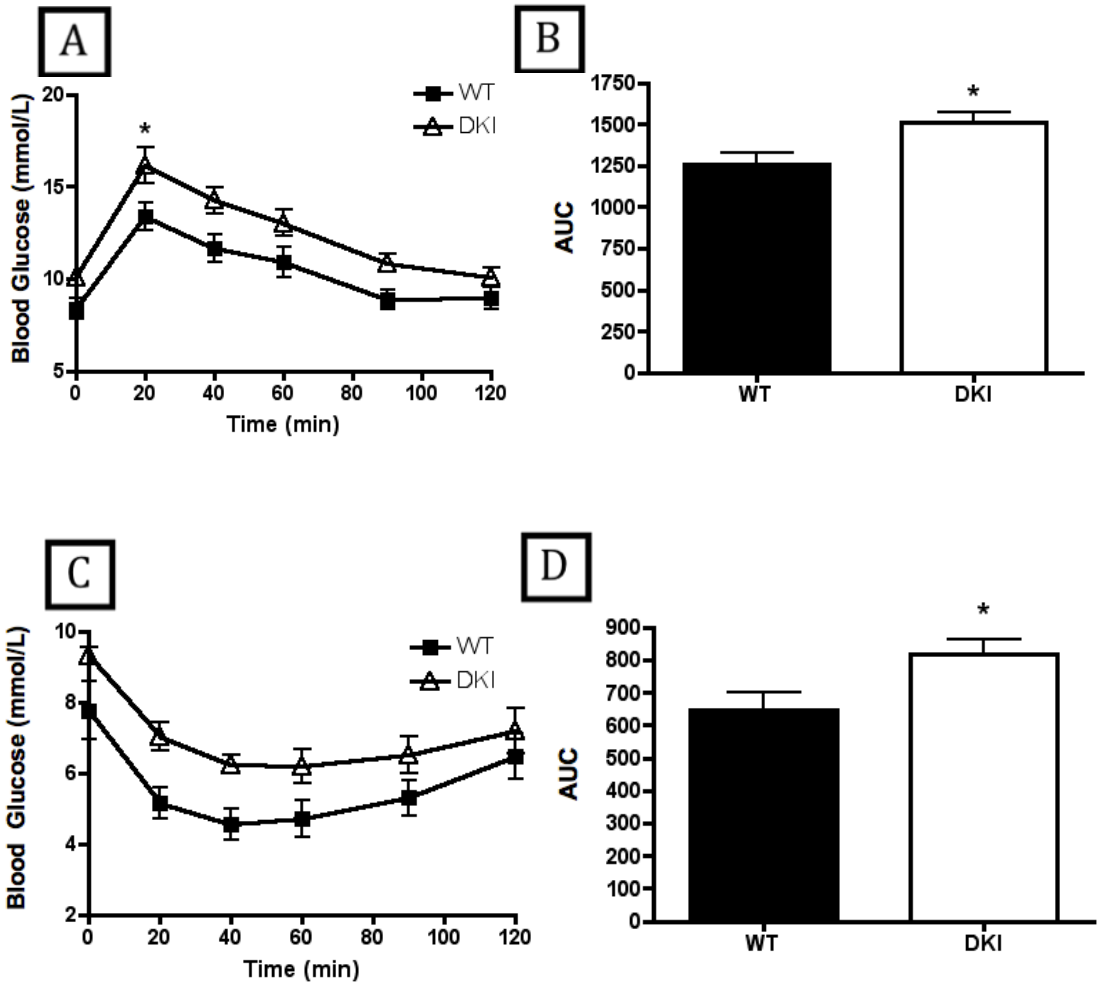


Figure 9. Glucose and Insulin Tolerance. (**A**) WT and ACC DKI response to a 1.0 grams of glucose/ kg of body weight infusion over a 2-hour time period at 16 weeks of age (N=8 per group). (**B**) WT and ACC DKI area under the curve of the response to 1.0 grams of glucose/kg of body weight at 16 weeks of age (N=8 per group). (**C**) WT and ACC DKI response to a 0.7 Units of insulin/ kg of body weight infusion over a 2-hour time period at 17 weeks of age (N=8). (**D**) WT and ACC DKI area under the curve of the response to 0.7 Units of insulin/kg of body weight at 17 weeks of age (N=8 per group). WT mice are represented by a square and ACC DKIs are represented by a white triangle. Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by repeated measures two-way ANOVA (**A,D**) and Student's t-tests (**B,D**). * Indicates significance versus the control with $P<0.05$. ** Indicates significance versus the control with $P<0.005$.

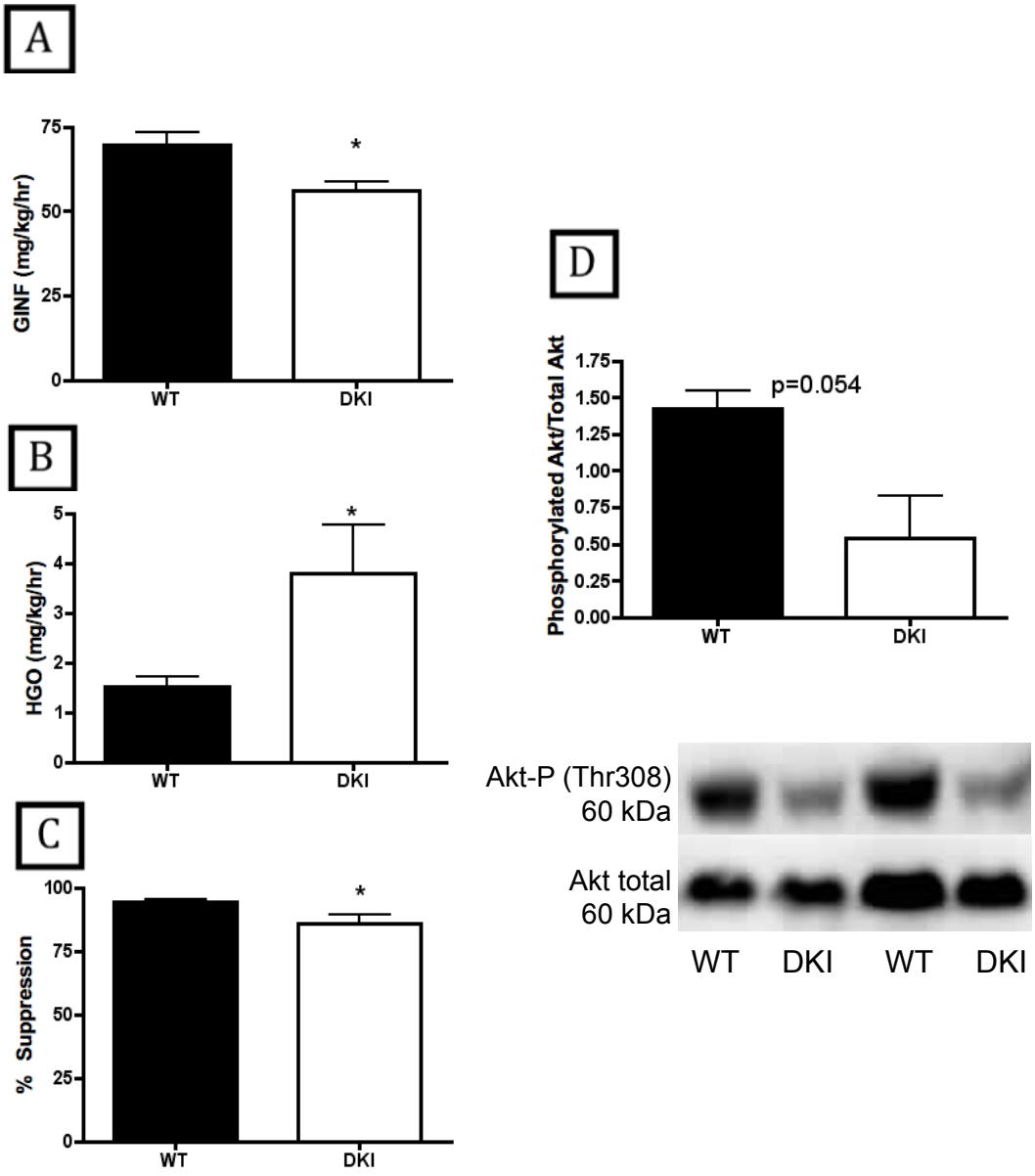


Figure 10. Hyperinsulinemic-Euglycaemic Clamp and Liver Akt. (**A**) Glucose infusion rate in WT and ACC DKI mice at 18 weeks of age (N=8 per group). (**B**) Percent suppression of glucose in WT and ACC DKI mice at 18 weeks of age (N=8 per group). (**C**) Hepatic glucose output of WT and ACC DKI mice at 18 weeks of age (N=8 per group). (**D**) WT and ACC DKI comparison of phosphorylated Akt expressed as a ratio to the total Akt in clamped liver (N=8 per group) and representative blots of WT and ACC DKI mice phosphorylated Akt (Akt-P) and and total Akt. Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests (**A-D**). * Indicates significance versus the control with $P<0.05$.

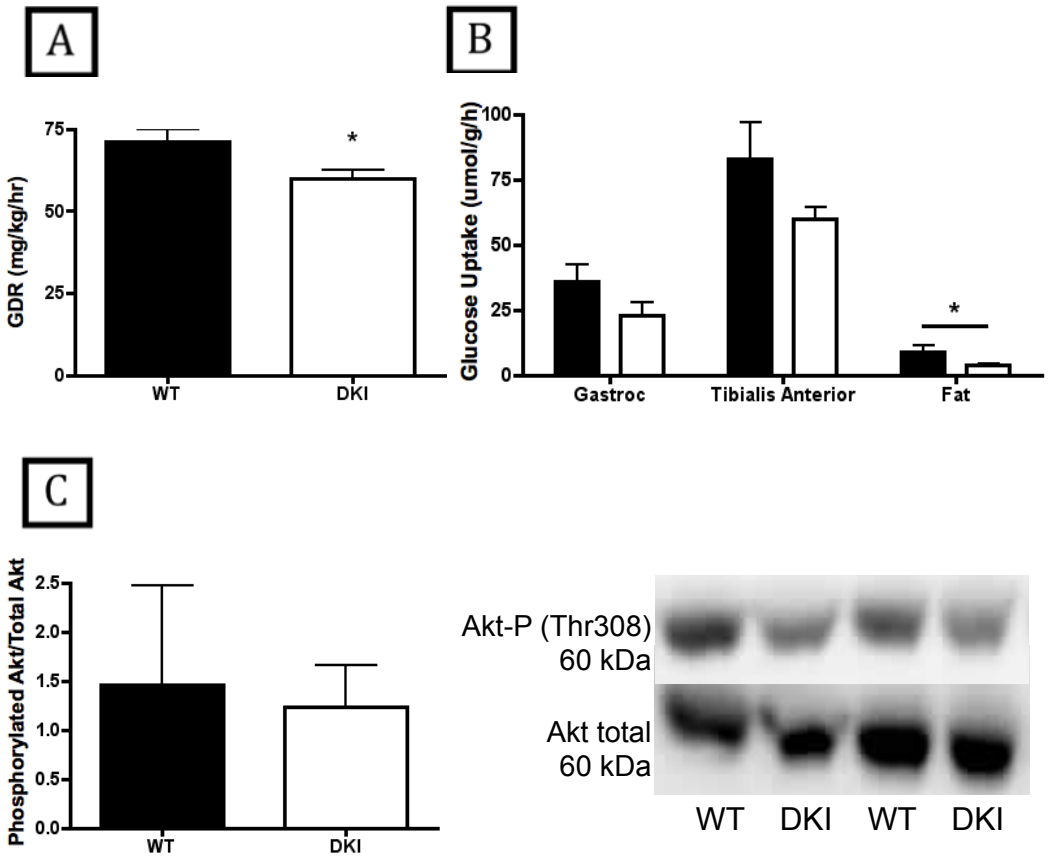


Figure 11. Glucose Disposal, 2-DG Glucose Uptake, and Muscle Akt. (**A**) Glucose disposal rate of WT and ACC DKI mice at 18 weeks of age (N=8 per group). (**B**) Glucose uptake in liver, gastrocnemius, TA, and fat tissue in WT and ACC DKI mice at 18 weeks of age (N=8 per group). (**C**) WT and ACC DKI comparison of phosphorylated Akt expressed as a ratio to the total Akt in clamped TA (N=8 per group) and representative blots of WT and ACC DKI mice phosphorylated Akt (Akt-P) and and total Akt. Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests (**A-C**). * Indicates significance versus the control with $P<0.05$.

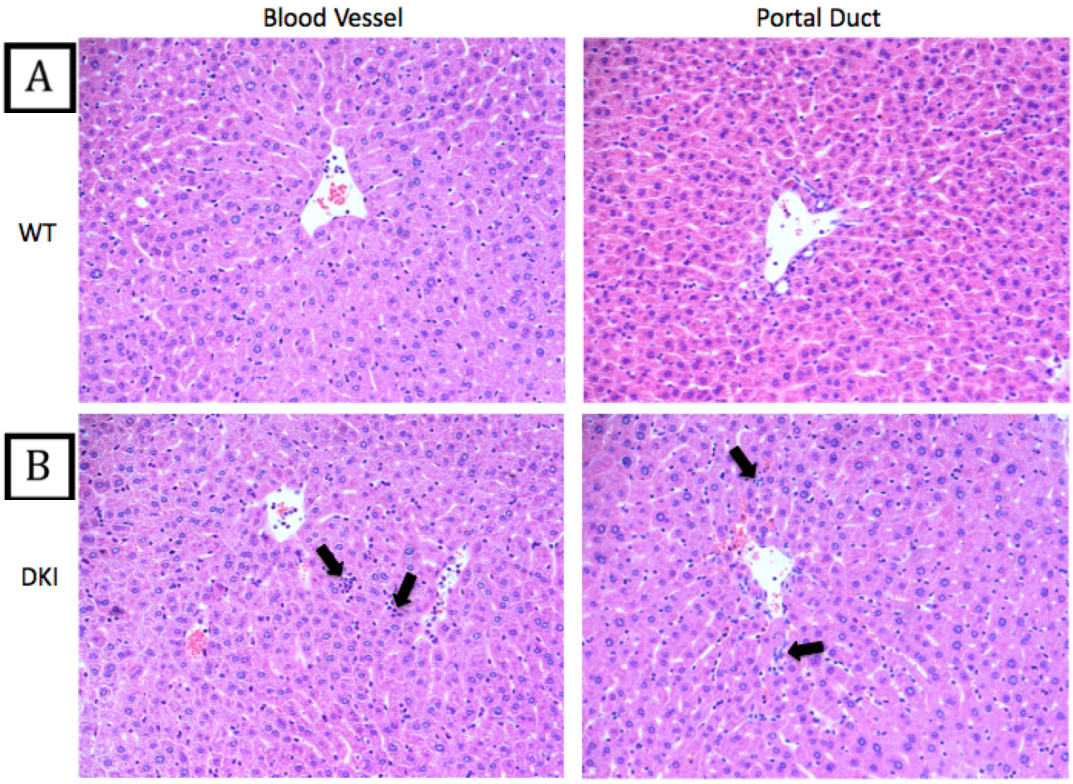


Figure 12. H&E Liver Slides. (**A**) Representative H&E stains for assessment of steatosis in WT mice (N=8 per group). Images show the region of the liver surrounding a blood vessel and portal duct. (**B**) Representative H&E stains for assessment of steatosis in ACC DKI mice (N=8 per group). Images show the region of the liver surrounding a blood vessel and portal duct. Arrows point to areas of neutrophil aggregation.

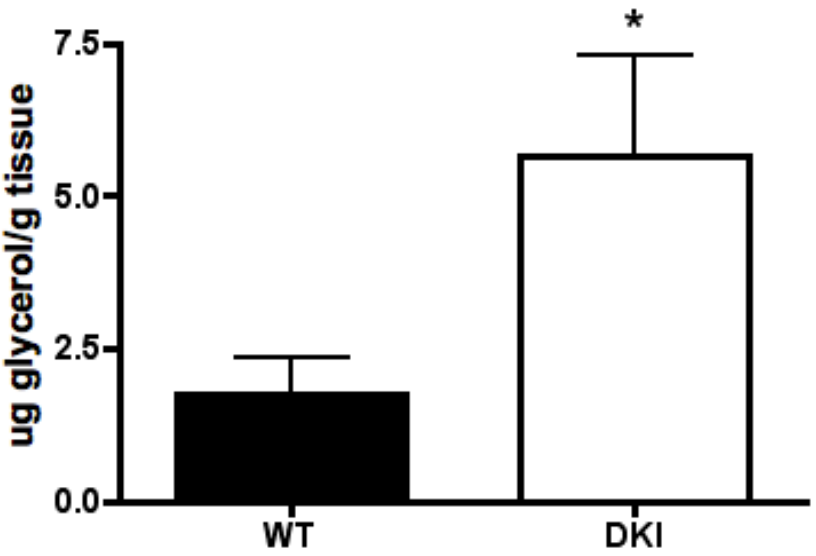


Figure 13. Triacylglycerol Levels. Hepatic levels of triaglycerols (TAG) expressed as μg of glycerol/g of tissue (N=8 per group). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests. * Indicates significance versus the control with $P<0.05$.

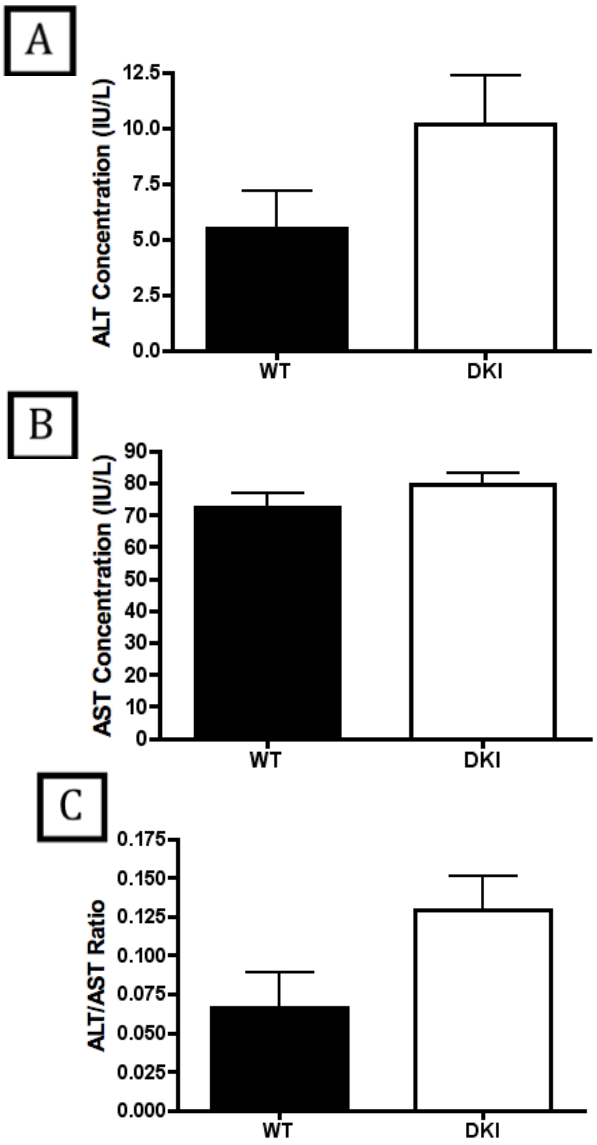


Figure 14. Serum ALT and AST Concentrations. (**A**) Graph representing alanine transaminase (ALT) levels in the serum of WT and ACC DKI mice (N=8 per group). (**B**) Graph representing aspartate transaminase (AST) levels in the serum of WT and ACC DKI mice (N=8 per group). (**C**) Graph representing ALT/ AST ratio of WT and ACC DKI mice (N=8 per group). Shown as WT (black bar) and ACC DKI (white bar). All values are expressed as the mean ±SEM and data was analyzed by Student's t-tests (**A-C**). * Indicates significance versus the control with $P<0.05$.

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