University of Alberta

New Insight into the Role of Myocardial Fatty Acid Uptake and Utilization in Health and Disease

by

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DEDICATION

I dedicate this thesis, along with everything else in my life, to my wife Karmen.

ABSTRACT

All mammalian cells rely on adenosine triphosphate (ATP) to maintain function and for survival. The heart has the highest basal ATP demand of any organ due to the necessity for continuous contraction. As such, the ability of the cardiomyocyte to monitor cellular energy status and adapt the supply of substrates to match the energy demand is crucial. However, despite having basal energy demands exceeding those of any other organ in the body, cardiomyocytes do not possess large reserves of substrates required to form high-energy. As such, the heart must rely on exogenous substrate supply to be transported into the cardiomyocytes in order to be catabolized and to produce ATP. Because of this, continuous transport of substrates such as fatty acids (FAs), glucose, and lactate into the cardiomyocytes is a key component of cardiac energy metabolism. During normal physiological conditions, this production of ATP is met almost entirely (>95%) through oxidative phosphorylation. Specifically, FA oxidation is responsible for the generation of 50–70% of ATP in a normal adult heart while only 20–30% of the energy provided is derived from glucose and less than 5% from other sources. Herein, we review the involvement of myocardial FA uptake and subsequent utilization as it relates to cardiac function in physiologic and pathophysiologic processes. Specifically, we examine the cardiomyocyte-specific role of CD36, a FA transport protein, during ischemia-reperfusion injury. Utilizing an inducible cardiomyocyte-specific CD36 ablation mouse model, we provide genetic evidence that reduced FA oxidation as a result of diminished CD36-mediated FA uptake improves post-ischemic cardiac efficiency and functional recovery. As such, targeting cardiomyocyte FA uptake and FA oxidation via inhibition of CD36 in the adult myocardium may provide therapeutic benefit during ischemia-reperfusion. Furthermore, we examine the cardiomyocyte-specific role of adipose triglyceride lipase (ATGL) overexpression during doxorubicin induced cardiac

dysfunction. Our data suggest that chronic reduction in myocardial triacylglycerol (TAG) content by cardiomyocyte-specific ATGL over-expression is able to prevent doxorubicin-induced cardiac dysfunction. We also examine the role of AMPK inhibitory phosphorylation of acetyl CoA carboxylase during high workload and ischemia-reperfusion injury. Our findings challenge the previously suggested role of AMPK-mediated ACC phosphorylation and inactivation as having a major role in the regulation of substrate metabolism and function in healthy and stressed myocardium. As well, since diabetes is one of the pathophysiologic processes known to cause alterations in FA metabolism in the myocardium, we further examined the effects of glucose-lowering medication on early outcomes of patients that had and acute coronary syndrome event. The work presented also emphasizes areas that require further investigation for the purpose of eventually translating this information into improved patient care.

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CHAPTER 1.

Introduction

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CHAPTER 1

Introduction

Fatty Acid Utilization in Health and Disease

Virtually all energy-mediated cellular functions are driven by the energy carrier adenosine triphosphate (ATP)[1]. The cell's ability to balance ATP production versus consumption often means the difference between cell survival and death. While all organs rely on the cellular production of ATP, the heart is unique in the fact that it must contract continuously and thus produces and utilizes vast quantities of ATP[2-4] in order to meet its extremely high energy demand. However, despite having basal energy demands exceeding those of any other organ in the body, cardiomyocytes do not possess large reserves of substrates required to form high-energy phosphates[3, 4]. As such, the heart must rely on exogenous substrate supply to be transported into the cardiomyocytes in order to be catabolized and to produce ATP. Because of this, continuous transport of substrates such as fatty acids (FAs), glucose, and lactate into the cardiomyocytes is a key component of cardiac energy metabolism[5]. Once transported into the cardiomyocyte, these substrates are catabolized to produce ATP. During normal physiological conditions, this production of ATP is met almost entirely (>95%) through oxidative phosphorylation[2-4, 6]. Specifically, FA oxidation is responsible for the generation of 50–70% of ATP in a normal adult heart while only 20–30% of the energy provided is derived from glucose and less than 5% from other sources. Long-chain FAs (LCFAs) serve a variety of roles in cellular homeostasis. In

addition to mitochondrial energy conversion, they act as an energy store after esterification into the triacylglycerol pool and are constituents of membrane phospholipids[7]. Moreover, FAs are involved in co- and post-translational modification of proteins[8, 9].

The contribution of FA and other energy substrates to ATP generation is tightly regulated, and there is a significant degree of flexibility and interdependence between energy substrates utilized[10, 11]. Various cardiac physiologic and pathologic states can cause alterations of the tightly regulated pathways of myocardial energy substrate metabolism, and these alterations can contribute to the progression of myocardial damage. Therefore, it stands to reason that if physiologic and pathologic myocardial states contribute to metabolic perturbations, perhaps the reverse is true. That is, perhaps manipulation of myocardial energy metabolism can be used to alter the natural history of physiologic and pathologic myocardial stress states. As such, this thesis will explore how modulation of myocardial FA uptake and subsequent utilization contribute to myocardial performance during a variety physiologic and pathologic stressors such as, increased cardiac workload, ischemia/reperfusion (I/R) injury, and doxorubicin-induced cardiac dysfunction. While the initial three studies presented in this thesis will address how manipulation of cardiac energy metabolism during stress may benefit the heart in mouse models, the last chapter will examine whether diabetic patients using ATP-sensitive potassium channel (K_{ATP}) inhibitors are at risk during an acute coronary event. Together, the work presented in this thesis provides significant insight into physiologic and pathophysiologic processes that alter myocardial metabolism as well as examine if manipulation of FA metabolism and treatment of disease that cause alterations in FA metabolism may alter the natural history of these processes in order to benefit patients.

FA Uptake and Utilization in the Heart

Overview of fatty acid transport

As the cardiomyocyte is not capable of *de novo* synthesis of FAs and possesses limited amounts of stored cytoplasmic FA, the cardiomyocyte relies heavily on exogenous FA transport to provide substrates for the eventual production of ATP[12]. FAs are delivered to the heart from two main sources: (1) FAs that are contained in circulating triacylglycerol (TAG)-rich lipoproteins (very low density lipoproteins (VLDL) and chylomicrons)[13, 14]; and (2) non-esterified FA; referred to as free FAs[15] (FFAs). Transport of TAG-rich lipoproteins from the capillary lumen to the cardiomyocyte is postulated to occur via lipoprotein lipase (LPL) catalyzed hydrolysis of the TAG core[16-18] and lipoprotein receptor-mediated endocytosis[19-21]. FFAs cross into the cardiomyocyte by two distinct mechanisms[22, 23]: (1) passive non-protein mediated flip flop mechanism[22, 24-26] and (2) protein-mediated active transport[22, 26-28].

Fatty acid uptake

As stated above, FFAs originating from either albumin or lipoprotein-TAG enter the cardiomyocyte either by passive diffusion, or via a protein carrier mediated pathway[29]. Although passive diffusion contributes to the transport of FAs into the

cardiomyocyte, the extremely high metabolic demand of the heart makes free diffusion of FAs inadequate to maintain function. Due to this inadequacy, protein-mediated transport of FAs is especially important in the heart[23]. A number of cardiac FA transporters have been identified, including: (1) FA binding protein ($FABP_{pm}$)[26, 27]; (2) FA transport protein (FATP) 1-6[28, 30] and (3) CD36[15, 31-34]. Genetic ablation of these proteins from mouse models indicates that these proteins accomplish the majority of FA transport into the cardiomyocyte[26, 35, 36].

$FABP_{PM}$ is a 40 kDa peripheral membrane protein at the outer leaflet of the plasma membrane[37-39]. Studies of overexpression of $FABP_{PM}$ in skeletal muscle have shown increases in FA sarcolemmal transport[26]. As well, using an inhibitory antibody to $FABP_{PM}$ has shown decreased FA uptake[27, 39]. The importance of $FABP_{PM}$ to myocellular FA uptake has also been shown through work in the cardiomyocyte[27]. As well, FATP1, which is present in the heart but is also strongly expressed in adipose tissue[40, 41], and FATP 6, which has heart-specific expression[30], are the two members of the FATP family found in the cardiomyocyte. Studies have shown that in mice exhibiting cardiomyocyte-specific overexpression of FATP1 there were increased rates of palmitate esterification and oxidation, along with decreased rates of glucose utilization[42]. Moreover, in mice with whole-body FATP1 ablation, insulin-stimulated TAG synthesis was blunted, and diet-induced insulin resistance was prevented[43]. As well, FATP6 localizes to the sarcolemma of cardiac myocytes where it co-localizes with CD36[30]. This fact suggests that a novel mechanism may exist for the uptake of FFAs into cardiac myocytes using FATP6[30]. Taken together, FATP 1/6 appear to play an important role in FA uptake into the cardiomyocyte.

CD36, an 88 kDa protein, is the most investigated member of the myocardial FA transport proteins. Loss of function and gain of function mouse studies have provided evidence that CD36 is responsible for 50-60% of myocardial FA uptake[44-47]. CD36 appears to translocate to the sarcolemma in response to various cellular stimuli[34]. This translocation between intracellular endosomes and the sarcolemma seems to be important in the regulatory control of FA uptake[48]. The precise mechanism of CD36-facilitated LCFA uptake is incompletely understood and different mechanisms have been proposed. Current popular opinion supports the idea that CD36 and $FABP_{pm}$ act in concert as acceptors of FFA[49]. This increases their local concentration and thus increases flip-flop passive diffusion of FFA into the cell[50]. As well, CD36 may act as a transporter itself and may bring FFA from the interstitium to FATPs, thereby increasing the rate of FA transport[30, 51]. Also, instead of functioning as classical pores/channels, CD36 and $FABP_{pm}$ have been suggested to mediate LCFA uptake by accelerating FA dissociation from albumin[52, 53]. FFA uptake across the lipid bilayer might also be facilitated by esterification-coupled influx, referred to as 'metabolic trapping'[53].

The role of CD36 in myocardial FA metabolism has also been shown in patients with genetic CD36 deficiency. When investigated with a FA radioactive tracer, these patients have been shown to have a reduction in myocardial FA uptake, consistent with the data produced in mouse models[54]. This is direct evidence that studies on CD36 via genetically altered mice may be translatable to our patients and lead to advances in patient care. Studies in this thesis will investigate the role of inducible cardiomyocyte-specific CD36 ablation in functional recovery following ischemia and reperfusion and the results will be described in chapter 3.

Intracellular control of FA transport

Once inside the cardiomyocyte, FAs need to be transported to their site of conversion to fatty-acyl CoA. Long chain FAs are converted into fatty-acyl CoA esters by one of the acyl CoA synthetases (ACSL1-6) [55, 56] . Although it is not clear which isozyme of ACSL is responsible for most of the conversion or where in the cell this occurs, significant evidence indicates that this reaction takes place on the cytoplasmic side of the mitochondrial outer membrane[56]. This transport of FA through the cytoplasm to the outer mitochondrial membrane is accomplished via cytoplasmic FABP ($FABP_C$) [57-59] . Previous studies have shown that in heart-type $FABP_C$ null mice, FA binding in cytoplasm was reduced, along with FFA uptake and FA β-oxidation[60]. The mechanisms of transfer of FAs from the inner-leaflet bilayer to FABPC and then subsequently to ACSL are not completely understood. However, when FAs are esterified via ACSL, they are removed from the concentration gradient. The importance of ACSL has recently been shown in work on ACSL deficient mice[56]. These mice were shown to have impaired myocardial FA β-oxidation[56]. Once FAs are converted to fatty acyl-CoAs, they can then proceed down either of two major pathways: (1) further esterification to form TGs and other intermediates[15, 55, 61] (2) transport into the mitochondria for eventual oxidation[61, 62].

Myocardial triacylglycerol metabolism

The heart does not maintain large energy reserves within the cardiomyocytes[2-4]. However, when FA substrate supply exceeds oxidation, the heart can store FAs in

TAGs, which can be later hydrolyzed in times of need[63-66]. TAG molecules are mainly produced through the glycerol phosphate pathway involving sequential acyltransferase and phosphatase reactions[67]. Overall, the formation of TAGs starts with an initial step regulated by glycerol-3-phosphate acyltransferase (GPAT) and the successive reactions ending with 1,2-diacylglycerol (DAG) converted to TAGs by diacylglycerols acyltransferase (DGAT) [68-70] . GPAT initiates TAG synthesis by catalyzing the acylation of glycerol-3-phosphate[67]. Obese mice with whole body GPAT ablation have reduced TAG content highlighting the importance of GPAT in TAG synthesis in the myocardium[71]. Following GPAT acylation, the resulting lysophosphatidate is further acylated by acyl-glycerol-3-phosphate acyltransferase (AGPAT) to phosphatidate, which is subsequently dephosphorylated to DAG by phosphatidic acid phosphatase (lipin). The role of lipin in the cardiomyocyte has been explored in the whole body loss of function mouse model in which, mice with lipin-1 deficiency exhibited a greater than 80% reduction myocardial PAP activity[72]. The final step in myocardial TAG synthesis is the acylation of DAG, which is catalyzed by DGATs. Two DGAT enzymes (DGAT1 and DGAT2) have been identified and both are expressed in the heart[73].

Similar to the multi-enzyme stepwise process of TAG synthesis, TAGs stored in lipid droplets, are hydrolyzed via the successive action of multiple enzymes including adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL) [67] . The breakdown of TAG is activated during times of increased cellular ATP demand as TAG supply fatty acyl-CoAs as a substrate for subsequent ATP production[74, 75]. The first step in this process is the hydrolysis of TAGs to DAG and the rate-limiting enzyme controlling this is ATGL[76, 77]. Previous work in our lab has shown that mice with inducible cardiomyocyte-specific ATGL ablation demonstrate

myocardial steatosis along with increased myocardial TAG content (unpublished data). Further work in our lab also showed that cardiomyocyte-specific ATGL over-expression exhibited chronically reduced myocardial TAG content[78]. This work provides evidence, with both loss of function and gain of function studies, of the key role of ATGL in myocardial TAG content[78]. Furthermore, these studies revealed that genetic manipulation of the intramyocardial TAG pool altered overall cardiac metabolism and function, therefore suggesting that the regulation of cardiac TAG metabolism plays a critical role in healthy and diseased hearts. Studies in this thesis will investigate the role of cardiomyocyte-specific ATGL overexpression in the doxorubicin-induced cardiac dysfunction and the results will be described in chapter 4.

Mitochondrial FA uptake

To generate ATP, the fatty acyl-CoAs that are either broken down from the TAG storage pool or are immediately targeted for catabolism must be transported into the mitochondria to undergo β-oxidation. FA entry into the mitochondria occurs through the carnitine palmitoyl-transferase (CPT) system initiated by the rate-limiting enzyme, CPT-1[79-81]. CPT-1 catalyzes the conversion of fatty acyl-CoA to acylcarnitine, which is subsequently transported across the mitochondrial membrane.

Adenosine monophosphate-activated protein kinase (AMPK), a well-described energy sensing kinase, regulates mitochondrial FA uptake via CPT-1. AMPK accomplishes this regulation by preventing the production of malonyl CoA via the inhibitory phosphorylation of acetyl CoA carboxylase (ACC) [80, 82-88] . This set of enzymes is known as the AMPK-ACC-malonyl CoA axis. AMPK phosphorylates the two

isoforms of acetyl coenzyme A carboxylase (ACC 1 and 2) at two well-characterized serine residues (Ser79 and 221) and subsequently inhibits ACC activity[82-85]. As ACC converts acetyl CoA to malonyl CoA, AMPK-mediated inhibition of ACC results in a decrease in malonyl CoA levels. Malonyl CoA is a potent endogenous inhibitor of CPT-1 and the reduction of malonyl CoA levels secondary to AMPK activation removes inhibition of CPT-1 and subsequently increases fatty acyl-CoA transport into the mitochondria for β-oxidation. Studies in this thesis will investigate the influence of AMPK inhibitory phosphorylation of ACC on myocardial metabolism and function and the results will be described in chapter 5.

As stated above, CPT-1 converts fatty acyl-CoA to acylcarnitine. This acylcarnitine is then exchanged for carnitine across the mitochondrial membrane by carnitine:acylcarnitine translocase (CT). Therefore, CT is not only important for acylcarnitine shuttle into the mitochondrial matrix but CT also provides cytoplasmic carnitine for CPT-1[89]. Upon entry to the mitochondrial matrix, CPT-2 catalyzes the reaction that converts acylcarnitine to its original fatty acyl-CoA compound[90].

The identification of the presence of CD36 on the mitochondrial membrane has lead to controversy as to the role of FA transport proteins in mitochondrial FA uptake[91-93]. Although it has been shown that CD36 exists at the mitochondrial membrane in mouse skeletal muscle[91-94], a contrary study showed no evidence of CD36 within the mitochondria[94]. Regardless of the existence of CD36 on the mitochondrial membrane, further studies suggest that the presence or absence of CD36 in isolated mitochondria has no effect on mitochondrial FA uptake[95]. However, studies in skeletal muscle of mice with whole body CD36 ablation showed that FA entry into the mitochondria is significantly decreased in CD36 deficient mice when compared to controls[96].

Therefore, whether or not CD36 in the mitochondria regulates mitochondrial FA uptake remains to be clarified.

FA β-oxidation

The fatty acyl-CoA compound in the mitochondrial matrix undergoes FA β-oxidation. FA β-oxidation is a cycle that involves four enzymes, acyl CoA dehydrogenase, enoyl CoA hydratase, 3-OH acyl CoA dehydrogenase, and 3-ketoacyl CoA thiolase (3-KAT) [97] . Each enzyme has multiple isoforms in the mitochondria for different fatty acyl-CoA chain lengths. Each cycle of FA β-oxidation shortens the FA acyl-CoA compound by 2 carbons while producing acetyl CoA, flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH).

The cycle has numerous levels of control including transcriptional regulation and feedback inhibition[98]. The transcriptional control of the enzymes of FA β-oxidation is regulated primarily by nuclear receptor transcription factors that include PPAR-α[11, 99, 100]. PPAR-α is a member of a ligand-activated nuclear receptor superfamily[11]. PPAR-α forms a heterodimer with the retinoid X receptor and binds to the PPAR response element found on the promoter region of target genes to increase their expression[11, 101]. PPAR-α target genes constitute a comprehensive set of genes that participate in many if not all aspects of lipid utilization[102] (see [103] for review). This includes very long-chain acyl CoA dehydrogenase (VLCAD), long-chain acyl CoA dehydrogenase (LCAD), medium-chain acyl CoA dehydrogenase (MCAD), 3-KAT, mitochondrial uncoupling (mitochondrial thioesterase [MTE]-1 and uncoupling proteins

[UCP]2/3) [100, 102] . In addition, PPAR-α negatively regulates glucose oxidation by up-regulating gene expression of pyruvate dehydrogenase kinase (PDK)-4[100]. As well, in terms of control via feedback inhibition, mitochondrial acetyl CoA accumulation has been shown to inhibit 3-KAT.

Glucose Uptake and Utilization in the Heart

Glucose uptake

Glucose transport in the cardiomyocyte is the first step in carbohydrate metabolism and is mainly dependent on insulin levels and contractile activity[104-108]. Glucose movement into the cardiomyocyte is primarily mediated through sarcolemmal membrane protein facilitated transport[104, 107-113]. Members of the glucose transporter (GLUT) family that belong to a large family of twelve transmembrane segment transporters are predominantly responsible for glucose transport[112-115]. In the cardiomyocyte, insulin-independent glucose transport occurs mainly via GLUT1[116, 117]. The translocation of GLUT4, which is the foremost glucose transporter in the cardiomyocyte[116, 118, 119], is predominantly thought to be insulin-dependent.

Glycolysis

Once glucose has entered the myocellular cytoplasm it is converted to glucose-6-phosphate by hexokinase, which can then enter into multiple different pathways including glycolysis, glycogen synthesis, or the pentose phosphate pathway. Only glycolysis will be discussed in this thesis. Glucose-6-phosphate is subsequently converted to fructose-6-phosphate in a reaction catalyzed by phosphoglucose isomerase. The next step in glycolysis is the rate-determining step catalyzing the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate by phosphofructokinase-1 (PFK1). AMPK activation promotes the activity of PFK-1 via phosphorylation and activation of PFK2[120]. PFK2 catalyzes the reaction of fructose-6-phosphate (F6P) to fructose 2,6 bisphosphate (F2, 6BP) [121] . An increase in accumulation of F2, 6BP is an activator of PFK1[120, 121], the enzyme responsible for the conversion of F6P to F1, 6BP, the rate limiting step of the glycolysis pathway[121]. As such, activation of AMPK plays a major role regulating glycolysis in the heart.

Following the reaction of PFK1, the six carbon fructose 1,6-bisphosphate molecule undergoes a series of steps forming two separate three carbon molecules that get converted to 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate (GAP) by GAP dehydrogenase (GAPDH). As this reaction is dependent on the reduction of NAD^+ this step in glycolysis is regulated by the reduction-oxidation status of the cardiomyocyte. Following its formation, 1,3-bisphosphoglycerate undergoes a series of glycolytic steps ultimately being converted to pyruvate in a reaction catalyzed by pyruvate kinase. Glycolysis produces a relatively small amount of the overall ATP generated by the cardiomyocyte, however, glycolysis is unique in that ATP generation does not require oxygen. Therefore, as discussed later in this introduction, the role of glycolysis is emphasized during myocardial ischemia.

Glucose oxidation

As stated above, under normal physiological conditions approximately 50 - 70 % of acetyl CoA-derived ATP originates from FAs and 20 - 30% is derived from glucose[2-4, 61, 122]. Acetyl CoA derived from glucose is produced via the conversion of pyruvate to acetyl CoA. Pyruvate that has entered the mitochondria is decarboxylated to acetyl CoA, CO_2 and NADH by the pyruvate dehydrogenase (PDH) complex. Pyruvate decarboxylation catalyzed by PDH is the key step in the ability for glucose to be completely oxidized. As such, this key step in glucose oxidation is under strict regulation in the cardiomyocyte. PDH is regulated by transcriptional control and post-translational modification by phosphorylation[123, 124]. Inhibitory phosphorylation of PDH is catalyzed by PDH-kinase (PDK), which can be reversed by activating dephosphorylation by PDH-phosphatase (PDHP)[123, 125-127]. Therefore, regulation of PDK is another level of control over PDH activity. As such, in the cardiomyocyte and PDK has been shown to be under negative feedback inhibition via ADP content as well as pyruvate levels in the heart.

The Interaction Between Fatty Acid and Glucose Metabolism

The heart balances between FA and glucose utilization for the production of ATP. The relationship between glucose and FA cycles is known as the Randle cycle[125, 126]. As stated above, PDH is under strict regulation in the cardiomyocyte, and the

control of PDK is another level PDH regulation. As such, it has been shown that high levels of acetyl CoA, derived from both FAO and glucose oxidation, can upregulate PDK activity in the cardiomyocyte, which will decrease the rate of glucose oxidation [128] . Increased levels of FA derived acetyl CoA also effect glucose utilization since citrate, a tricarboxylic acid intermediate, is a PFK1 inhibitor[129, 130]. Therefore, the Randle cycle shows that the primary influence of increased FAO on the cardiomyocyte is decreased glucose utilization. Correspondingly, the Randle cycle also describes the reciprocal relationship where, rises in glucose uptake and subsequent utilization decrease overall FA utilization in the cardiomyocyte. That is, studies have shown that the final enzyme in FAO, 3-KAT, is negatively inhibited by glucose-derived acetyl CoA production[131]. Another example of the increase in glucose oxidation causing a decrease in FAO is the increase in malonyl CoA content during times of high glucose oxidation. This occurs as a result of increased glucose derived acetyl CoA production leading to the conversion of acetyl CoA to acetyl-carnitine that is shuttled to the cytoplasm by CT. This cytoplasmic acetyl-carnitine is then converted back to acetyl-CoA which is the substrate used by ACC to form malonyl CoA, which is a potent inhibitor of CPT-1 and thus decreases FAO[11].

The concept of the Randle cycle is still a major area of interest in cardiac metabolism, 50 years after Randle's original published findings. This thesis will focus on genetically modified mouse models that test the Randle's hypotheses as well as use them to generate hypotheses on ways to modify metabolism to gain functional improvements during myocardial health and disease.

Physiologic Regulation of Myocardial FA Uptake and Utilization

Increased myocardial workload, such as that seen with exercise, may result in the heart increasing cardiac output greater than six fold above basal levels [132] . This increase in myocardial work results in an increase in exogenous substrate uptake by the cardiomyocyte. It has also been shown that increased myocardial work also causes an increase in FA and glucose uptake as well as an overall increase in oxidative phosphorylation, both FA β-oxidation and glucose oxidation [133-135] . As well, it has previously been shown that during high work loads in the heart, malonyl CoA content is significantly decreased, suggesting one mechanism by which enhanced FA β-oxidation occurs [87, 136] . However, the precise role of the influence of AMPK inhibitory phosphorylation of ACC on myocardial metabolism and function during times of increased myocardial work is unknown and will be further explored in chapter 5 of this thesis.

Regulation of FA uptake and transport occurs at every level and ultimately affects the metabolic state and function of the cardiomyocyte. Regulation first starts with the systemic control of circulating sources of cardiac FA. That is, the regulation of circulating FFA, VLDL-TAGs and chylomicron (CM)-TAGs. Circulating levels of FFA in the vasculature has a large range during normal physiology. The fed, fasted and exercise states all manipulate FFA through physiologic regulation. Control of lipolysis in adipose tissue is a fundamental determinant of systemic FA supply. A wide range of hormones and other substances have been recognized as regulators of lipolysis, but insulin and catecholamines appear to be central to systemic lipolytic regulation [137] . In the fed state, insulin release has an anti-lipolytic effect thereby decreasing circulating

FFA [137] , whereas, during fasting, insulin release is down regulated thus removing inhibitory control of lipolysis and increasing circulating FFA [137] . As well, during exercise, β adrenergic effects stimulate lipolysis and increase circulating FFA [137] . The main target of regulation of circulating FFA is the inhibition of activation of two in vivo hormones; HSL and ATGL [138, 139] . The effects of these two systemic stimuli on circulating FFAs ultimately also alter cardiac energy status. That is, changes in FFA concentration, directly correlate with rates of myocardial FA β-oxidation in the acute setting[11]. Plasma TAG levels are determined by the balance between the rate of production of chylomicrons and VLDL in the intestine and liver, respectively, and their rate of clearance in peripheral tissues [140] . In normal individuals the majority of TAG input into the plasma is of dietary origin [140] .

FA transporters themselves are also highly regulated. For instance, studies have shown that the well-known AMPK activator, activator 5-amino-4-imidazolecarboxamide riboside (AICAR), regulates the translocation of $FABP_{PM}$ from intracellular pools to the plasma membrane [141] . This suggests that at times when there is a requirement for an enhanced ATP production, such as increased myocardial workload, the rate of LCFA oxidation is regulated in part by $FABP_{PM}$ translocation to the sarcolemma. Furthermore, although the mechanisms involved in regulating CD36-mediated FA transport are poorly understood, in the cardiomyocyte, contraction-induced increase in FA transport is accompanied by concurrent translocation of CD36 from an intracellular depot to the sarcolemmal membrane. In addition, others have shown that activators of AMPK such as leptin, oligomycin, AICAR, and dipyridamole, induce the translocation of CD36 to the sarcolemmal membrane in the heart, further suggesting that FA uptake is enhanced during times of increased myocardial workload [141-143] . However, it should be noted

that in contrast to these findings, other studies have suggested that increased AMPK activity is not essential for increased FA transport and eventual utilization [144] . This has been demonstrated through work in AMPK kinase dead (KD) mice, where CD36 translocation to the sarcolemmal membrane has been shown to be independent of AMPK activity in skeletal muscle [145] . Whether or not these AMPK-independent mechanisms of CD36 translocation are specific to skeletal muscle only or are also found in the heart is currently unknown.

Another FA transporter, FATP, is regulated by numerous mechanisms in normal physiology including nutrients, hormones and cytokines. Interestingly, AMPK activation in the adipocyte has been suggested to occur as a result of a FATP-dependent mechanism that causes an increase in the AMP/ATP ratio [146] . This finding suggests that FA transporter activation may actually be upstream of AMPK [146] . Although this upregulation of AMPK via FA transporters has not yet been demonstrated in the cardiomyocyte, it does highlight the fact that FA transporters can influence AMPK to alter intracellular substrate utilization just as AMPK targets FA transporters to alter substrate uptake. The extent and/or importance of this reciprocal relationship has not yet been fully explored and is thus poorly understood, however, the degree by which the cardiomyocyte can vary FA uptake during normal physiology highlights the relative importance of substrate flux in heart.

Myocardial Ischemia

Acute Coronary Syndrome

Myocardial ischemia results from an imbalance in myocardial oxygen supply and demand. Multiple mechanisms can cause an ACS event but the most common cause is inadequate coronary blood flow. Reduction in coronary blood flow is primarily due to narrowing of the coronary arteries by atherosclerotic disease. The progression of atherosclerosis resulting in a significant coronary artery lesion most commonly presents in the sixth to seventh decade of life[147]. Atherosclerosis is the accumulation of extra-cellular lipid between the intima and media of the vessel wall. This accumulation is characterized by a fibrous cap with large fibrofatty deposit underneath[147]. Acute coronary syndrome (ACS) develops when there is disruption of the fibrous cap resulting in a stimulus for thrombogenesis. This acute decrease in coronary artery lumen diameter, and thus coronary artery blood flow, leads to an ACS event.

Acute coronary syndrome (ACS) constitutes a clinical syndrome with three major subsets; 1) Unstable Angina (UA), 2) non-ST-elevation myocardial infarction (NSTEMI) and 3) ST-elevation myocardial infarction (STEMI). UA is defined by positive biomarkers of necrosis in the appropriate clinical setting (chest discomfort or angina equivalent) not associated with electrocardiographic (EKG) changes. An NSTEMI is UA along with ST-segment depression or prominent T-wave inversion in the absence of ST-segment elevation on EKG, and STEMI is UA with ST-segment elevation on EKG[147].

The Role of FA Uptake and Utilization during Ischemia and Reperfusion

During ischemia, many changes in myocardial energy metabolism also occur, which are primarily adaptive responses to the reduction in the availability of oxygen (Fig. 1N) [148-151] . The predominant metabolic change that occurs when the myocardium is

dealing with an inadequate supply of oxygen is a decrease in overall oxidative phosphorylation (both glucose and FA oxidation) [148-151] . Since oxidative phosphorylation is the primary means of myocardial ATP generation under non-ischemic conditions[2-4, 6], the cardiomyocyte attempts to compensate for this reduction in ATP production by increasing anaerobic metabolism and thus increasing flux through the glycolytic pathway [149] . However, as a result of increased glycolysis and an uncoupling of glycolysis from glucose oxidation, there is an increase in pyruvate conversion to lactate by lactate dehydrogenase (LDH) as well as excess proton accumulation [104, 150, 152-154] . Moreover, it has been proposed that the ATP generated from glycolysis is used by the cell to maintain intracellular ionic homeostasis instead of being used for contractile function and this likely contributes to impaired cardiac function during ischemia [149, 150, 152-154] .

While prolonged ischemia without reprieve will eventually lead to infarction and necrosis, reperfusion of the myocardium before infarction takes place can maintain cardiomyocyte viability [151, 155, 156] . Similar to the ischemic period, cellular reperfusion is also marked by metabolic alterations [151, 155-158] . For example, during reperfusion, FA oxidation recovers much quicker than glucose oxidation and FAs become the primary substrate for oxidative phosphorylation [2, 66, 159, 160] . Moreover, this increase in FA oxidation suppresses glucose oxidation by way of the Randle cycle[125, 126], which also leads to uncoupling of glycolysis and glucose oxidation [151, 154] . As in ischemia, the reperfusion period following ischemia has also been shown to have increased lactate formation, proton accumulation and a more acidic intracellular environment [148, 151, 160] . The extent to which these metabolic consequences contribute to myocardial reperfusion injury is as yet unclear. However, the concept that

proton production resulting from the uncoupling of glycolysis from glucose oxidation contributes to cardiac inefficiency will be explored in chapter 3 of this thesis.

Doxorubicin Induced Alterations in Myocardial FA Uptake and Utilization

Anthracyclines such as, doxorubicin are an effective class of antineoplastic agents [161] . Despite its efficacy in the treatment of a variety of cancers, the clinical use of doxorubicin is limited by cardiac side effects [162, 163] . Previous work in cultured cardiomyocytes, animals and humans have shown that doxorubicin alters myocardial FA uptake and utilization [164-168] . Doxorubicin has been shown to alter serum free FA levels in a rodent model [167, 169] . Furthermore, using ^{125}I-beta-methyl-branched FA (^{125}I-BMIPP), Wakasugi et al. showed in a rodent model that FA uptake decreased during the development of doxorubicin-induced cardiac dysfunction [170] . As well, in isolated cardiomyocytes others have shown that doxorubicin administration significantly reduced palmitate oxidation possibly by diminishing CPT-1 function [164] . Although numerous studies have shown doxorubicin-induced alteration in FA uptake and utilization in the heart, these studies have their own inherent limitations. Much of the conclusions drawn about doxorubicin-induced FA alterations in the heart are done on isolated cardiomyocytes [164, 171] . Although this has been an invaluable tool to answer many cardiac questions, due to the hearts constant state of work, conclusions drawn about metabolism in the heart when the cardiomyocyte is not beating against a workload are difficult to extrapolate to the *in vivo* environment. Furthermore, no studies to date have perfused doxorubicin treated hearts to understand definitively the metabolic profile of a heart subject to doxorubicin. As well, while many studies have examined the

effect of FA uptake and oxidation in the cardiomyocyte, no studies to date have examined the role of myocardial TAG metabolism in the contribution to doxorubicin-induced alterations in metabolism and function. Since previous studies have suggested that cardiomyocyte TAG catabolism plays an important role in regulating cardiac function in pathological conditions [67, 172] this thesis will further examine if manipulation of the myocardial TAG pool can be used as an approach to treat doxorubicin-induced metabolic and functional alterations in the heart.

The Role of FA Utilization in the Heart during Diabetes

Cardiac dysfunction in type 2 diabetes

The intramyocardial accumulation of lipids and/or lipid metabolites such as TAG, ceramides, DAG, and long chain acyl CoAs leading to cardiac dysfunction is known as 'cardiac lipotoxicity' [173] . Lipotoxic cardiomyopathy leads to a number of pathologic consequences including apoptosis, autophagy, fibrosis, and impairment in systolic and diastolic function[174-176]. This cardiac steatosis is a result of uncoupling between supply/uptake of free FA into the heart and subsequent utilization. This pattern of lipid accumulation in the heart is seen in insulin resistance and diabetes. This concept of lipid accumulation seen in diabetes leading to cardiac dysfunction has been explored in numerous mouse models[177-179]. Our lab and others have developed mouse models of proteins involved in FA metabolism in either loss of function or gain of function genetic manipulations [45, 78, 180, 181] . The results show that genetic manipulation leading to

increased myocardial FA uptake and utilization leads to a similar phenotype as that seen in diabetic hearts, highlighting the role of FAs in cardiac dysfunction during diabetes. One reason for the lack of awareness of cardiac lipotoxicity as a clinical entity is due to the fact that lipotoxic cardiomyopathy in humans often will occur concurrently with ischemic heart disease. Therefore, we tend to focus on the mechanical problem that we can offer mechanical therapy for (percutaneous coronary intervention or coronary artery bypass graft surgery) without giving as much attention to the possibility that another issue, a metabolic issue, may cause the cardiac dysfunction in our severe coronary artery disease patients.

FA Uptake in the Cardiomyocyte during Diabetes

Initial systemic adaptation to the mismatch in FA uptake and utilization is to store excess energy in the form of increasing adipose cellular volume. However, continual increase in adipose volume can eventually lead to FFA 'leak' into the circulation[179, 182, 183]. Although FFA concentration is a primary determinant of the rate of myocardial FA β-oxidation, there are other mechanisms involved. For instance diabetes is associated with increased levels of $FABP_C$[184] and CD36[185] in the cardiomyocyte. Heyliger et al. provided circumstantial evidence that in the diabetic rat heart, protein-mediated binding of FAs to the sarcolemma is elevated[186]. Studies conducted on diabetic mice revealed substantially increased CD36 levels in cardiac endothelium[187]. Furthermore, our lab has previously shown that increased CD36 expression promotes cardiac lipotoxicity in aged mouse hearts. This study further highlights the role that CD36 plays in myocardial substrate uptake and eventual metabolism. In chapter 3 this

thesis will further explore whether manipulation of cardiomyocyte-specific CD36 can alter myocardial function during pathologic stress.

FA Storage and Oxidation in the Cardiomyocyte during Diabetes

Diabetes is associated with increased changes in myocardial FA utilization. Growing evidence also supports the notion that diabetic changes in cardiac FA handling lead to eventual significant changes in function[188]. FAs that are taken up into heart and are primarily oxidized or stored as TAGs. As long as the FA uptake and utilization remains appropriately balanced, metabolic dysregulation does not occur. However, these processes are unbalanced toward lipid accumulation in insulin-resistant cardiomyocytes[189]. Further studies in our lab has shown that diabetes induced lipotoxicity can be prevented with the forced overexpression of ATGL in the cardiomyocyte, suggesting that manipulation of cardiac TAG metabolism can be used as an approach to treat metabolic perturbations in the heart [181] . As well, this increase in intracellular lipid accumulation does not seem to be simply attributable to a concomitant reduction in FA β-oxidation[190]. For example, in hearts of high fat fed rats, db/db mice, obese Zucker rats, and ZDF rats FAO is reduced slightly[189], unaltered[191, 192], or increased[193]. It is evident that mitochondrial overload of lipid is at least one factor leading to insulin resistance. Therefore, it is logical to speculate that enhancing FA β-oxidation without increased ATP consumption does not increase insulin sensitivity. Rather, correcting the “mismatch” between β-oxidation and TCA cycle activity by lowering β-oxidation may alleviate insulin resistance.

Altered Ionic Homeostasis in Hearts during Diabetes

Due to the significant increase in FA uptake and utilization during diabetes, there is a subsequent significant decrease in glucose oxidation via the well-described Randle cycle [125, 126] . One hypothesis relating the functional decline of the heart with diabetes is the concept of increased uncoupling of glycolysis in the cytoplasm from glucose oxidation in the mitochondria [194] . When this occurs, Brownlee has hypothesized that glycolytic intermediates are shuttled toward pathologic pathways leading to altered ionic homeostasis and myocardial contractile dysfunction [195] . As well, this alteration in cardiomyocyte ionic homeostasis and energetic status leads to alterations in myocardial ATP-sensitive potassium (K_{ATP}) channels.

The Role of K_{ATP} Channels in Cardiac FA Metabolism

Cardiac K_{ATP} channels

K_{ATP} channels were originally discovered in the heart of guinea pigs [196] , and are present in a number of metabolically active tissues including pancreatic islet cells, skeletal muscle, vascular smooth muscle, and the brain [197-200] . K_{ATP} channels are formed as a hetero-octamer complex of the sulphonylurea receptor (SUR) and an inward rectifier K^+ channel (Kir) [201, 202] . The SUR regulatory subunits and the pore-forming Kir subunits both have two isoforms [202-204] . While the isoforms of the SUR and Kir subunits of K_{ATP} channels have some organ-specificity with distinct electrophysiological

and pharmacological properties, the heart predominantly expresses Kir6.2 and SUR2A [205] . With the aid of pharmacologic inhibitors (e.g.: sulphonylureas) and openers (nicorandil) various roles of K_{ATP} channels emerged. For example, in the pancreatic beta cell, sulphonylurea-binding leads to inhibition of K_{ATP} channels, which alters the resting potential of the cell, leading to calcium influx and stimulation of insulin secretion [206] . However, the organ-specific role of K_{ATP} channels has been better defined with genetically engineered loss of function and gain of function mouse models of K_{ATP} channels. Kir6.2 knock out mice have emphasized the critical role of K_{ATP} channels in ischemic preconditioning (discussed below) [207] , while Kir6.2 transgenic mice that are ATP-insensitive have demonstrate tolerance to reduced ATP sensitivity in the cardiomyocyte compared to pancreatic beta cells [208] . Further work on Kir6.1 knock out mice show that these mice are prone to spontaneous ST elevations and sudden death [209] . In the heart K_{ATP} channels are involved in action potential duration, mediation of ST segment elevation, metabolic sensing and cardioprotection during ischemia. This thesis will focus on the latter two topics below.

K_{ATP} channels and the regulation of cardiac FA metabolism

K_{ATP} channel function is directly linked to cellular energetics. Since myocardial K_{ATP} channels are components of the cardiomyocyte energetic network, it stands to reason that the principle substrate for energy utilization in the heart, FAs, is linked to K_{ATP} channel function. Indeed it has been shown that LCFA as well as fatty-acyl CoA compounds modulate K_{ATP} channel function [210] . As well, in the mitochondria, Garlid et al. suggest open mitochondrial K_{ATP} channels alter mitochondrial architecture. That is,

an open K_{ATP} channel causes inner mitochondrial swelling which leads to greater efficiency in transfers between mitochondrial and cellular ATPases. This increase in myocardial efficiency protects cardiac function during ischemia and reperfusion [211] . Mitochondrial swelling has been shown to be beneficial to oxidative phosphorylation and ATP production [212].

K_{ATP} channels, ischemic preconditioning and diabetes

The most prominent role of myocardial K_{ATP} channels is ischemic preconditioning (IPC) and in protection of the cardiomyocyte during ischemia [213] . IPC describes the adaptation of the myocardium to ischemic stress preceded by short periods of ischemia and reperfusion [213] . Murray et al. showed that mice that were preconditioned with short periods of ischemia and reperfusion before a prolonged infarct had a significantly decreased infarct size compared to controls [213] . The role of K_{ATP} channels in IPC has been elucidated in loss of function studies. Suzuki et al. demonstrated that the significant reduction in infarct size seen in control mice that underwent IPC prior to a prolonged ischemic injury is not seen in K_{ATP} channel knock out mice [214] . That is, mice that lack K_{ATP} channels, have similar response to a prolonged ischemic insult whether they underwent IPC or not, suggesting that K_{ATP} channels play a critical role in IPC protection during ischemia [214] . Furthermore, The activation of the myocardial K_{ATP} channels is a key mediator of the ST segment elevation response to transmural ischemia.

This key role of K_{ATP} channels in myocardial protection during an ischemic event has lead to questions about whether diabetic patients with coronary artery disease

should take insulin lowering medications that inhibit K_{ATP} channels as this may inhibit the patients ability to gain the protection of IPC. Tomai et al. showed that patients on the insulin secretagogue, glibenclamide, had impairment of IPC during coronary angiography [215] . Consistent with the benefit of K_{ATP} channel activation, during ischemia and reperfusion, Gori et al. has shown that sildenafil prevented I/R induced endothelial dysfunction by opening K_{ATP} channels in humans [216] . Given the critical protective role of the K_{ATP} channels, this thesis will explore whether diabetic patients who are taking K_{ATP} channel blockers may have an adverse clinical outcome in the setting of ACS.

Hypothesis and Aims

General Hypothesis

Since the healthy adult heart obtains approximately 50 - 70 % of the myocardial acetyl CoA-derived ATP from FA, it stands to reason that manipulation of this process may alter the natural history and cardiac dysfunction during physiologic and pathophysiologic processes. This thesis examines three key aspects of FA utilization in the cardiomyocyte, that is, FA uptake, FA storage and FA oxidation. As well, we examine the role of the three elements of FA utilization in numerous physiologic and pathologic conditions in the heart including, increased workload, ischemia-reperfusion injury, doxorubicin-induced cardiac dysfunction and diabetes. As well, we examine the role of drugs used in the treatment of diabetes, a pathologic condition that alters myocardial FA metabolism, and how they may affect patient outcomes. Therefore, I hypothesize that manipulation of FA uptake and utilization can be used as an approach to optimize cardiac function during physiologic and pathologic stress.

Specific Aims

The specific aims of this thesis are described within the individual chapters pertaining to the experimental results.

Specific Aim 1

To use short-term inducible cardiomyocyte-specific CD36 ablation in order to test the hypotheses that: 1) ic*CD36*KO mouse hearts have reduced FA uptake, utilization and storage and 2) this alteration in cardiac substrate utilization leads to improved post-ischemic functional recovery. These studies will allow us to determine if the concept of a combined strategy of limiting FA uptake and partially inhibiting FAO is a beneficial therapeutic approach to reducing ischemic injury [22, 217] .

Specific Aim 2

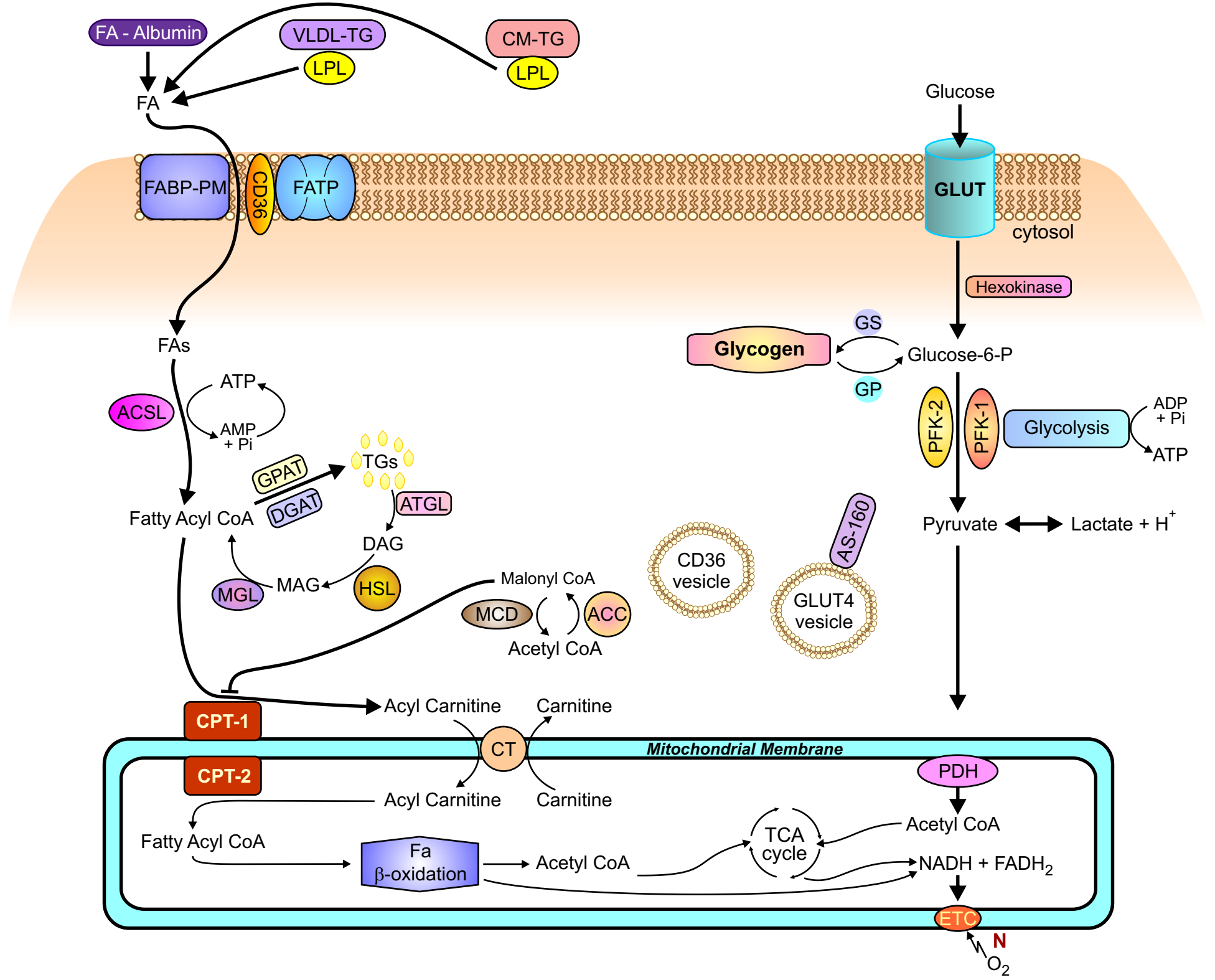
To use cardiomyocyte-specific ATGL overexpression in order to examine: (1) the effects of doxorubicin administration on myocardial exogenous substrate metabolism and TAG metabolism and (2) determine if manipulation of the intramyocardial TAG pool could be used as an approach to treat doxorubicin-induced cardiac dysfunction.

Specific Aim 3

To use ACC double knock-in mutation in order to understand the importance of the AMPK-ACC-malonyl CoA axis in the control of myocardial metabolism which will help define the mechanisms that control FAO in the heart as well as resolve controversies surrounding the role that AMPK activation plays in controlling myocardial injury in response to cardiac stress.

Specific Aim 4

To determine whether diabetic patients who had an ACS event while taking K_{ATP} channel inhibitors were at higher risk of death or heart failure than patients who were not exposed to KATP channel inhibitors in order to examine whether sulphonylureas impair the cardioprotective response to ischemia that is mediated by K_{ATP} channels.


FA - Albumin
VLDL-TG
LPL
CM-TG
LPL
FA
Glucose
FABP-PM
CD36
FATP
GLUT
cytosol
Hexokinase
FAs
GS
Glycogen
Glucose-6-P
GP
ATP
ACSL
AMP
+ Pi
PFK-2
PFK-1
Glycolysis
ADP
+ Pi
ATP
GPAT
TGs
DGAT
ATGL
Fatty Acyl CoA
DAG
Pyruvate
Lactate + H+
MGL
MAG
HSL
Malonyl CoA
CD36
vesicle
AS-160
GLUT4
vesicle
MCD
ACC
Acetyl CoA
CPT-1
Acyl Carnitine
Carnitine
CT
Mitochondrial Membrane
CPT-2
PDH
Acyl Carnitine
Carnitine
Acetyl CoA
Fatty Acyl CoA
Fa
β-oxidation
Acetyl CoA
TCA
cycle
NADH + FADH2
ETC
N
O2

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CHAPTER 2.

Materials and Methods

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Materials

Primary antibodies utilized for immunoblotting (described below) in this thesis were anti-CD36 (produced in-house as previously described[1]), anti-FATP1 (#14497; Santa Cruz Biotechnology), anti-ACSL1 (#4047; Cell Signaling), anti-ATGL (product number 2138; Cell Signaling, Beverly, MA), anti-perilipin-5 (number 03-GP31; American Research Products, Belmont, MA), anti-phospho(Ser660)HSL (product number 4126; Cell Signaling), anti-HSL (product number 4170; Cell Signaling), anti-ACC (product number 07-303; EMD Millipore, Billerica, MA), anti-AMPKα2 (number 19131; Santa Cruz Biotechnology, Dallas, TX), anti-phospho ACC (product number 3661; Cell Signaling) and anti-phospho AMPK (product number 2531; Cell Signaling) antibodies. Horseradish peroxidase (HRP)-labeled secondary antibodies were obtained from Santa Cruz Biotechnology. Protease inhibitor cocktail and Phosphatase inhibitor cocktail set IV were purchased from Sigma-Aldrich (St. Louis, Missouri) and Calbiochem (Gibbstown, NJ) respectively. Radioisotope chemicals were purchased from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). These chemicals included [U-^{14}C]glucose, D-[5-^{3}H]glucose, [9,10-^{3}H-(*N*)]Oleic acid and [9,10-^{3}H-(*N*)]palmitic acid. For immunoblotting, Trans-Blot®Transfer Medium (pure nitrocellulose) from BioRad (Richmond, California), FUJI Medical X-ray films from Mandel Scientific (Guelph, Ontario), and Western Lightning® Chemiluminescence Reagents Plus kit from Perkin

Elmer Life and Analytical Sciences (Woodbridge, Ontario) were used. Ecolite® Aqueous Counting Scintillation fluid was purchased from MP Biomedicals (Solon, Ohio). For UPLC analysis of long chain CoA esters a C18 column, 10cm x 2.1 mm and, 2.7 μm particle size, from Supelco. Unifilter P81 96-well filter plates were purchased from Whatman (Florham Park, New Jersey). Hyamine hydroxide (1 M in methanol solution) was obtained from NEN Research Products (Boston, Massachusetts). For determination of serum free fatty acids, HR-series non-esterified free FA (NEFA) determination kits from Wako Pure Chemicals Industries, Ltd (Osaka, Japan) were used. For the analysis of adenine nucleotides by UPLC, a Supelcosil™ LC-18-T Super Guard cartridge, 5 µm particle size, 2 x 4 mm and a Supelcosil™ LC-18-T, 5 µm particle size, 250 x 4.6 mm column were obtained from Supelco/Sigma-Aldrich (St. Louis, Missouri).

Methods

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with the Canadian Council on Animal Care guidelines. The University of Alberta Health Sciences Animal Welfare Committee approved all animal procedures.

Inducible Cardiomyocyte-Specific CD36 Knock Out Mice

The targeting vector (Fig. 2-1A) was engineered to generate flox/flox CD36 mice for creation of the inducible CD36 cardiomyocyte-specific mutant. This vector contains 3 Lox-P and 2 FRT sites, which are sites for Cre and FLP recombinases, respectively. The vector targets mouse CD36 exons 2 and 3, and contains a Neomycin resistance gene cassette (NEO) for selection in embryonic stem (ES) cells. We previously successfully targeted exon 2, which contains the translation start site (black arrow), to create total*CD36*KO mice[2]. An important difference between our previous strategy[2] and this one is that the murine CD36 gene in this case was identified and cloned from a C57Bl/6 bacterial artificial chromosome (BAC) library and transfected into C57Bl/6 ES cells. The resulting mice are 100% C57Bl/6 and this avoids the potential contribution of strain differences to phenotype ("passenger effect"). The use of the Cre-Lox and FLP-FRT systems allowed for selection of ES cells in G418, identification of the homologously recombined allele, and then removal of the NEO cassette, using FLP recombinase (an extra LoxP site was engineered around the NEO cassette in case FLP recombinase failed). This precludes any potential contribution of the NEO cassette to normal expression and regulation of the endogenous allele prior to cell/tissue specific removal of the exons of interest. After FLP mediated removal of NEO, Lox-P sites remain encompassing exons 2 and 3. When Cre recombinase is expressed, excision of these exons occurs.

After transfection by electroporation and selection using G418, ES cell genomic DNA was isolated from surviving colonies and Southern blot analysis using a probe that would differentiate the homologously recombined allele from the endogenous allele was

performed and potential clones identified (data not shown). PCR and Southern blot confirmed that the integration was through homologous recombination and not random integration, by using probes/primers outside the targeting vector (data not shown). The individual positive clones were also sequenced for confirmation of integration of all 3 Lox-P sites. Five ES clones positive for homologous recombination by Southern blotting were identified; only one was confirmed by sequencing to have all 3 Lox-P sites. This clone, 186, was injected into 129svj blastocysts and resulted in chimeras with significant male skewing. (This is of note because the ES cells were male derived.) High level male chimeras were mated to C57Bl/6 mice, and black pups were born (offspring of the 129svj genome are agouti), indicating germ line transmission of our ES clone. These mice were brother-sister mated to generate mice for subsequent mating to Cre lines.

We devised a specific PCR strategy to differentiate flox/flox from flox/wt (Fig. 2-1B) and then mated against the tamoxifen-inducible myosin heavy polypeptide 6, cardiac muscle alpha Cre mouse (cardiomyocyte specific, Jackson Labs strain 005657, B6.FVB(129)-Tg(Myh6-cre/Esr1*)1Jml/J, 15x back crossed to C57Bl/6). Mice used for experimental purposes were housed on a 12-h light/12-h dark cycle with ad libitum access to chow diet (#5001, Lab Diet, St. Louis, MO; 13.5% kcal from fat) and water. Both male and female mice exhibited similar baseline metabolic alterations as a result of CD36 deletion and therefore both sexes were used for experimentation.

Cardiomyocyte-Specific ATGL Overexpressing Mice

ATGL over-expressing (MHC-ATGL) mice were generated as described previously[3]. MHC-ATGL mice were generated in the laboratory of R. Zechner using

the α-myosin heavy chain (αMHC) promoter (GenBank accession no. U71441), and was fused to the full-length murine Pnpla2 cDNA as described by Haemmerle et al. [4]. Construction of the ATGL targeting vector was carried out in the plasmid pBS II SK+[4]. The targeting vector was then inserted into B6D2 zygotes and transferred to pseudopregnant females [4]. Subsequent genotyping of the offspring was done by DNA extraction from mouse tail tips and polymerase chain reaction analysis[4]. Animals with elevated ATGL expression were identified and homozygous pairs were formed to allow congenic lines of over-expressors and WT littermate animals to breed[4]. Throughout the rest of this thesis, ATGL overexpressing mice will simply be referred to as MHC-ATGL.

Acetyl-CoA Carboxylase Double Knock-in Mutant

This transgenic strain was generated in the laboratory of Dr. Greg Steinberg and Dr. Bruce Kemp. ACC1-S79A and ACC2-S212A knock-in mice were generated by OzGene Pty Ltd. The targeting strategy is summarized in Fig 2-2. Chimeric WT/KI$^{flox\text{-}Acc1(S79A)}$ mice were crossed with the OZ-Cre deleter strain. Genomic DNA from two litters was purified, *Bam*HI digested, resolved, transferred to nylon, and probed with probe 1 (Fig. 2-2A). Chimeric WT/KI$^{flox\text{-}Acc2(S212A)}$ mice were crossed with the OZ-Cre deleter strain. Genomic DNA from two litters was purified, *Bgl*II digested, resolved, transferred to nylon, and probed with probe 2 (Fig 2-2B). For ACC1 KI, the desired WT (10.8 kb) and Cre-deleted KI locus (6.5 kb) bands are observed for two progeny (A1 & A2). The filter was stripped and re-probed with a Cre-specific probe (bottom panel) to confirm transmission of the Cre-transgene. For ACC2 KI, the desired WT (10.7 kb) and Cre-deleted KI locus (9.4 kb) bands are observed for 5 progeny (A15 to A19). The filter was

stripped and re-probed with a Cre-specific probe (bottom panel) to confirm transmission of the Cre transgene (A15, A16 and A18) (Fig. 2-2C).

Tamoxifen Administration and CD36 Ablation

Tamoxifen (T5648, Sigma) dissolved in corn oil was administered orally to adult 12-16 week-old control and littermate ic*CD36*KO mice at a dose of 100 mg/kg/day for 5 consecutive days. All experiments were performed 4-6 weeks following final tamoxifen administration. This time point was chosen to avoid any confounding influence from possible transient cardiomyopathy due to tamoxifen-induced nuclear Cre translocation [3] in male and female ic*CD36*KO and littermate control mice.

Induction of doxorubicin-induced cardiac dysfunction

Mice were divided into 4 groups: a) WT + saline (n=5), b) WT + doxorubicin (n=7), c) MHC-ATGL + saline (n=5) and d) MHC-ATGL + doxorubicin (n=7). Doxorubicin (Sigma) was administered weekly at a cumulative dose of 32 mg/kg via intraperitoneal (i.p.) injections at 8 mg/kg body weight for 4 weeks. The corresponding volume of saline was administered to the control groups. Previous reports have shown left ventricular (LV) dysfunction and remodelling without overt heart failure with this doxorubicin dose, route of administration and frequency of injection [5]. Thus we chose this protocol of LV dysfunction rather than heart failure to mimic a clinically relevant scenario since the incidence of heart failure in recent doxorubicin regimen trials is less than 2.1% [6].

Echocardiography and Dobutamine Stress Echocardiography

Mice were mildly anesthetized using isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada). To assess *in vivo* heart function under high workload conditions, dobutamine was administered at a dose of 4mg/kg via intraperitoneal injections during echocardiography. Ten minutes after dobutamine injection, *in vivo* function was assessed, and hearts were harvested and frozen for subsequent biochemical analysis.

M-mode echocardiographic images were used to measure left ventricular wall thickness in systole and diastole, as well as left ventricular chamber diameters in systole and diastole. These measurements were used to calculate left ventricular volumes in systole and diastole as well as ejection fraction, fractional shortening, LV mass, and cardiac output. Tissue Doppler echocardiographic images were used to measure the E-wave and A-wave of diastolic filling. These measurements allow for the calculation of IVRT, MV deceleration rate and ratio's of E-waves and A-waves.

Isolated Working Heart Perfusions

Hearts were perfused aerobically in working mode with Krebs-Henseleit buffer containing, 5 mmol/liter glucose, and 50 μU/ml insulin and either 1.2 mmol/L palmitate prebound to 3% delipidated bovine serum albumin (BSA) or 0.8 mmol/liter oleate prebound to 3% delipidated BSA as described previously[3]. Mice were euthanized in the fed state, and hearts were dissected and subsequently perfused. Preload and afterload pressure were set to 11.5 and 50 mmHg, respectively. To assess *ex vivo* heart function under high workload conditions, hearts were perfused for an initial period of 30 min at normal workload followed by a 30-min perfusion at 80 mmHg afterload with buffer containing an additional 300 nmol/liter isoproterenol. To assess *ex vivo* heart function during ischemia and reperfusion, hearts were aerobically perfused for 30 minutes, or aerobically perfused for 30 minutes followed by 18 minutes of global no flow ischemia and 40 minutes of reperfusion. At the end of aerobic perfusion, high workload perfusions or reperfusion, hearts were immediately frozen in liquid N_2 with a Wollenberger clamp and stored at -80°C as described previously[3]. Following perfusion, atria were removed and ventricles were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

Measurement of Mechanical Function in Isolated Working Mouse Hearts

Heart rate and peak systolic pressure and developed pressure (mmHg) were measured with a Gould P21 pressure transducer (Harvard Apparatus) connected to the aortic outflow line. Cardiac output and aortic flow (mL/min) was measured with Transonic T206 ultrasonic flow probes in the preload and afterload lines, respectively. Diastolic pressure (mmHg) was calculated as the difference between peak systolic pressure and

developed pressure. Mean arterial pressure was calculated as the sum of 2/3 of the diastolic pressure and 1/3 of the peak systolic pressure. The rate pressure product was calculated as the product of heart rate and the peak systolic pressure. Coronary flow was calculated as the difference between the cardiac output and aortic flow. Cardiac power was calculated as the product of the difference in peak systolic pressure and preload by cardiac output. Data were collected utilizing an MP100 system from AcqKnowledge (BIOPAC Systems, Inc USA).

Measurement of Glycolysis, Glucose and Fatty Acid Oxidation

Glycolysis, glucose oxidation and FA (palmitate or oleate) oxidation rates were measured by perusing hearts with either a combination of [9,10-^{3}H]palmitate/[9,10-^{3}H]oleate and [U-^{14}C]glucose (for determination of FAO and glucose oxidation) or [U-^{14}C]glucose and [5-^{3}H]glucose (for determination of glucose oxidation and glycolysis) as previously described[3]. Glycolysis rates were quantified by collecting $^{3}H_2O$ derived from [5-^{3}H]glucose, glucose oxidation rates were quantified by collecting $^{14}CO_2$ derived from [U-^{14}C]glucose and FAO rates were quantified by collecting $^{3}H_2O$ derived from [9,10-^{3}H]palmitate/[9,10-^{3}H]oleate at 10 minute intervals as described previously[3]. $^{3}H_2O$ used in the measurement of FAO and glycolysis was measured by 'vapor transfer method' [7]. A microcentrifuge tube containing 200 μL of sample was placed in a scintillation vial containing 500 μL of water. This capped tube was placed in a 50 ^{o}C oven for 24hours and then placed in a 4 ^{o}C fridge which caused evaporation followed by condensation of $^{3}H_2O$. The microcentrifuge tubes are removed and scintillation fluid is added for subsequent counting of radioactive $^{3}H_2O$. $^{14}CO_2$ was used to measure glucose

oxidation rates. Briefly, gaseous $^{14}CO_2$ released is trapped in a hyamine hydroxide solution while $^{14}CO_2$ in solution is trapped on filter paper saturated by hyamine hydroxide [7].

Analysis of Serum Free Fatty Acids

Non-esterified free FA (FFA) concentrations were determined in serum collected from fed mice using a HR Series NEFA-HR free FA determination kit (Wako Diagnostics, Germany). Blood was taken at the time of euthanization and kept on ice. Whole blood was centrifuged for 10 min at 4,000 rpm at 4°C and serum collected and stored at -80°C until time of analysis. The series of reactions used to quantify FFA in serum utilize the catalyzation of fatty acyl-CoA compounds by acyl-CoA synthetase (ACSL). The fatty acyl CoA compounds formed then react with acyl-CoA oxidase to form H_2O_2. The H_2O_2 produced along with HRP cause the 3-methyl-N-ethyl-N(∫-hydroxyethyl)-aniline (MEFA) and 4-aminoantipyrine to oxidize and form a purple color that has a wavelength at 550nm.

Tissue Homogenization and Immunoblot Analysis

Frozen hearts were ground using mortar and pestle. Tissues were homogenized in lysis buffer comprised of 20mM Tris, 5mM ethylenediaminetetraacetic acid (EDTA), 10mM sodium pyrophosphate, 100mM sodium fluoride, 1% nonylphenyl polyethylene glycol, and 10 μL/mL each of protease inhibitor (P8340, Sigma), phosphatase inhibitor

(524628, Calbiochem) and sodium orthovanadate (S6508, Sigma). Tissue lysates were then centrifuged at 12,000xg at 4°C for 20 minutes. Protein concentration in an aliquot of the supernatant was determined using the Bradford protein assay kit from Biorad. Supernatants were stored at -80°C until further processing. Denatured samples of cell homogenates were subjected to SDS-PAGE and proteins were transferred onto a nylon membrane. Subsequent immunoblotting to determine phosphorylation and expression of target proteins. Immunoblots were developed using the Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). All densitometric data were corrected against Actin protein expression and total protein loading visualized via Memcode (Pierce) staining.

Lipid Analysis and FA Uptake Measurements

Lipids were extracted from lysate according to the method of Folch [8]. Aliquots of the organic phase were transferred to fresh tubes, and the organic solvent was evaporated under a nitrogen stream. To determine TAG content, lipid extracts were resuspended in ice-cold 1% Triton X-100 by brief sonication, and TAG concentration was measured using a colorimetric kit assay (product number 2780-400H Infinity TAG reagent; Thermo Fisher Scientific) [3]. Quantification of long-chain acyl coenzyme A (acyl-CoA) species and ceramides was performed by using Water's Acquity Ulta-high performance liquid chromatography (UPLC) as described previously [3]. FA uptake was determined as described previously [3] using hearts that were perfused with buffer

containing 1.2 mmol/L [9,10-^{3}H]palmitate prebound to 3% delipidated BSA, 5 mmol/liter [U-^{14}C]glucose, and 50 μU/mL insulin for 60 min [9-11].

Nucleotide and Malonyl CoA Measurements

Malonyl CoA and nucleotides were extracted from 20 to 30 mg of frozen ventricular tissue by homogenization in ice-cold 6% (vol/vol) perchloric acid-1 mmol/liter dithiothreitol-0.5 mmol/liter EGTA. Homogenates were spun at 12,000 x g for 5 min at 4°C. Half of the homogenate was then used for quantification of malonyl CoA content by Ultra-high performance liquid chromatography (UPLC) and the other half of the homogenate was used for nucleotide extractions. The homogenate used for nucleotide measurements required neutralization of pH using K_2CO_3. Samples were kept on ice for an additional 30 minutes followed by centrifugation at 10,000 x g for 2 min at 4°C. Nucleotide concentrations in supernatants were determined by UPLC.

Gene expression analysis

Gene expression analysis was performed by quantitative reverse transcriptase PCR as previously described [12]. The following primer and probe sequences were used for quantitative PCR analysis (FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine): *Pnpla*2 forward, 5**'**-GGTCCTCCGAGAGATGTGC-3**'**;

*Pnpla*2 reverse, 5’ TGGTTCAGTAGGCC- ATTCCTC-3’; *Pnpla*2 probe, 5’-FAMCAGGGCTACAGAGATGGACTTCGATTCCTT-TAMRA-3’ [3].

Histology

Mouse hearts fixed in 10% formalin were prepared and stained with hematoxylin and eosin (H&E). Nuclei in the sections were first stained with hematoxylin for 5 minutes and then rinsed with water. The section is placed in 0.3% acid alcohol in order to leach out non-specific background stain and then re-rinsed in water. Sections are finally counterstained with eosin for 2 minutes, followed by dehydration and mounting.

Statistical Analysis

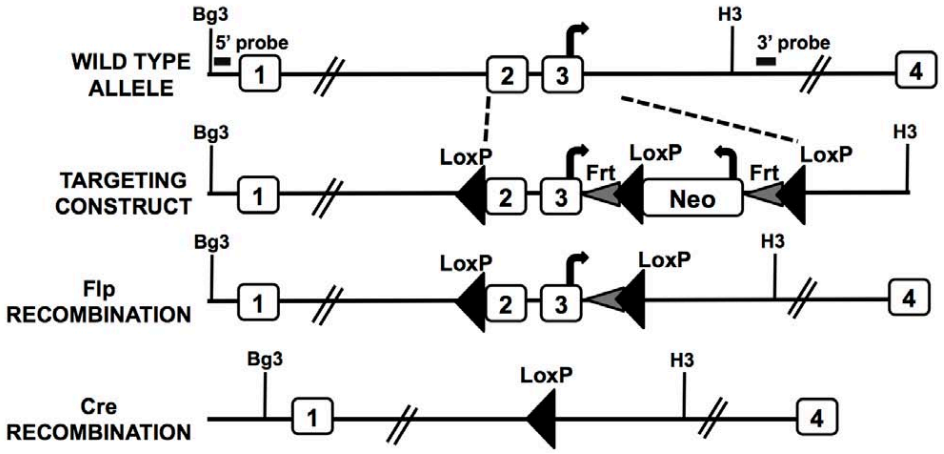
In our cohort study categorical data were summarized as percentages and odds ratios, and differences were tested with the χ^2 test; continuous variables were summarized as medians and interquartile ranges, and differences were tested with the Wilcoxon rank-sum test. We used multivariable logistic regression to evaluate the association between DM and death/HF in the 30 days after ACS, adjusting for baseline differences and prognostically important covariates using a backward, stepwise selection method with $p \leq 0.10$ used as an exit criteria. We chose logistic regression over Cox proportional hazards analysis given the short timeline (30 days). All results are

presented as unadjusted and adjusted ORs with their respective 95% CIs. All tests were 2-sided, with the level of significance set at $p<0.05$, and all analyses were performed with SAS version 9.3(SAS Institute Inc, Cary, NC).

For studies done with mice, results are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student's t-test. *P*-values of less than 0.05 were considered statistically significant.

Figure 2-1. Generation and characterization of the ic*CD36*KO mice. A. A targeting vector (Floxed) was created to generate floxed CD36 ES cells by homologous recombination (WT=endogenous allele). Both LoxP (black arrows) & FRT (grey arrows) sites were engineered into the genome. FRT sites flank the NEO gene, which was inserted such that ES cells could be selected with G418. NEO was subsequently removed with FLP recombinase. Injection of floxed CD36 (FCD36) ES cells into blastocysts led to generation of FCD36 mice. Exons 2 & 3, generating a null allele, were subsequently be removed by cre recombinase excision. Bg3 and H3 represent BglIII and HindIII restriction enzyme sites, respectively. Black arrows represent the direction of transcription/translation. **B.** Agarose gel of PCR results from DNA of offspring from a Fl/+ x Fl/+ cross. The floxed (FL) allele is about 35bp larger than the endogenous allele.

A


Bg3
5' probe
H3
3' probe
WILD TYPE ALLELE
1
2
3
4
Bg3
LoxP
Frt
LoxP
Neo
Frt
LoxP
H3
TARGETING CONSTRUCT
Bg3
LoxP
LoxP
H3
Flp RECOMBINATION
Bg3
LoxP
H3
Cre RECOMBINATION

B

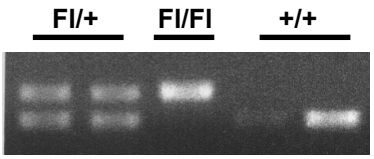
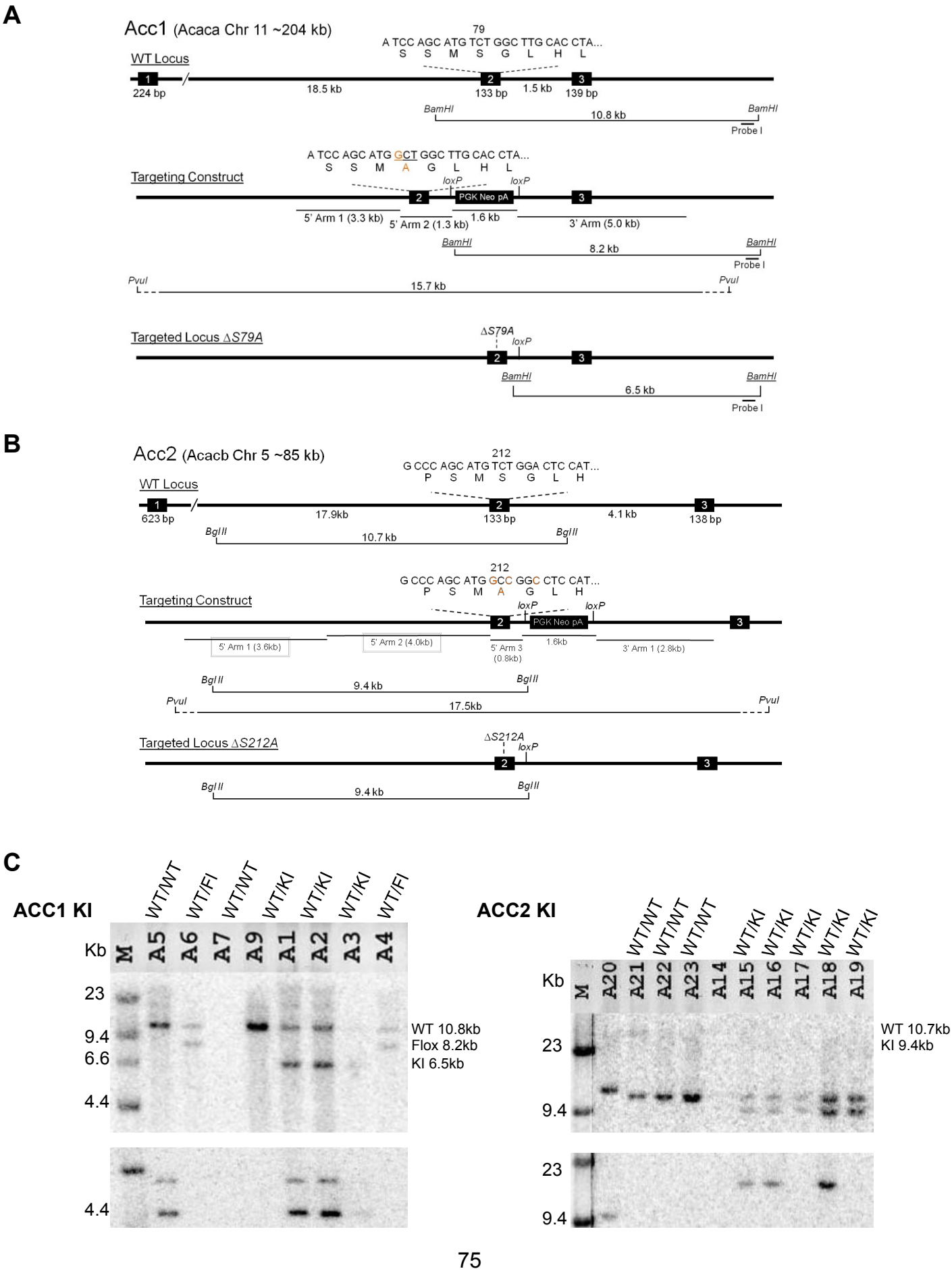

Fl/+
Fl/Fl
+/+

Figure 2-2. Generation of ACC1 and ACC2 KI mice. **A.** Chimeric WT/KI$^{flox\text{-}Acc1(S79A)}$ mice were crossed with the OZ-Cre deleter strain. Genomic DNA from two litters was purified, *Bam*HI digested, resolved, transferred to nylon, and probed with probe 1. **B.** Chimeric WT/KI$^{flox\text{-}Acc2(S212A)}$ mice were crossed with the OZ-Cre deleter strain. Genomic DNA from two litters was purified, *Bgl*II digested, resolved, transferred to nylon, and probed with probe 2. **C.** For ACC1 KI, the desired WT (10.8 kb) and Cre-deleted KI locus (6.5 kb) bands are observed for two progeny (A1 & A2). The filter was stripped and re-probed with a Cre-specific probe (bottom panel) to confirm transmission of the Cre-transgene. For ACC2 KI, the desired WT (10.7 kb) and Cre-deleted KI locus (9.4 kb) bands are observed for 5 progeny (A15 to A19). The filter was stripped and re-probed with a Cre-specific probe (bottom panel) to confirm transmission of the Cre transgene (A15, A16 and A18).


A
Acc1 (Acaca Chr 11 ~204 kb)
79
A TCC AGC ATG TCT GGC TTG CAC CTA...
S S M S G L H L
WT Locus
1
2
3
224 bp
18.5 kb
133 bp
1.5 kb
139 bp
BamHI
10.8 kb
BamHI
Probe I
A TCC AGC ATG GCT GGC TTG CAC CTA...
S S M A G L H L
Targeting Construct
loxP
loxP
PGK Neo pA
5' Arm 1 (3.3 kb)
5' Arm 2 (1.3 kb)
1.6 kb
3' Arm (5.0 kb)
BamHI
8.2 kb
BamHI
Probe I
PvuI
15.7 kb
PvuI
Targeted Locus ΔS79A
ΔS79A
loxP
BamHI
6.5 kb
BamHI
Probe I
B
Acc2 (Acacb Chr 5 ~85 kb)
212
G CCC AGC ATG TCT GGA CTC CAT...
P S M S G L H
WT Locus
623 bp
17.9kb
133 bp
4.1 kb
138 bp
BglII
10.7 kb
BglII
212
G CCC AGC ATG GCC GGC CTC CAT...
P S M A G L H
Targeting Construct
loxP
loxP
PGK Neo pA
5' Arm 1 (3.6kb)
5' Arm 2 (4.0kb)
5' Arm 3 (0.8kb)
1.6kb
3' Arm 1 (2.8kb)
BglII
9.4 kb
BglII
PvuI
17.5kb
PvuI
Targeted Locus ΔS212A
ΔS212A
loxP
BglII
9.4 kb
BglII
C
ACC1 KI
WT/WT
WT/FI
WT/WT
WT/KI
WT/KI
WT/KI
WT/FI
Kb
M
A5
A6
A7
A9
A1
A2
A3
A4
23
9.4
6.6
4.4
WT 10.8kb
Flox 8.2kb
KI 6.5kb
4.4
ACC2 KI
WT/WT
WT/WT
WT/WT
WT/KI
WT/KI
WT/KI
WT/KI
WT/KI
Kb
M
A20
A21
A22
A23
A14
A15
A16
A17
A18
A19
23
9.4
WT 10.7kb
KI 9.4kb
23
9.4

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CHAPTER 3.

Cardiomyocyte-Specific Ablation of CD36 Improves Post-ischemic Functional Recovery

My role in this work involved breeding and maintenance of the mouse colony, performing all the experiments (except those noted below), analysis and interpretation of the data as well as the writing of the manuscript. Technical staff performed UPLC and echocardiography in the CVRC core facility.

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CHAPTER 3.

Cardiomyocyte-Specific Ablation of CD36 Improves Post-ischemic Functional Recovery

Abstract

Background: Although pre-clinical evidence has suggested that partial inhibition of myocardial FA oxidation (FAO) and subsequent switch to greater glucose oxidation for ATP production can prevent ischemia/reperfusion (I/R) injury, controversy about this approach persists. For example, mice with germline deletion of the fatty acid transporter CD36, exhibited either impaired or unchanged post-ischemic functional recovery despite a 40-60% reduction in FAO rates. Because there are limitations to cardiac studies utilizing whole body CD36 knockout (total*CD36*KO) mice, we have now generated an inducible and cardiomyocyte-specific CD36 KO (ic*CD36*KO) mouse to better address the role of cardiomyocyte CD36 and its regulation of FAO in post-ischemic functional recovery.

Methods: Mice with tamoxifen-inducible, cardiomyocyte-specific, CD36 deficiency (ic*CD36*-/-) were generated by interbreeding *CD36*-flox mice with B6.Cg-Tg(Myh6-cre/Esr1)1Jmk/J mice. To examine cardiac function and fatty acid metabolism following I/R, ex vivo working hearts from control and ic*CD36*KO mice were either aerobically perfused for 30 minutes (controls) or subjected to 18 minutes of global no-flow ischemia and 40 minutes of aerobic reperfusion (I/R). Perfusion conditions consisted of Krebs-Henseleit solution containing 1.2 mmol/L [U

3H]palmitate, 5 mmol/L [U-14C]glucose, 2.5 mmol/L calcium, and 100 uU/mL insulin at a preload pressure of 11.5 mm Hg and afterload pressure of 50 mm Hg.

Results: Four to six weeks following CD36 ablation, hearts from ic*CD36*KO mice had significantly decreased FA uptake compared to controls, which was paralleled by significant reductions in intramyocardial triacylglycerol content. Analysis of cardiac energy metabolism using *ex vivo* working heart perfusions showed that reduced FAO rates were compensated by enhanced glucose oxidation in hearts from ic*CD36*KO mice. In contrast to the total*CD36*KO mice, hearts from ic*CD36*KO mice exhibited significantly improved functional recovery following ischemia/reperfusion (18 minutes of global no-flow ischemia followed by 40 minutes of aerobic reperfusion). This improved recovery was associated with lower calculated proton production prior to and following ischemia compared to controls. Moreover, the amount of ATP generated relative to cardiac work was significantly lower in hearts from ic*CD36*KO mice compared to controls, indicating significantly increased cardiac efficiency in hearts from ic*CD36*KO mice.

Conclusions: These data provide genetic evidence that reduced FAO as a result of diminished CD36-mediated FA uptake improves post-ischemic cardiac efficiency and functional recovery. As such, targeting cardiomyocyte FA uptake and FAO via inhibition of CD36 in the adult myocardium may provide therapeutic benefit during ischemia-reperfusion.

Key Words: Ischemia and reperfusion, lipids, metabolism, efficiency

Introduction

Myocardial ischemia occurs as a consequence of insufficient blood flow and subsequent oxygen delivery to the myocardium, resulting in a variety of clinical conditions ranging from mild angina to myocardial infarction. Myocardial injury and contractile dysfunction are directly correlated with the length and severity of an ischemic event. Although many mechanisms contribute to ischemic injury (see [1] for review), there is clear evidence that contractile dysfunction during and after myocardial ischemia is mediated, at least in part, by the predominant type of energy substrate metabolized by the heart[2, 3].

In the healthy heart, mitochondrial oxidation of long-chain fatty acids (FAs) provides the majority of ATP needed for proper cardiac function[4]. Notwithstanding this, pre-clinical evidence has suggested that partial inhibition of myocardial FA oxidation (FAO) and subsequent switch to greater glucose oxidation for ATP production can prevent ischemia/reperfusion (I/R) injury (see[2, 4] for reviews). Consistent with this finding, partial inhibitors of FAO in clinical use, such as trimetazidine[5, 6] and ranolazine[7-10], have shown to improve and preserve cardiac function in patients suffering from ischemic heart disease and I/R injury[11, 12]. However, more recent reports suggest that mechanistically both drugs may act via alternative pathways and thus exert their beneficial effects independent of partial inhibition of FAO[12-14]. There is also a growing body of evidence suggesting that partial inhibition of myocardial FAO may actually contribute to cardiac dysfunction[15, 16] as a result of mismatch between FA uptake into the cardiomyocyte and subsequent utilization, leading to excessive and pathological lipid accumulation. It thus remains unclear as to whether partial inhibition of FAO is truly beneficial to the injured or diseased myocardium.

To address the potential limitations inherent in existing pharmacological therapies aimed at optimizing myocardial energetics, genetically modified mouse models designed to partially inhibit FA uptake and/or oxidation could be utilized. One such model is the whole body CD36 knock out (total*CD36*KO) mouse[17]. CD36 is a transmembrane sarcolemmal protein involved in facilitating approximately 50% of cardiomyocyte FA uptake and is consequently responsible for controlling 40-60% of FAO rates in the working mouse heart[18-20]. Although total*CD36*KO mice appeared to be suitable to address how partial inhibition of FA uptake and oxidation can influence ischemic injury, conflicting reports about the extent of myocardial I/R injury in these mice have emerged[21, 22]. The precise reason(s) for the different outcomes is not known, but may include perfusion conditions, mouse genetic background, type of FA used, age of mice, etc. Additionally, germline deletion of CD36 modifies a variety of metabolic pathways in multiple tissues[23] and subsequently whole body metabolism, which may make it difficult to determine the effects of cardiomyocyte-specific CD36-mediated alterations in metabolism on I/R injury. Moreover, compensatory alterations resulting from chronic CD36 ablation in other metabolic processes within the cardiomyocyte may have occurred during development that could influence I/R injury.

To overcome the challenges inherent to the total*CD36*KO mouse, we have generated a cardiomyocyte-specific and tamoxifen-inducible CD36 KO (ic*CD36*KO) mouse. Using short-term inducible cardiomyocyte-specific CD36 ablation we tested the hypotheses that: 1) ic*CD36*KO mouse hearts have reduced FA uptake, utilization and storage and 2) this alteration in cardiac substrate utilization leads to improved post-ischemic functional recovery. These studies will allow us to determine if the concept of a combined strategy of limiting FA uptake and partially inhibiting FAO is a beneficial therapeutic approach to reducing ischemic injury[24, 25].

Methods

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with the Canadian Council on Animal Care guidelines. The University of Alberta Health Sciences Animal Welfare Committee and the Institutional Animal Care and Use Committee of the Cleveland Clinic approved all animal procedures. Mice were housed in a temperature controlled environment with a 12:12 hr reversed light/dark cycle with ad libitum access to chow diet (product number 5001 from Lab Diet, St. Louis, MO, with 13.5% kcal from fat) and water.

Transgenic Mice

ic*CD36*KO mice were generated as described in Chapter 2.

Tamoxifen administration and CD36 ablation.

Tamoxifen (T5648, Sigma) dissolved in corn oil was administered orally to adult 12-16 week-old control and littermate ic*CD36*KO mice at a dose of 100 mg/kg/day for 5 consecutive days as described in Chapter 2.

Echocardiography

Mice were mildly anesthetized using 0.75% isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics) as described in Chapter 2[26, 27].

Heart perfusions

Hearts were perfused in the working heart mode at 11.5 mm Hg preload and 50 mm Hg afterload with Krebs-Henseleit buffer containing 1.2 mmol/L palmitate prebound to 3% delipidated bovine serum albumin (BSA), 5 mmol/L glucose, and 50 μU/mL insulin. Hearts were aerobically perfused for 30 minutes, or aerobically perfused for 30 minutes followed by 18 minutes of global no flow ischemia and 30 minutes of reperfusion. At the end of aerobic perfusion or reperfusion, hearts were immediately frozen in liquid N_2 with a Wollenberger clamp and stored at -80°C as described previously[26]. For metabolic measurements, palmitate and glucose were labeled using either a combination of [9,10-^{3}H]palmitate and [U-^{14}C]glucose (for determination of FAO and glucose oxidation) or [U-^{14}C]glucose and [5-^{3}H]glucose (for determination of glucose oxidation and glycolysis) as previously described[26]. For isolation of adult ventricular cardiomyocytes, hearts were perfused in Langendorff mode as described in Chapter 2[28].

Lipid analysis and FA uptake

Serum free FA levels were measured using a colorimetric assay kit (#999–34691; Wako). For measurement of cardiac lipid concentrations, frozen hearts were ground using mortar and pestle and powdered tissue was homogenized in lysis buffer comprised of 20 mmol/L Tris, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1% nonylphenyl polyethylene glycol, and 10 μL/mL each of protease inhibitor (P8340, Sigma), phosphatase inhibitor (524628, Calbiochem) and sodium orthovanadate (S6508, Sigma). Lipids were extracted and triacylglycerol (TAG) content was determined as previously described[26]. Quantification of long-chain acyl coenzyme A (acyl-CoA) species was performed by using Waters Acquity Ultra-high performance liquid

chromatography as described previously[29]. FA uptake was determined as described previously[26] using hearts that were perfused with buffer containing 1.2 mmol/L [9,10-^{3}H]palmitate prebound to 3% delipidated BSA, 5 mmol/liter [U-^{14}C]glucose, and 50 μU/mL insulin for 60 min. .

Histology

Hematoxylin and eosin (H & E) stains of paraffin-embedded sections were visualized using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Retiga 1300i FAST 1394 CCD camera (QImaging, Surrey, BC, Canada) as described in Chapter 2[26].

Immunoblot analysis

Denatured samples of cardiac homogenates were subjected to SDS-PAGE and proteins were transferred onto a nylon membrane. Subsequent immunoblotting to determine expression of target proteins was employed. Primary antibodies used for immunoblotting were anti-CD36 (produced in-house as previously described[30]), anti-FATP1 (#14497; Santa Cruz Biotechnology), and anti-ACSL1 (#4047; Cell Signaling) antibodies. Immunoblots were developed using the Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). All densitometric data were corrected against total protein loading visualized via Memcode (Pierce) staining as described in Chapter 2[29].

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student's t test as described in Chapter 2.

Results

Inducible cardiomyocyte-specific CD36 ablation does not alter cardiac morphology.

A Cre/loxP recombination system was employed to generate ic*CD36*KO mice and mice were studied 4-6 weeks following tamoxifen administration. Four weeks post tamoxifen administration, CD36 protein expression was markedly reduced in both ventricles (Fig. 3-1A) and isolated cardiomyocytes (Fig. 3-1B) from ic*CD36*KO mice compared to control mice, confirming ablation of CD36 in ic*CD36*KO mice. Ablation of CD36 was restricted to the cardiomyocytes as CD36 protein expression in other tissues, such as skeletal muscle, was unchanged (Fig. 3-1C).

To assess the impact of relatively short-term adult cardiomyocyte-specific CD36 ablation on other proteins involved in FA uptake, we examined protein expression of FA transport protein 1 (FAPT1), and long-chain acyl-CoA synthetase isoform 1 (ACSL1). FATP1, along with CD36, acts to transport FAs across the cell membrane in the cardiomyocyte[31] while ACSL1 catalyzes the synthesis of acyl-CoA from long-chain FAs and is a necessary mediator of FA import[32]. Despite significant reduction in CD36 expression, there was no compensatory increase in FATP1 and ACSL1 protein expression (Fig. 3-1D) in hearts from ic*CD36*KO mice. Histological analysis of ventricular sections stained with hematoxylin and eosin showed no evidence of abnormal cardiomyocyte morphology or collagen deposition in hearts from ic*CD36*KO mice compared to controls (Fig. 3-1E).

Inducible cardiomyocyte-specific CD36 ablation results in diminished myocardial fatty acid utilization.

Using *ex vivo* working heart perfusions, cardiac metabolism and performance were directly assessed. Consistent with the normal morphology of the hearts from ic*CD36*KO mice, we found that heart rate (HR; Fig. 3-2A), HR x peak systolic pressure (PSP; Fig. 3-2B) and cardiac power (Fig. 3-2C) was not different between genotypes. In addition, cardiac output and coronary flow rates were unchanged between genotypes (Table 3-1). Importantly, since cardiac function was not different between genotypes, evaluation of baseline cardiac substrate metabolism could be performed in the absence of changes in myocardial energetic demand.

As shown in figure 3-2D, ablation of CD36 in cardiomyocytes corresponded to a significant reduction in FA uptake in hearts from ic*CD36*KO mice compared to controls. Consistent with this, FAO rates were also decreased by 48% in hearts from ic*CD36*KO mice compared to controls (Fig. 3-2E). In agreement with the well-described Randle cycle[33, 34], glucose oxidation rates were correspondingly increased by 68% in hearts from ic*CD36*KO mice compared to controls (Fig. 3-2F), yet there was no significant change in glycolytic rates (Fig. 3-2G). Despite this switch to greater mitochondrial oxidation of glucose vs. FAs in hearts from ic*CD36*KO mice (Fig. 3-2H - 56% of TCA cycle acetyl-CoA production), these alterations in myocardial substrate metabolism did not significantly reduce total TCA cycle acetyl CoA production (Table 3-1) or total ATP production from the exogenously supplied substrates (Fig. 3-2I).

Consistent with the significant decrease in FA uptake observed, hearts from ic*CD36*KO mice also had a significant reduction in myocardial TAG content (Fig. 3-2J) and a trend toward reduced myocardial total long-chain acyl CoA content (Fig. 3-2K) compared to controls. These data show that the reduction in FAO in hearts from ic*CD36*KO mice was adequate in relation to the reduction in CD36-mediated FA uptake thereby avoiding excessive and pathological accumulation of myocardial lipids[35]. In contrast to the total*CD36*KO mouse model[36], short-

term cardiomyocyte-specific CD36 ablation did not lead to alterations in serum concentrations of free FAs (Fig. 3-2L), suggesting that our *ex vivo* metabolic data more closely represent *in vivo* cardiac metabolism.

Loss of cardiomyocyte-specific CD36 does not alter *in vivo* cardiac function at baseline.

To determine the functional consequences of inducible cardiomyocyte-specific CD36 ablation under normal physiologic conditions *in vivo*, we performed transthoracic echocardiography on mildly anesthetized mice. Measures of systolic and diastolic function were not altered between genotypes (Table 3-2). In fact, none of the parameters that we measured were different between genotypes, including ejection fraction, fractional shortening, isovolumic relaxation time, E/A ratio, mitral valve deceleration time, E/E', and Tei index (Table 3-2). Consistent with unchanged cardiac function, hearts from ic*CD36*KO mice had no structural or morphological abnormalities: the ratio of ventricular weight to tibia length was unchanged (7.34 ± 0.4 g/m, n=7 vs 6.7 ± 0.3 g/m, n=5, p=0.30), and there were no differences in the thickness of the interventricular septum (IVS) and left ventricular posterior wall (LVPW, Table 3-2). Together, these data indicate that despite alterations in substrate metabolism, hearts from ic*CD36*KO mice were neither functionally compromised nor had compensatory structural remodeling.

Inducible cardiomyocyte-specific CD36 ablation improves myocardial functional recovery following ischemia.

Because hearts from ic*CD36*KO mice have a metabolic profile that has been proposed to improve cardiac efficiency post-ischemia[5, 37, 38], we hypothesized that hearts from ic*CD36*KO mice would demonstrate improved post-ischemic functional recovery. To test this hypothesis, *ex vivo* perfused working hearts from control and ic*CD36*KO mice were subjected to 30 minutes of aerobic perfusion, followed by 18 minutes of global no-flow ischemia and 40 minutes of aerobic reperfusion as previously described[39, 40]. Upon reperfusion following

ischemia, hearts from control mice exhibited a significant reduction in cardiac function as represented by HR x PSP (Fig. 3-3B) and cardiac power (Fig. 3-3C) compared to the pre-ischemic phase. In contrast, hearts from ic*CD36*KO mice had significantly improved recovery of cardiac function compared to controls (Fig. 3-3B-E) in the absence of changes in HR between genotypes (Fig. 3-3A). Importantly, these data suggest that hearts from ic*CD36*KO mice also exhibit improved post-ischemic functional recovery when compared to the total*CD36*KO mice, which showed either unchanged or decreased cardiac function compared to controls during reperfusion[21, 22].

In order to determine if cardiac efficiency contributed to improved post-ischemic functional recovery in hearts from ic*CD36*KO mice, we also measured myocardial substrate metabolism during the reperfusion period. As expected in the injured myocardium following ischemia, overall oxidative metabolism (FAO and glucose oxidation) was lower (Fig. 3-3F-G) compared to the aerobic period (Fig. 3-2E-F) in both genotypes. Interestingly, although FAO and glucose oxidation rates decreased to the same levels in hearts from both control and ic*CD36*KO mice (Fig. 3-3F-G), glycolytic rates did not increase to compensate for this loss in ATP production (Fig. 3-3H). Indeed, ATP production from exogenously provided substrates decreased by 47% and 38% in control and ic*CD36*KO genotypes, respectively, during reperfusion (Fig. 3-3I) compared to pre-ischemic aerobic values (Fig. 3-2I). This impaired energetic status is consistent with the fact that the hearts are performing significantly less work during reperfusion.

Despite the aforementioned metabolic changes, the amount of ATP generated relative to cardiac work was dramatically and significantly lower in hearts from ic*CD36*KO mice compared to controls (Fig. 3-3J), indicating that hearts from ic*CD36*KO mice require less ATP per unit work and exhibit significantly increased metabolic efficiency. In agreement with the concept that

proton production resulting from the uncoupling of glycolysis from glucose oxidation contributes to cardiac inefficiency post-ischemia[41, 42], calculated proton production during reperfusion, was decreased in the more efficient hearts from ic*CD36*KO mice compared to controls (Fig. 3-3K). Moreover, the calculated proton production prior to ischemia was significantly lower in hearts from ic*CD36*KO mice compared to controls (data not shown), further supporting the concept that increased cardiac efficiency in hearts from ic*CD36*KO mice could help explain improved post-ischemic functional recovery.

Table 3-1. Functional and Metabolic Parameters of *Ex Vivo* Perfused Working Hearts From Control and icCD36KO Mice

Parameters		Control	ic*CD36*KO
Pressure			
	Peak Systolic Pressure, mmHg	58 ± 0.8	60 ± 0.7
	Diastolic Pressure, mmHg	45 ± 0.7	47 ± 0.8
	Developed Pressure, mmHg	12.7 ± 0.9	12.7 ± 0.8
	Mean Arterial Pressure, mmHg	50 ± 0.6	51 ± 0.7
Flow rates			
	Cardiac Output, ml/min	6.6 ± 0.6	7.0 ± 0.3
	Coronary Flow, ml/min	2.5 ± 0.1	2.6 ± 0.1
Mitochondrial Acetyl CoA Production			
	Glucose	1991 ± 277	3369 ± 326*
	Palmitate	5543 ± 758	2986 ± 502*
	Total	7535 ± 652	6355 ± 549

Data are mean ± SEM of n = 21-26 for pressure and flow rates.
Data are mean ± SEM of n = 14-15 for mitochondrial Acetyl CoA Production.
*$p < 0.05$

Table 3-2. Echocardiographic Parameters From Control and ic*CD36*KO Mice

Parameters		Control	ic*CD36*KO
Global Performance			
	Body Weight, g	30.9 ± 1.3	29.1 ± 0.4
	Heart Rate, bpm	416 ± 14	428 ± 22
	Tei Index	0.75 ± 0.06	0.75 ± 0.04
Systolic Function			
	Ejection Fraction, %	56.5 ± 2.2	56.8 ± 4.0
	Fractional Shortening, %	29.1 ± 1.4	29.5 ± 2.6
Diastolic Function			
	Isovolumic Relaxation Time, ms	19.8 ± 1.0	19.6 ± 1.0
	E/A ratio	1.16 ± 0.04	1.01 ± 0.07
	Mitral Valve Deceleration Time, ms	13.1 ± 1.8	14.2 ± 1.2
	E/E'	20.1 ± 1.9	27.0 ± 2.5
Wall Measurements			
	Interventricular Septal Thickness - Diastole, mm	0.73 ± 0.02	0.76 ± 0.04
	Interventricular Septal Thickness - Systole, mm	1.07 ± 0.03	1.09 ± 0.09
	Left Ventricular Internal Diameter - Diastole, mm	3.68 ± 0.10	3.80 ± 0.11
	Left Ventricular Internal Diameter - Systole, mm	2.61 ± 0.10	2.69 ± 0.16
	Left Ventricular Posterior Wall - Diastole, mm	0.71 ± 0.02	0.72 ± 0.04
	Left Ventricular Posterior Wall - Systole, mm	1.02 ± 0.03	1.05 ± 0.07

Data are mean ± SEM of n = 6-7.

Table 3-3. Functional and metabolic Parameters of Ex-Vivo Reperfused Working Hearts From Control and icCD36KO Mice

Parameters		Control	ic*CD36*KO
Pressure			
	Peak Systolic Pressure, mmHg	31 ± 4.2	46 ± 4.3*
	Diastolic Pressure, mmHg	26 ± 3.1	38 ± 3.5*
	Developed Pressure, mmHg	5.0 ± 1.3	7.9 ± 1.2
	Mean Arterial Pressure, mmHg	28 ± 3.4	41 ± 3.8*
Flow rates			
	Cardiac Output, ml/min	1.9 ± 0.5	3.2 ± 0.4*
	Coronary Flow, ml/min	1.4 ± 0.2	2.0 ± 0.2*
Mitochondrial Acetyl CoA Production			
	Glucose	626 ± 163	1108 ± 285
	Palmitate	3076 ± 556	2707 ± 393
	Total	3634 ± 642	3815 ± 587

Data are mean ± SEM of n = 13-17 for pressure and flow rates.
Data are mean ± SEM of n = 6 for mitochondrial Acetyl CoA Production.
*$p < 0.05$

Figure 3-1. Characterization of the ic*CD36*KO mice. A. Immunoblot analysis was performed using ventricular homogenates. Levels of CD36 were quantified by densitometry and normalized against total protein loading visualized via Memcode (Pierce) staining (n = 3, *p < 0.05). **B.** Immunoblot analysis of CD36 protein expression in isolated adult cardiomyocytes (CM) from control and ic*CD36*KO mice (n = 3, *p < 0.05). **C**. Immunoblot analysis of CD36 protein expression in skeletal muscle (n = 3). **D.** Immunoblot analysis of FATP1 and ACSL1 protein expression was performed using ventricular homogenates. **E.** Representative histological images of apical heart sections stained with H&E and Masson's trichrome stain at x40 magnification. Scale bars indicate 67 μm.

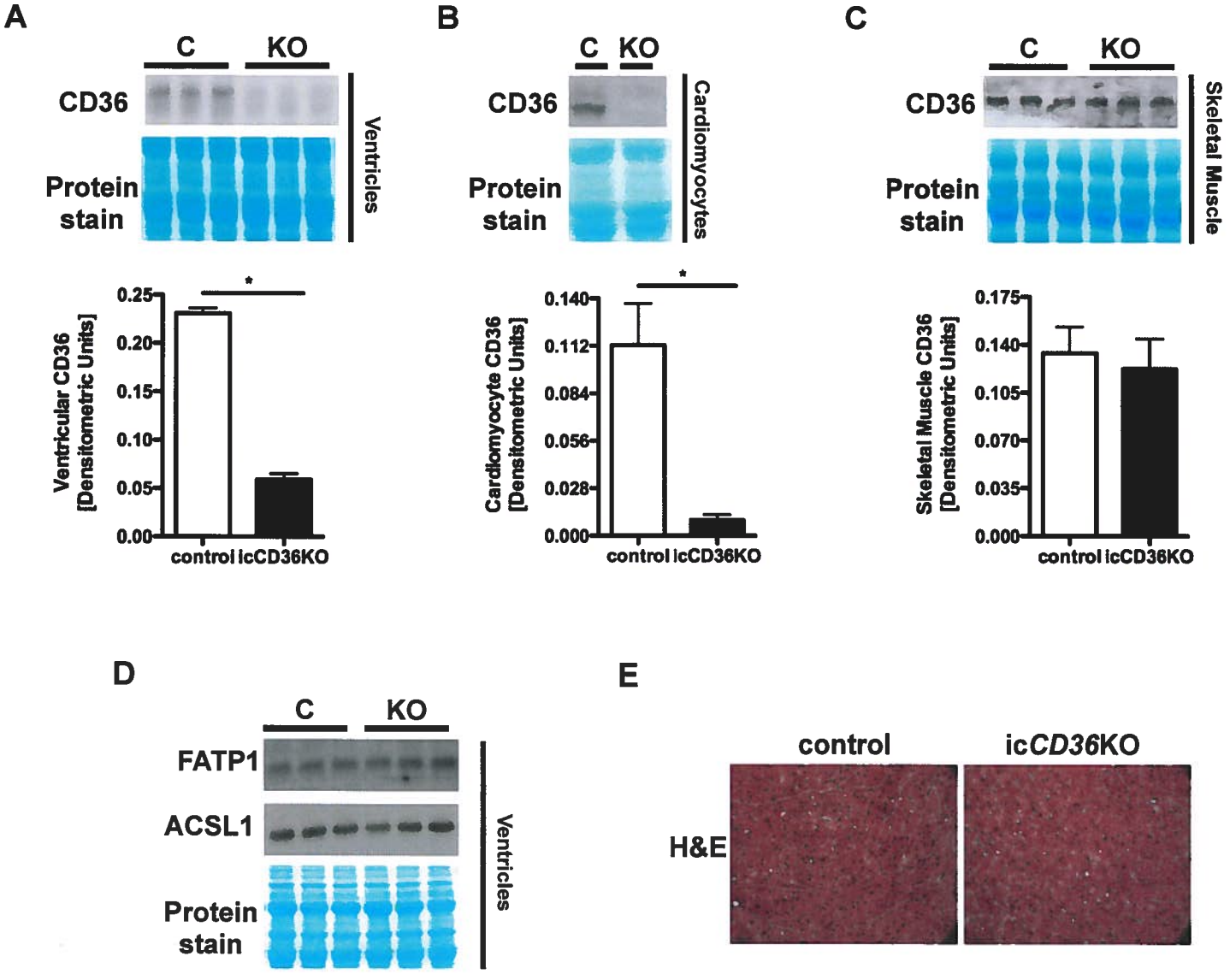

A
C
KO
CD36
Protein stain
Ventricles
Ventricular CD36 [Densitometric Units]
0.25
0.20
0.15
0.10
0.05
0.00
*
control
icCD36KO
B
C
KO
CD36
Protein stain
Cardiomyocytes
Cardiomyocyte CD36 [Densitometric Units]
0.140
0.112
0.084
0.056
0.028
0.000
*
control
icCD36KO
C
C
KO
CD36
Protein stain
Skeletal Muscle
Skeletal Muscle CD36 [Densitometric Units]
0.175
0.140
0.105
0.070
0.035
0.000
control
icCD36KO
D
C
KO
FATP1
ACSL1
Protein stain
Ventricles
E
control
icCD36KO
H&E

Figure 3-2. *Ex vivo* myocardial function and energy metabolism. A. Heart rate (*n* = 21 – 26). **B.** Rate pressure product (*n* = 21 – 26). **C.** Cardiac Power (*n* = 21 – 26). **D.** FA uptake (*n* = 4). **E.** Palmitate oxidation rates (*n* = 14 - 15). **F.** Glucose oxidation rates (*n* = 21 – 26). **G.** Glycolysis rates (*n* = 7 – 11). **H.** Krebs cycle acetyl-CoA production (*n* = 14 – 15) **I.** ATP production via exogenous substrates (glycolysis - *n* = 7 – 11, glucose - *n* = 21 – 26, palmitate *n* = 14 – 15) **J.** Myocardial triacylglycerol (TAG) content (*n* = 3 - 4) **K.** Total long-chain acyl-CoA content (*n* = 5 – 6) **L.** Serum free FA content (*n* = 5 - 7).

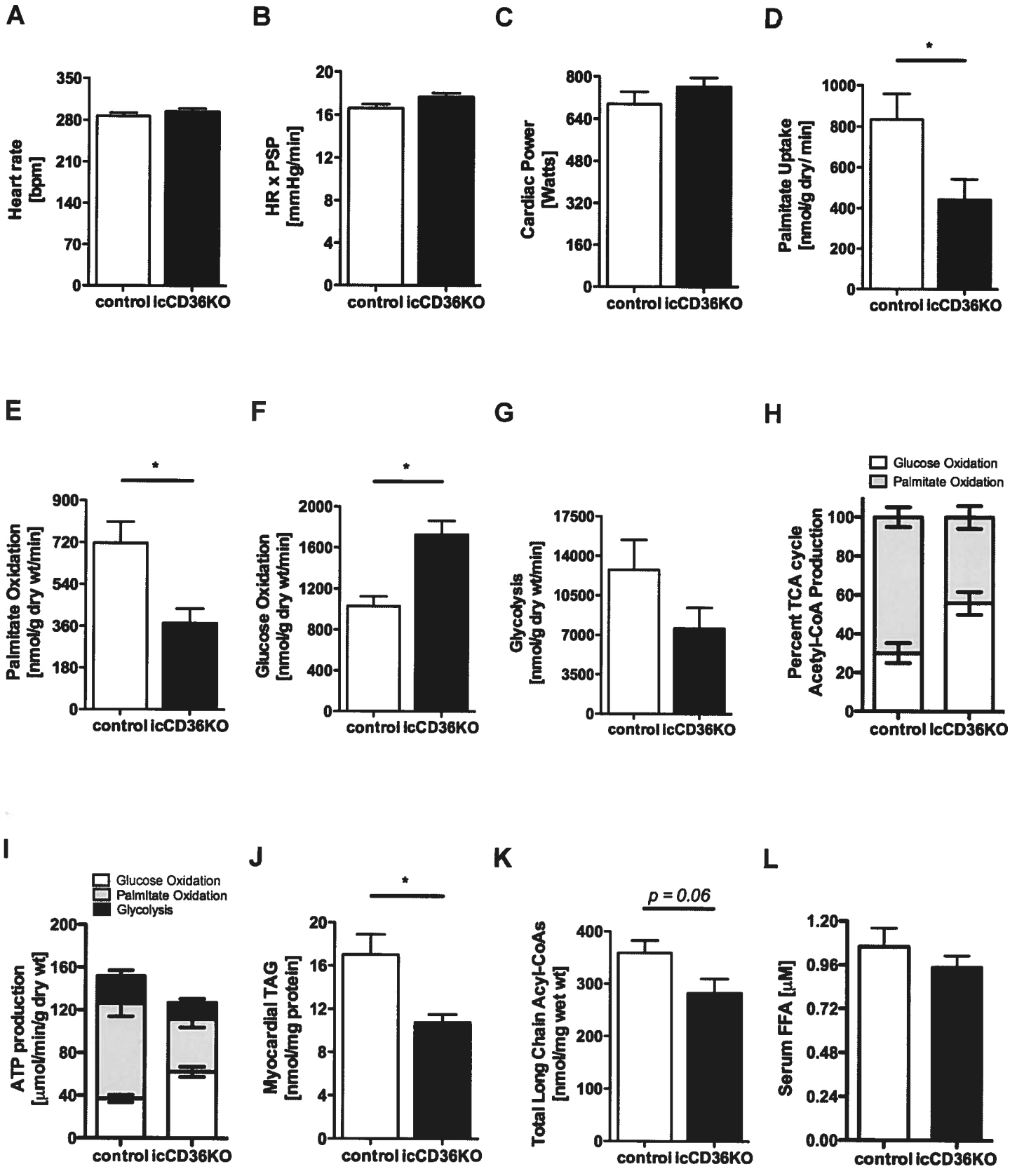
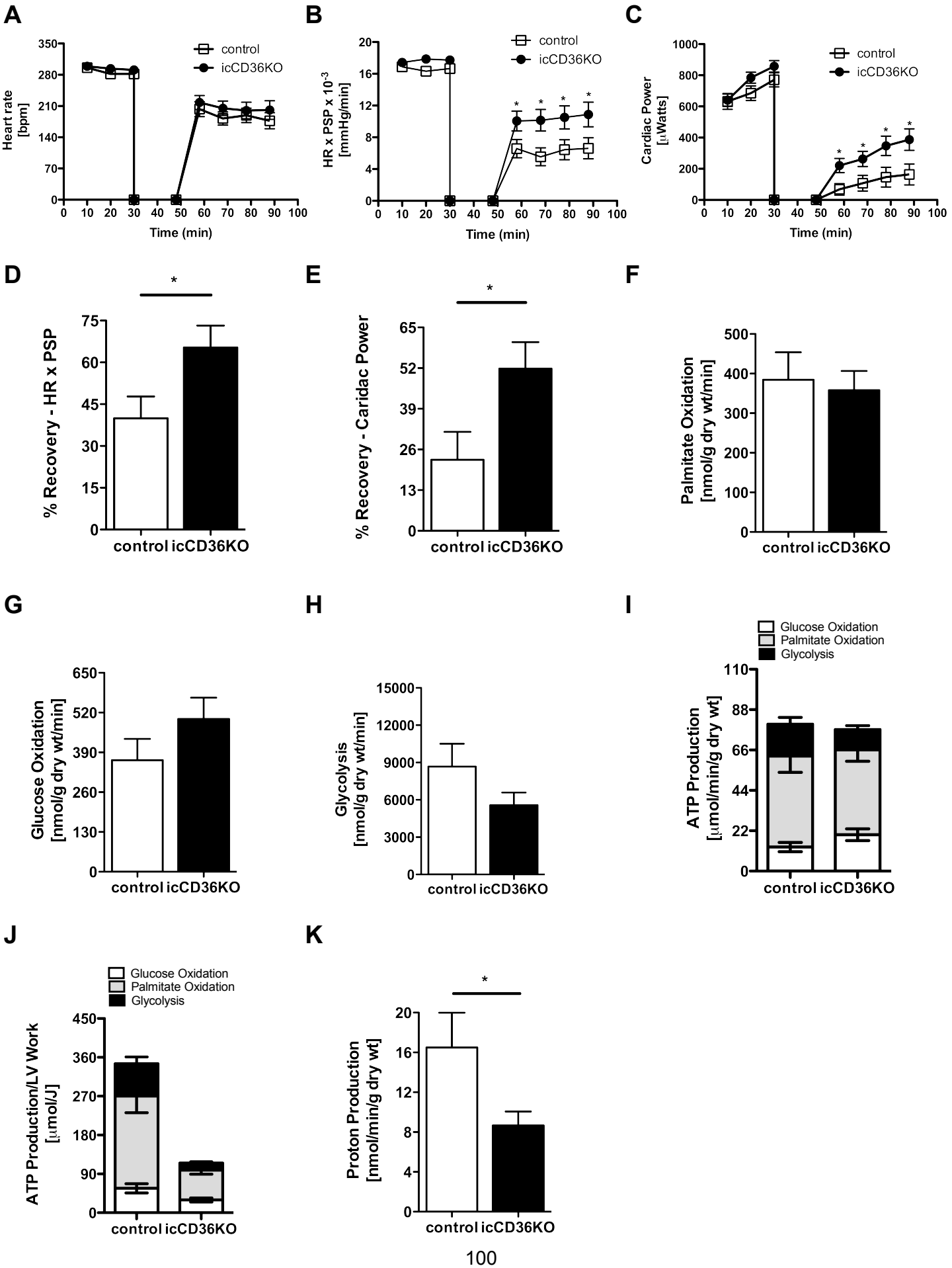

A
Heart rate [bpm]
350
280
210
140
70
0
control icCD36KO
B
HR x PSP [mmHg/min]
20
16
12
8
4
0
control icCD36KO
C
Cardiac Power [Watts]
800
640
480
320
160
0
control icCD36KO
D
*
Palmitate Uptake [nmol/g dry/ min]
1000
800
600
400
200
0
control icCD36KO
E
*
Palmitate Oxidation [nmol/g dry wt/min]
900
720
540
360
180
0
control icCD36KO
F
*
Glucose Oxidation [nmol/g dry wt/min]
2000
1600
1200
800
400
0
control icCD36KO
G
Glycolysis [nmol/g dry wt/min]
17500
14000
10500
7000
3500
0
control icCD36KO
H
Glucose Oxidation
Palmitate Oxidation
Percent TCA cycle Acetyl-CoA Production
100
80
60
40
20
0
control icCD36KO
I
Glucose Oxidation
Palmitate Oxidation
Glycolysis
ATP production [µmol/min/g dry wt]
200
160
120
80
40
0
control icCD36KO
J
*
Myocardial TAG [nmol/mg protein]
20
16
12
8
4
0
control icCD36KO
K
p = 0.06
Total Long Chain Acyl-CoAs [nmol/mg wet wt]
400
300
200
100
0
control icCD36KO
L
Serum FFA [µM]
1.20
0.96
0.72
0.48
0.24
0.00
control icCD36KO

Figure 3. ***Ex vivo*** **myocardial function, energy metabolism and efficiency in the reperfused myocardium.** **A.** Heart rate (*n* = 13 – 17). **B.** Rate pressure product (*n* = 13 – 17). **C.** Cardiac power (*n* = 13 – 17). **D.** Percent recovery in rate pressure product (*n* = 13 – 17). **E.** Percent recovery in cardiac power (*n* = 13 – 17). **F.** Palmitate oxidation rates during reperfusion (*n* = 6). **G.** Glucose oxidation rates during reperfusion (*n* = 13 – 17). **H.** Glycolysis rates during reperfusion (*n* = 7 – 11). **I.** ATP production via exogenous substrates during reperfusion (glycolysis - *n* = 7 – 11, glucose - *n* = 13 – 17, palmitate *n* = 6). **J.** ATP production via exogenous substrates per left ventricular work during reperfusion (glycolysis - *n* = 7 – 11, glucose - *n* = 13 – 17, palmitate *n* = 6). **K.** Proton production during reperfusion (*n* = 7 – 11).


A
Heart rate [bpm]
control
icCD36KO
Time (min)
B
HR x PSP x 10-3 [mmHg/min]
control
icCD36KO
Time (min)
C
Cardiac Power [μWatts]
control
icCD36KO
Time (min)
D
% Recovery - HR x PSP
control icCD36KO
E
% Recovery - Caridac Power
control icCD36KO
F
Palmitate Oxidation [nmol/g dry wt/min]
control icCD36KO
G
Glucose Oxidation [nmol/g dry wt/min]
control icCD36KO
H
Glycolysis [nmol/g dry wt/min]
control icCD36KO
I
Glucose Oxidation
Palmitate Oxidation
Glycolysis
ATP Production [μmol/min/g dry wt]
control icCD36KO
J
Glucose Oxidation
Palmitate Oxidation
Glycolysis
ATP Production/LV Work [μmol/J]
control icCD36KO
K
Proton Production [nmol/min/g dry wt]
control icCD36KO

Discussion

We have previously shown that global loss of CD36 and the subsequent partial inhibition of cardiomyocyte FAO do not negatively impact the ability of the heart to recover from an ischemic insult[39]. More recently, a selective CD36 ligand was shown to reduce myocardial FA uptake *in vivo* and lessen post-ischemic myocardial injury[25]. Although the latter suggests that inhibition of CD36 in the heart may be of benefit during ischemia, the protective effects induced by the CD36 ligand could occur secondary to reducing plasma FA levels[25]. In contrast to the two aforementioned studies, another study showed that genetic ablation of CD36 resulted in impaired post-ischemic functional recovery[22]. Although the reasons for the conflicting outcomes in the genetic whole body CD36 KO model are unknown, there are inherent limitations in the use of mice with germline deletion of CD36, including alterations in whole body metabolism[23] and potential compensatory adaptations that may have occurred over the lifetime of the mouse. Since there are currently no pharmacological CD36 inhibitors that can specifically limit myocardial FA uptake and subsequently impart a partial inhibition of FAO, and in order to address the limitations of our previous study, we created and utilized a mouse model of adult inducible cardiomyocyte-specific CD36 deficiency.

In this model, Lox P sites were engineered around exons 2 and 3. As in the total*CD36*KO mouse, we targeted CD36 exon 3, which has the translation start site and codes for the N-terminal transmembrane domain and is essential to proper membrane expression. We then crossed the floxed mouse to a tamoxifen-inducible cardiomyocyte-specific Cre mouse. Both these strains are C57Bl/6 congenic, eliminating the potential for confounding influences from the “passenger gene” effect. Our strategy proved successful as CD36 was efficiently and specifically deleted from cardiomyocytes 4 weeks following tamoxifen administration, and this

occurred without compensatory changes in two functionally interacting proteins, FATP1 and ACSL1[31, 32]. Histological examination of hearts from ic*CD36*KO mice showed no abnormal cardiomyocyte morphology or collagen deposition. In addition, gravimetric and echocardiographic assessment of heart weight and chamber wall thickness, respectively, showed no induction of cardiac hypertrophy in our ic*CD36*KO mouse model, in contrast to mice with germline deletion of CD36[43]. Whether the absence of cardiac hypertrophy in the ic*CD36*KO mice is simply a reflection of the relatively short duration of CD36 ablation or whether it is due to cardiomyocyte-specific CD36 ablation or the lack of systemic changes observed in the whole body CD36 KO mouse, is currently unknown.

To assess whether short-term cardiomyocyte-specific CD36 ablation resulted in myocardial metabolic or functional alterations, we performed *ex vivo* working heart perfusions along with *in vivo* echocardiography. Hearts from ic*CD36*KO mice exhibited a significant decrease in FA uptake, confirming that CD36 is a major mediator of FA transport in the cardiomyocyte. Consistent with impaired FA uptake, both TAG storage and FAO rates were significantly reduced in hearts from ic*CD36*KO mice compared to controls. Furthermore, in agreement with the metabolic processes involved in the Randle cycle[33, 34], impaired FAO rates led to increased rates of glucose oxidation in the hearts from ic*CD36*KO mice compared to controls. Despite these dramatic changes in myocardial metabolism, no evidence of cardiac dysfunction was observed in perfused hearts or by *in vivo* echocardiography, showing that the hearts were not energetically compromised. As the metabolic profile induced by CD36 ablation has been proposed to be beneficial for the heart in the setting of I/R by lessening intramyocardial proton accumulation and improving myocardial efficiency[21], we sought to test whether cardiomyocyte-specific CD36 ablation lessens myocardial ischemic injury.

Since it has been shown that contractile dysfunction during and after myocardial ischemia can be lessened by stimulating glucose oxidation either directly or secondary to

inhibition of FAO[5, 37, 44], there was a strong rationale for hypothesizing that hearts from ic*CD36*KO mice would have improved post-ischemic functional recovery compared to controls. Indeed, post-ischemic functional recovery in hearts from ic*CD36*KO mice was significantly better than controls, and this was associated with lower calculated proton production prior to and following ischemia. Moreover, the amount of ATP needed per unit work was significantly lower in hearts from ic*CD36*KO mice compared to controls, indicating that the cardiac efficiency was significantly higher in hearts from ic*CD36*KO mice. Since the extent of proton accumulation in hearts from ic*CD36*KO mice was less than controls, our data are in alignment with previous studies that have argued that cardiac efficiency is increased in these situations due to the fact that the cardiomyocyte can direct ATP towards contractile function and not restoring ionic homeostasis in the post-ischemic period[5, 37, 44]. However, whether this is the only beneficial effect of CD36 ablation in the cardiomyocyte is as yet unknown and future work will have to be performed to fully characterize the mechanisms involved in CD36 ablation-mediated improved post-ischemic functional recovery.

In summary, the data presented herein show for the first time that cardiomyocyte-specific CD36 ablation can significantly reduce FA uptake, FAO, and TAG storage in healthy hearts as well as improve myocardial efficiency and functional recovery following an ischemic injury. Our findings are consistent with recent studies that have suggested that contractile dysfunction during and after myocardial ischemia can be reduced by stimulating glucose oxidation either directly or secondary to inhibition of FAO[37, 44]. Based on our findings, it is tempting to speculate that pharmacotherapy designed to specifically inhibit cardiomyocyte CD36 may represent a promising new approach to lessen myocardial I/R injury in patients.

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CHAPTER 4.

Cardiomyocyte-Specific Adipose Triglyceride Lipase Over-Expression Prevents Doxorubicin-Induced Cardiac Dysfunction in Female Mice

My role in this work involved performing all the experiments (except those noted below), analysis and interpretation of the data as well as the writing of the manuscript. Technical staff performed UPLC and echocardiography in the CVRC core facility.

Manuscript Status: *A version of this chapter has been accepted for publication in Heart.*

***Nagendran J**, Kienesberger PC, Pulinilkunnil T, Zordoky B, Young ME, Zechner R, Dyck JRB. Cardiomyocyte-Specific ATGL Over-Expression Prevents Doxorubicin-Induced Cardiac Dysfunction in Female Mice. Heart, 2013 – Accepted*

CHAPTER 4.

Cardiomyocyte-Specific Adipose Triglyceride Lipase Over-Expression Prevents Doxorubicin-Induced Cardiac Dysfunction in Female Mice

Abstract

Objectives: Anthracyclines such as doxorubicin are an effective class of antineoplastic agents. Despite its efficacy in the treatment of a variety of cancers, the clinical use of doxorubicin is limited by cardiac side effects. While it has been suggested that doxorubicin alters myocardial fatty acid metabolism, it is poorly understood whether this is the case and whether variations in myocardial triacylglycerol (TAG) metabolism contribute to doxorubicin-induced cardiotoxicity. Since TAG catabolism in the heart is controlled by adipose triglyceride lipase (ATGL), this study examined the influence of doxorubicin on cardiac energy metabolism and TAG levels as well as the consequence of forced-expression of ATGL in the setting of doxorubicin-induced cardiotoxicity.

Design and Setting: Wild type (WT) mice and mice with cardiomyocyte-specific ATGL over-expression were divided into two groups per genotype that received a weekly intraperitoneal injection of saline or doxorubicin for four weeks.

Results: Four weeks of doxorubicin administration significantly impaired *in vivo* systolic function (11% reduction in ejection fraction, $p<0.05$), which was associated with increased lung wet-to-dry weight ratio. Furthermore, doxorubicin-induced cardiac dysfunction *in vivo* was independent of changes in glucose and fatty acid oxidation in WT hearts. However, doxorubicin administration significantly reduced myocardial TAG content in WT mice ($p<0.05$). Importantly,

cardiomyocyte-specific ATGL over-expression and the resulting decrease in cardiac TAG accumulation attenuated the decrease in ejection fraction ($p<0.05$) and thus protected mice from doxorubicin-induced cardiac dysfunction.

Conclusions: Taken together, our data suggest that chronic reduction in myocardial TAG content by cardiomyocyte-specific ATGL over-expression is able to prevent doxorubicin-induced cardiac dysfunction.

***Keywords**:* Lipids, Systolic dysfunction, Basic science, Metabolic medicine

Introduction

Anthracyclines such as doxorubicin are an effective class of antineoplastic agents for the treatment of many human neoplasms, including breast cancer [1]. However, the clinical use of doxorubicin is limited by cardiac side effects [2]. Doxorubicin administration is known to cause cardiotoxicity that can develop into congestive heart failure (CHF) [2]. At present, medical management of heart failure due to doxorubicin administration is similar to that of all patients with left ventricular dysfunction and heart failure [2] and does not specifically target doxorubicin-induced cardiotoxicity. This is largely due to our lack of understanding of the mechanisms that lead to doxorubicin-induced cardiac dysfunction. Therefore, development of medical therapies and identification of novel pharmacologic targets that aid in the management of doxorubicin-induced cardiac dysfunction is paramount to the improvement of patient care and the long-term survival of these individuals.

Although a number of mechanisms have been proposed for doxorubicin-induced cardiac dysfunction, including enhanced oxidative stress, increased topoisomerase II activity, apoptosis, and collagen synthesis/degradation [3], targeting these pathways has yielded insufficient success in improving doxorubicin-induced cardiomyopathy [4]. Recent studies have associated alterations in myocardial energy metabolism with the cardiotoxic effects of doxorubicin [5]. These studies have demonstrated that overall oxidative phosphorylation in the cardiomyocyte, and adenosine triphosphate (ATP) production is decreased with doxorubicin administration. Furthermore, previous studies have suggested that myocardial substrate utilization is also altered with doxorubicin administration and that fatty acid (FA) oxidation decreases with doxorubicin-induced cardiotoxicity [6]. However, to date, no studies have examined the dynamic

myocardial triacylglycerol (TAG) pool as it pertains to substrate utilization during doxorubicin-induced cardiotoxicity. This is increasingly important since there is growing evidence that promotion of a hypolipidemic cardiomyocyte environment diminishes the cardiotoxic effects of doxorubicin-treated animals and patients [7]. As well, there is increasing evidence for the importance of the intramyocardial TAG metabolism as it pertains to overall cardiac energy metabolism in physiology and pathophysiology [8].

Despite these reported alterations in energy metabolism in doxorubicin-induced cardiomyopathy, the reported benefits of a hypolipidemic cardiomyocyte environment in diminishing the cardiac side effects of doxorubicin, and the critical role of TAG turnover in regulating overall FA utilization, no studies have specifically addressed whether myocardial TAG metabolism and accumulation are altered following doxorubicin administration and whether manipulating cardiac TAG metabolism could modify the natural history of doxorubicin-induced cardiac dysfunction. To examine this, the aims of this study were to: (1) investigate the effects of doxorubicin administration on myocardial exogenous substrate metabolism and TAG metabolism and (2) determine if manipulation of the intramyocardial TAG pool could be used as an approach to treat doxorubicin-induced cardiac dysfunction. As TAG catabolism in the heart is primarily controlled by adipose triglyceride lipase (ATGL) [9], this study examined whether maintaining a chronically low intramyocardial TAG pool, via forced-expression of ATGL specifically in cardiomyocytes, influences doxorubicin-induced cardiotoxicity. Our results show that maintaining a state of chronically low intramyocardial TAGs with concurrent administration of doxorubicin is sufficient to protect from doxorubicin-induced cardiac dysfunction.

Methods

Animals

ATGL over-expressing (MHC-ATGL) mice were generated as described previously in Chapter 2 [9]. A detailed description is presented in the Supplements. Female mice were chosen since female breast cancer is the most common cancer in North America and male breast cancer is a clinically rare entity [10]. Thus the study of cardiotoxic effects of doxorubicin is most appropriately conducted in female animals.

Induction of doxorubicin-induced cardiac dysfunction

Doxorubicin administration was performed as described in Chapter 2. Previous reports have shown left ventricular (LV) dysfunction without overt heart failure with this doxorubicin dose [11]. Thus we chose this protocol to mimic a clinically relevant scenario since the incidence of heart failure in recent doxorubicin regimen trials is less than 2.1% [12].

Echocardiography

Transthoracic echocardiography was performed as described previously [9]. A detailed description is presented in Chapter 2.

Heart Perfusions

Hearts were aerobically perfused in the working mode as previously described [9]. A detailed description is presented in Chapter 2.

Tissue homogenization and lipid analysis

Tissue homogenization, lysate protein assay, and tissue TAG content analysis was performed as described previously [9]. Quantification of long-chain acyl coenzyme A (acyl-CoA) species and ceramides was performed by UPLC [9]. A detailed description is presented in Chapter 2.

Immunoblot analysis

Immunoblot analysis was performed as described previously [9]. A detailed description is presented in Chapter 2.

Serum triacylglycerol analysis

Serum TAG concentration was determined using the 2780-400H Infinity TAG reagent (Thermo MA).

Gene expression analysis

Gene expression analysis was performed using quantitative reverse transcriptase PCR [9]. A detailed description is presented in Chapter 2.

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student's t-test. *P*-values of less than 0.05 were considered statistically significant.

Results

Four weeks of doxorubicin administration impairs in vivo systolic function but not diastolic function in WT mice

Doxorubicin administration of 8 mg/kg i.p. once a week for 4 consecutive weeks resulted in no observed mortality. While body weights were unchanged following saline administration in WT mice, a significant 8.9% loss in body weight was observed following doxorubicin administration (Fig. 4-1A), suggesting that this doxorubicin treatment regimen elicits a systemic response similar to previous reports [13]. We next determined whether 4 weeks of doxorubicin treatment led to morphological changes in the heart of WT mice. Indeed, doxorubicin-treated WT mice exhibited significant cardiac atrophy when compared to the saline-treated counterparts, as was evidenced by the decreased ratio of ventricular weight to tibia length in doxorubicin-treated WT mice (Fig. 4-1B). To further assess the effect of doxorubicin treatment on ventricular wall dimensions, we performed noninvasive transthoracic echocardiography. Consistent with the observed morphological changes, left ventricular posterior wall thickness in systole (Fig. 4-1C) and diastole (Fig. 4-1D) was significantly decreased in WT mice following doxorubicin administration, suggesting that 4 weeks of doxorubicin treatment causes significant thinning of the ventricular free walls. Importantly, echocardiographic analysis also showed an 11% reduction in the ejection fraction of doxorubicin-treated WT mice, suggesting systolic dysfunction (Fig. 4-1E and F). This decrease in systolic function was associated with a significant increase in the ratio of wet to dry lung weight (Fig. 4-1G) in WT mice treated with doxorubicin compared to the saline-treated controls. In contrast to the changes in systolic function, parameters of diastolic function such as mitral valve deceleration time, E/A ratio, E/E' and s/d ratio were unchanged between WT mice treated with doxorubicin and WT mice treated

with saline (Table 4-1). Taken together, these findings suggest that following 4 weeks of doxorubicin treatment, cardiac atrophy is paralleled by a selective decline in systolic function.

Doxorubicin-induced cardiac dysfunction in vivo is independent of changes in glucose and fatty acid oxidation in WT hearts

Previous work using neonatal rat cardiomyocytes, animal models and human studies, have suggested that doxorubicin administration decreases cardiomyocyte overall myocardial energy production by reducing oxidative phosphorylation rates [14]. However, this has not been directly measured in the working heart. Therefore, to determine whether alterations in myocardial energy metabolism could contribute to doxorubicin-induced cardiac dysfunction in WT mice, we measured myocardial glucose and oleate oxidation. This was accomplished by subjecting hearts to *ex vivo* perfusions in the working mode using radiolabeled substrates. Since heart rate (Fig. 4-2A) and left ventricular minute work (Fig. 4-2B) *ex vivo* were comparable between hearts from doxorubicin and saline treated WT mice, differences in substrate metabolism could be evaluated in the absence of alterations in energetic demand. In contrast to previous reports [14], our data show that rates of glucose (Fig. 4-2C) and oleate oxidation (Fig. 4-2D) were unchanged in *ex vivo* perfused working hearts from WT mice following doxorubicin treatment. Taken together, these findings demonstrate that 4 weeks of doxorubicin treatment does not lead to changes in mitochondrial oxidative metabolism of glucose and FAs, and that doxorubicin-induced cardiac dysfunction in WT hearts is independent from changes in oxidative substrate metabolism.

Doxorubicin administration reduces myocardial TAG content in WT mice

Since it has been recently shown that doxorubicin treatment causes cardiac lipotoxicity [15], we next examined whether changes in myocardial accumulation of toxic FA metabolites

and TAG could contribute to doxorubicin-induced cardiotoxicity. Interestingly, 4 weeks of doxorubicin administration did not lead to alterations in total long chain acyl-CoA (Fig. 4-3A) and ceramide (Fig. 4-3B) content in hearts from WT mice when compared to the saline-treated controls. In addition, the myocardial mRNA expression of enzymes involved in the synthesis of TAG in the myocardium [1-acyl-sn-glycerol-3-phosphate acyltransferase (aGPAT) and diacylglycerol acyltransferase-2 (DGAT2)], were not decreased with doxorubicin treatment in any of the groups (data not shown). However, doxorubicin administration significantly decreases serum TAG levels in WT mice (Fig. 4-3C). This finding is consistent with the significant decrease in intramyocardial TAG content in hearts from doxorubicin-treated WT mice (Fig. 4-3D). Interestingly, activating phosphorylation of hormone-sensitive lipase (HSL) at Serine 660, an enzyme involved in TAG catabolism, was similar between WT mice treated with saline and doxorubicin (Fig. 4-3E). In addition, protein expression of the lipid droplet coat protein, perilipin 5 (PLIN5), which has been shown to regulate TAG storage and catabolism [16], remained unchanged in hearts from WT mice following doxorubicin administration (Fig. 4-3F). Moreover, ATGL mRNA expression was unchanged (Fig. 4-3G) and there was an insignificant trend towards upregulation of ATGL protein expression in hearts from WT mice treated with doxorubicin (Fig. 4-3H). Together, these data suggest that cardiac dysfunction following 4 weeks of doxorubicin administration is associated with a reduction in myocardial TAG concentrations and is independent of alterations in known lipotoxic species such as long chain acyl-CoAs and ceramides.

Cardiomyocyte-specific ATGL over-expression protects from doxorubicin-induced cardiac dysfunction

Since doxorubicin-induced cardiomyopathy was associated with a significant decrease in circulating TAG levels and myocardial TAG content (Fig. 4-3C), we hypothesized that altering the intramyocardial TAG pool could influence the functional outcome of doxorubicin-induced

cardiotoxicity. To test this hypothesis, we utilized mice with cardiomyocyte-specific over-expression of ATGL (MHC-ATGL; Fig. 4-3E to G). This is a mouse model with chronically reduced myocardial TAG content (Fig. 3C) and PLIN5 protein expression (Fig. 4-3G), and unchanged levels of long chain acyl-CoAs (Fig. 4-3A) and ceramides (Fig. 4-3B) at baseline.

Following 4 weeks of doxorubicin administration, MHC-ATGL mice showed a significant 9.5% loss in body weight (Fig. 4-1A) that was similar to that observed in WT mice, suggesting that the systemic response to doxorubicin treatment was comparable between genotypes. However, when specifically assessing cardiac morphology, we observed that cardiomyocyte-specific ATGL over-expression ameliorated the significant doxorubicin-induced cardiac atrophy exhibited in WT mice, as was evidenced by unchanged ratio of ventricular weight to tibia length (Fig. 4-1B) and left ventricular posterior wall thickness in systole (Fig. 4-1C) and diastole (Fig. 4-1D). Importantly, echocardiographic analysis also showed unchanged ejection fraction in doxorubicin-treated MHC-ATGL mice, suggesting that ATGL over-expression protected from systolic dysfunction following 4 weeks of doxorubicin administration. In addition, this protection from *in vivo* systolic dysfunction also manifested itself in the preservation of the ratio of wet to dry lung weight (Fig. 4-1G) in MHC-ATGL mice treated with doxorubicin when compared to the saline-treated mice. Also, echocardiographic parameters of diastolic function were also unchanged in doxorubicin-treated MHC-ATGL mice.

Similar to WT hearts, oxidation rates of glucose and oleate were unchanged in *ex vivo* perfused working hearts form MHC-ATGL mice following doxorubicin treatment (Fig. 4-2C and D), corroborating that doxorubicin-induced cardiac dysfunction in WT mice and the protection from doxorubicin-induced cardiomyopathy in MHC-ATGL mice are not secondary to changes in mitochondrial oxidation of glucose and FAs. Taken together, these data suggest that following 4 weeks of doxorubicin treatment, cardiomyocyte-specific ATGL over-expression and the

resulting decrease in myocardial TAG content are sufficient to protect against doxorubicin-induced cardiac atrophy, systolic dysfunction, and pulmonary signs of CHF.

Table 4-1. Echocardiographic Parameters From WT and MHC-ATGL Mice

Parameters		WT		MHC-ATGL	
		Saline	Doxorubicin	Saline	Doxorubicin
Systolic Function					
	Fractional Shortening, %	28.1± 2.3	25.1 ± 2.0*	30.4 ± 1.9	29.5 ± 1.6
Diastolic Function					
	Isovolumic Relaxation Time, ms	18.2 ± 1.5	18.1 ± 0.9	14.3 ± 0.9	17.4 ± 1.1
	E/A ratio	1.47 ± 1.6	1.96 ± 0.2	1.65 ± 0.11	1.95 ± 0.2
	Mitral Valve Deceleration Time, ms	16.7 ± 1.7	15.9 ± 1.9	15.7 ± 1.3	18.0 ± 1.6
	E/E'	24.7 ± 1.6	21.8 ± 1.6	25.9 ± 2.4	28.6 ± 1.6
	Systolic/Diastolic ratio	0.51 ± 0.03	0.42 ± 0.04	0.46 ± 0.05	0.47 ± 0.03
Wall Measurements					
	Left Atrial Diameter, mm	1.94 ± 0.2	1.81 ± 0.1	2.28 ± 0.2	1.99 ± 0.1
	Interventricular Septal Thickness - Diastole, mm	0.88 ± 0.14	0.65 ± 0.01	0.76 ± 0.04	0.70 ± 0.01
	Interventricular Septal Thickness - Systole, mm	0.99 ± 0.09	0.85 ± 0.02	1.01 ± 0.06	1.02 ± 0.02
	Left Ventricular Internal Diameter - Diastole, mm	3.29 ± 0.66	3.70 ± 0.88	3.54 ± 0.04	3.57 ± 0.09
	Left Ventricular Internal Diameter - Systole, mm	2.40 ± 0.39	2.84 ± 0.12	2.46 ± 0.08	2.52 ± 0.10

Data are mean ± SEM of 4 to 7 WT and MHC-ATGL mice (30 to 33 weeks old)

*$p < 0.05$ for Saline vs. Doxorubicin values between mice of the same genotype

Figure 4-1. Effects of weekly doxorubicin administration at 8 mg/kg i.p. for 4 weeks on myocardial performance and left ventricular geometry in WT and MHC-ATGL mice. **A.** Percent initial body weight; **B.** Ratio of ventricle weight to tibia length. **C.** Left ventricular posterior wall thickness in systole (LVPWs); **D.** Left ventricular posterior wall thickness in diastole (LVPWd); **E.** Representative M-mode images; **F.** Ejection fraction; **G.** Wet to dry lung weight ratio. Values are mean ± SEM of n=4 to 7 female mice in each group (30 to 33 weeks old). *$p < 0.05$, saline vs. doxorubicin.

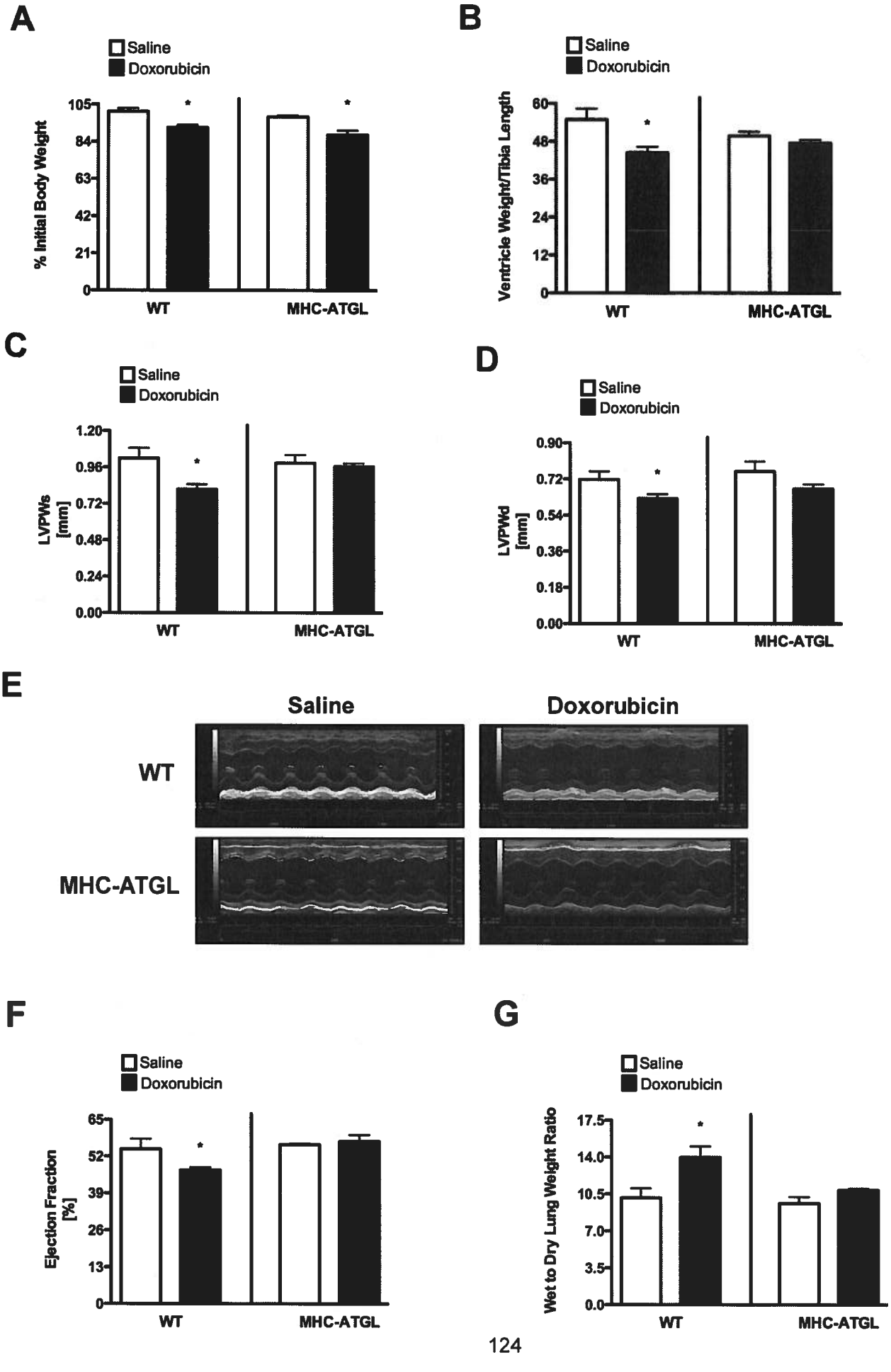

A
Saline
Doxorubicin
% Initial Body Weight
105
84
63
42
21
0
WT
MHC-ATGL
B
Saline
Doxorubicin
Ventricle Weight/Tibia Length
60
48
36
24
12
0
WT
MHC-ATGL
C
Saline
Doxorubicin
LVPWs [mm]
1.20
0.96
0.72
0.48
0.24
0.00
WT
MHC-ATGL
D
Saline
Doxorubicin
LVPWd [mm]
0.90
0.72
0.54
0.36
0.18
0.00
WT
MHC-ATGL
E
Saline
Doxorubicin
WT
MHC-ATGL
F
Saline
Doxorubicin
Ejection Fraction [%]
65
52
39
26
13
0
WT
MHC-ATGL
G
Saline
Doxorubicin
Wet to Dry Lung Weight Ratio
17.5
14.0
10.5
7.0
3.5
0.0
WT
MHC-ATGL

Figure 4-2. Cardiac energy metabolism of *ex vivo* perfused working hearts from saline and doxorubicin treated WT and MHC-ATGL mice. A. Heart rate. **B.** Left ventricular minute work. **C.** glucose oxidation rates. **D.** oleate oxidation rates. Values are mean ± SEM of n=4 to 7 female mice in each group (30 to 33 weeks old).

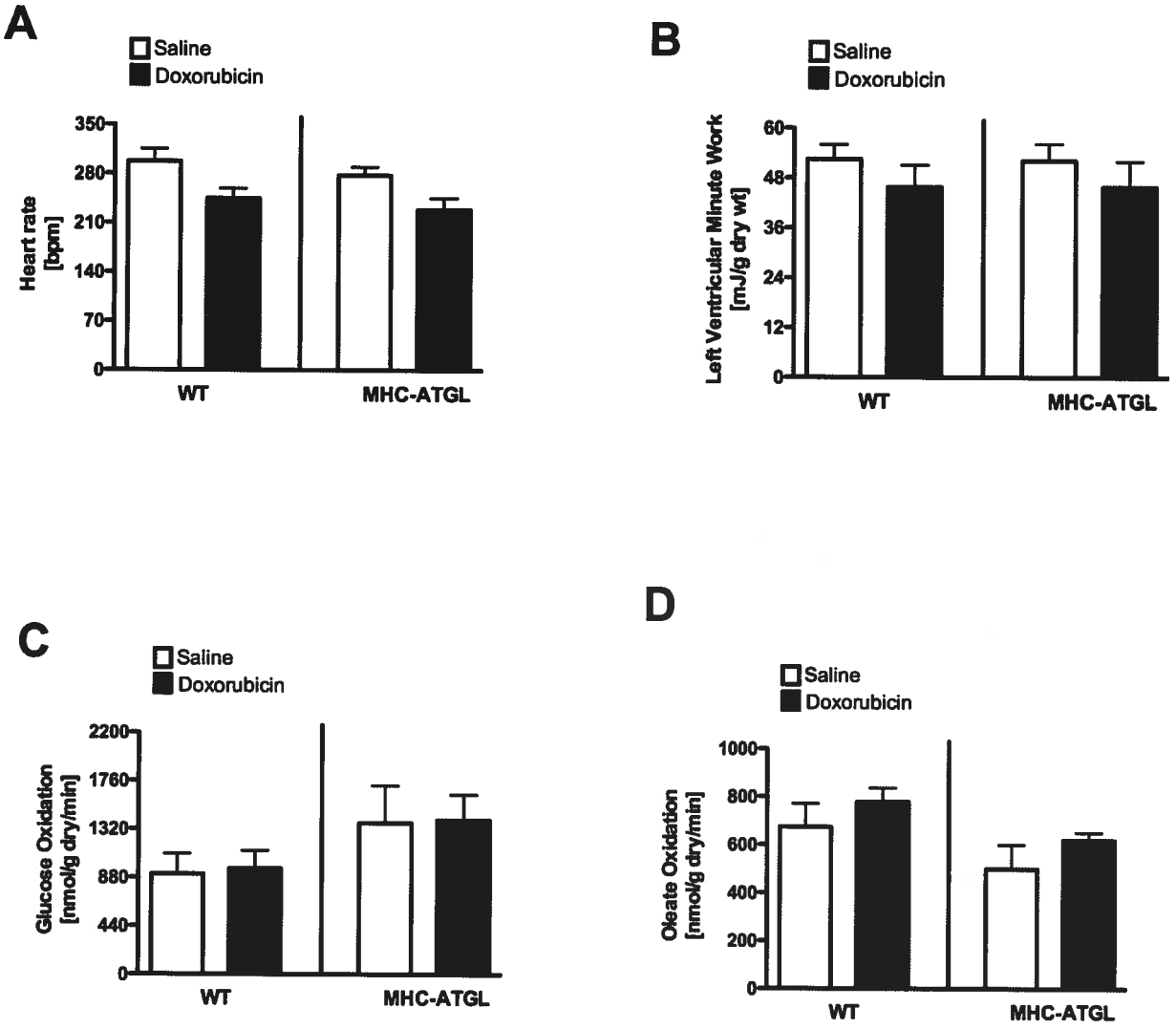
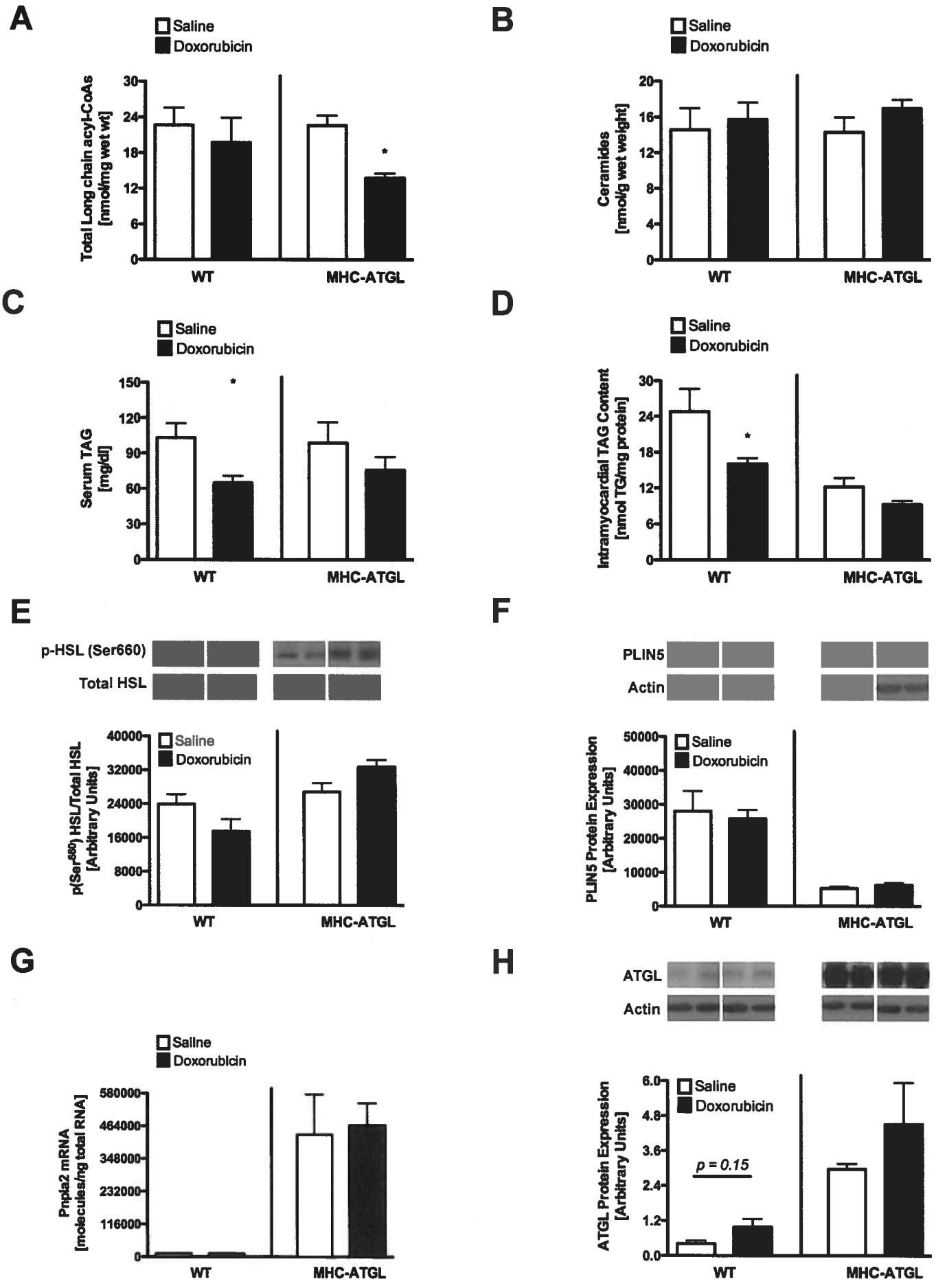

A
Saline
Doxorubicin
Heart rate [bpm]
350
280
210
140
70
0
WT
MHC-ATGL
B
Saline
Doxorubicin
Left Ventricular Minute Work [mJ/g dry wt]
60
48
36
24
12
0
WT
MHC-ATGL
C
Saline
Doxorubicin
Glucose Oxidation [nmol/g dry/min]
2200
1760
1320
880
440
0
WT
MHC-ATGL
D
Saline
Doxorubicin
Oleate Oxidation [nmol/g dry/min]
1000
800
600
400
200
0
WT
MHC-ATGL

Figure 4-3. Effects of doxorubicin on myocardial lipotoxic intermediates and triacylglycerol (TAG) metabolism in WT and MHC-ATGL mice following saline or doxorubicin administration. A. Myocardial total long-chain acyl-CoA content and **B.** ceramide content. **C.** Serum TAG content. **D.** Intramyocardial TAG content. **E.** Immunoblot analysis was performed using ventricular homogenates. Levels of phosphorylated HSL (Ser660) were quantified by densitometry and normalized against total HSL. **F.** Immunoblot analysis of PLIN5 protein expression. Levels of PLIN5 were quantified by densitometry and normalized against actin protein expression. **G.** Cardiac *Pnpla2 (Atgl)* mRNA expression. **H.** Immunoblot analysis of ATGL protein expression. Levels of ATGL were quantified by densitometry and normalized against actin protein expression. Values are mean ± SEM of n=4 to 7 female mice in each group (30 to 33 weeks old). *$p < 0.05$, saline vs. doxorubicin.


A
Saline
Doxorubicin
Total Long chain acyl-CoAs [nmol/mg wet wt]
30
24
18
12
6
0
*
WT
MHC-ATGL
B
Saline
Doxorubicin
Ceramides [nmol/g wet weight]
20
16
12
8
4
0
WT
MHC-ATGL
C
Saline
Doxorubicin
Serum TAG [mg/dl]
150
120
90
60
30
0
*
WT
MHC-ATGL
D
Saline
Doxorubicin
Intramyocardial TAG Content [nmol TG/mg protein]
30
24
18
12
6
0
*
WT
MHC-ATGL
E
p-HSL (Ser660)
Total HSL
Saline
Doxorubicin
p(Ser660) HSL/Total HSL [Arbitrary Units]
40000
32000
24000
16000
8000
0
WT
MHC-ATGL
F
PLIN5
Actin
Saline
Doxorubicin
PLIN5 Protein Expression [Arbitrary Units]
50000
40000
30000
20000
10000
0
WT
MHC-ATGL
G
Saline
Doxorubicin
Pnpla2 mRNA [molecules/ng total RNA]
580000
464000
348000
232000
116000
0
WT
MHC-ATGL
H
ATGL
Actin
Saline
Doxorubicin
ATGL Protein Expression [Arbitrary Units]
6.0
4.8
3.6
2.4
1.2
0.0
p = 0.15
WT
MHC-ATGL

Discussion

We investigated whether doxorubicin-induced cardiac dysfunction is accompanied by changes in myocardial exogenous substrate metabolism, lipotoxicity, and TAG accumulation. Additionally, we also studied whether manipulation of the intramyocardial TAG pool could be used as a novel method to ameliorate doxorubicin-induced cardiac dysfunction. As expected, doxorubicin administration resulted in cardiac atrophy that was paralleled by a selective decline in systolic function and precipitating pulmonary signs of CHF in WT mice. In contrast, mice that maintain a chronically low intramyocardial TAG pool via forced-expression of ATGL were resistant to doxorubicin-induced cardiac atrophy, LV systolic dysfunction and were protected from the pulmonary signs of CHF. These data show that forced expression of ATGL and a chronically decreased myocardial TAG pool protects from doxorubicin-induced cardiomyopathy.

In order to investigate the mechanisms by which increased ATGL expression, leading to a decreased myocardial TAG pool, protects from doxorubicin-induced cardiomyopathy, we examined myocardial substrate metabolism. Indeed, an increasing number of studies suggest that alterations in myocardial exogenous substrate metabolism are associated with doxorubicin-induced cardiac dysfunction [14]. Specifically, previous work has suggested that doxorubicin treatment decreases FA and glucose oxidation and the expression of enzymes involved in FA and glucose metabolism [14]. However, it is still unclear if doxorubicin treatment changes myocardial oxidative phosphorylation in the intact working heart and whether this is preceded or followed by changes in cardiac function. Therefore, in order to understand the role that myocardial exogenous substrate metabolism plays in doxorubicin-induced cardiac dysfunction, we examined myocardial FA and glucose oxidation rates using *ex vivo* working heart perfusions. In contrast to previous reports [14], FA and glucose oxidation rates were unchanged and thus

not associated with the cardiac dysfunction induced by this doxorubicin treatment protocol in WT and MHC-ATGL mice. This suggests that the doxorubicin-induced decrease in cardiac contractility develops independently from changes in mitochondrial oxidation of glucose and FAs. These findings also show that the ability of cardiomyocyte-specific ATGL over-expression to prevent doxorubicin-induced cardiac dysfunction is not due to alterations in cardiac exogenous substrate metabolism or impaired oxidative phosphorylation. Together, these findings are particularly importance as they highlight that mechanisms other than impaired myocardial substrate metabolism [14, 17] are contributing to doxorubicin-induced cardiac dysfunction. That said, overall doxorubicin damage to the hearts is less severe in the present study compared to previous studies [6]. Indeed, the doxorubicin administration protocol used in the present study does not cause overt heart failure but rather cardiac dysfunction, so as to more closely mimic the clinical scenario. Another, difference in our model compared to some of the previous work is that we have measured cardiac energy metabolism in the perfused working heart, rather than in isolated cardiomyocytes [6]. Together, our data suggest that alterations in energy metabolism may occur as a consequence of impaired function as opposed to causing cardiac dysfunction.

As alterations in myocardial exogenous substrate metabolism did not appear to be responsible for doxorubicin-induced cardiac dysfunction or the beneficial effects of ATGL over-expression, we examined other possible mechanisms. Recent studies have suggested that doxorubicin treatment leads to systemic lipotoxicity [15] and that this may contribute to doxorubicin-induced cardiotoxicity. To address this, we examined the effects of doxorubicin administration on intramyocardial long chain acyl-CoA and ceramides content in WT and MHC-ATGL mice, as these are lipotoxic to the cardiomyocyte [18]. In contrast to previous work that reported increased accumulation of lipotoxic intermediates with the administration of doxorubicin

in cultured cells [19], we found that these lipotoxic FA metabolites were unaltered by doxorubicin administration in hearts from WT mice. Similar to WT mice, MHC-ATGL mice treated with doxorubicin did not show altered accumulation of intramyocardial ceramide content. However, hearts from MHC-ATGL mice treated with doxorubicin showed a significant decrease in long chain acyl-CoA content compared to hearts from saline-treated MHC-ATGL mice or doxorubicin-treated WT mice. These latter findings suggest that a decrease in myocardial long chain acyl-CoA content induced by over-expression of ATGL may be partially responsible for preventing doxorubicin-induced cardiotoxicity and cardiac dysfunction. In fact, our data are entirely consistent with previous work in a rat model of doxorubicin-induced cardiotoxicity showing that a reduction of cardiomyocyte lipid accumulation diminishes the cardiac side effects of doxorubicin [7].

Although forced expression of ATGL may protect from doxorubicin-induced cardiac dysfunction via reduced long chain acyl-CoA content, we also investigated whether alterations in TAG levels and/or TAG catabolism may be involved. This is particularly important since there is growing evidence indicating that a hypolipidemic cardiomyocyte environment diminishes the cardiotoxic effects of doxorubicin-treated animals and patients [7]. In addition, previous studies have suggested that cardiomyocyte TAG catabolism plays an important role in regulating cardiac function in pathological conditions [8, 20], further supporting the notion that TAG levels may be important in doxorubicin-induced cardiotoxicity. Based on this rationale, we assessed intramyocardial TAG content as well as expression or phosphorylation of enzymes involved in TAG catabolism (HSL and ATGL) and storage (PLIN5). Interestingly, doxorubicin treatment led to a significant decrease in intramyocardial TAG content without significant changes to HSL activating phosphorylation at Ser660 or expression of ATGL and PLIN5 in WT mice. Because of these findings and the observation that myocardial FA oxidation was unchanged following

doxorubicin treatment, it is tempting to speculate that rather than enhanced TAG catabolism, reduced FA uptake and incorporation into newly formed TAG contributes to the decrease in TAG accumulation following doxorubicin treatment. Although this decrease in intramyocardial TAG levels in WT mice treated with doxorubicin may suggest that this contributes to cardiac dysfunction, a more dramatic reduction in cardiac TAG levels in MHC-ATGL mice was associated with preserved cardiac function. Although we generated a sophisticated mouse model that specifically decreases cardiac TAG levels, it is possible that other mechanisms are contributing to the protective effects from doxorubicin-induced cardiac dysfunction. As such, further studies are required to elucidate these potential mechanisms. However, based on these findings, we propose that the reduction in myocardial TAG accumulation in WT hearts is an insufficient adaptive response to doxorubicin treatment and that more pronounced lowering of intramyocardial TAGs induced by ATGL over-expression contributes to preventing doxorubicin-induced cardiac dysfunction. Therefore, observed decrease of intramyocardial TAG content in hearts treated with doxorubicin is an adaptive response that is further enhanced by ATGL over-expression.

This is the first study to assess the effects of doxorubicin administration on myocardial oxidative metabolism of glucose and FAs in the *ex vivo* perfused working heart as well as TAG accumulation. In contrast to previous reports, our findings demonstrate that doxorubicin-induced cardiac dysfunction occurs in the absence of alterations in myocardial glucose and FA oxidation. More importantly, our results show that manipulation of intramyocardial TAG metabolism leading to a chronically decreased myocardial TAG pool protects from doxorubicin-induced cardiomyopathy. Moreover, these findings suggest that the reduction in myocardial TAG accumulation in WT hearts is an adaptive, albeit insufficient response to doxorubicin treatment. Therefore, novel pharmacological targets aiming at reducing myocardial TAG concentrations

may provide new avenues for the treatment of cardiomyopathy in cancer patients undergoing anthracycline chemotherapy by producing a hypolipid environment within cardiomyocytes.

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CHAPTER 5.

Myocardial function during physiological and pathological stress is independent of metabolic changes induced by AMPK-mediated inhibitory phosphorylation of ACC

My role in this work involved in performing all the experiments (except those noted below), analysis and interpretation of the data as well as the writing of the manuscript. Technical staff performed UPLC and echocardiography in the CVRC core facility.

Manuscript Status*: A version of this manuscript is currently in preparation to be submitted for publication as an original article*

CHAPTER 5.

Myocardial function during physiological and pathological stress is independent of metabolic changes induced by AMPK-mediated inhibitory phosphorylation of ACC

Abstract

Background: During physiological and pathological stress the energy sensor adenosine monophosphate-activated protein kinase (AMPK) is suggested to play an important role in the regulation of myocardial substrate metabolism and function via its effect on its downstream target acetyl coenzyme A carboxylase (ACC). However, whether these effects require AMPK-mediated phosphorylation and inactivation of ACC remains to be definitively established. Therefore, the purpose of this study was to demonstrate whether AMPK-mediated inactivation of ACC influences myocardial metabolism and function during physiological stress induced by either increased myocardial workload or pathological stress induced by ischemia/reperfusion (I/R).

Materials and Methods: A double knock-in (DKI) mouse (ACC-DKI) model was generated in which the AMPK phosphorylation sites Ser79 on ACC1 and Ser221 on ACC2 were mutated to alanine, leading to the inability of AMPK to phosphorylate and inactivate ACC. Ex-vivo working heart perfusions were employed to assess cardiac metabolism and function.

Results: ACC-DKI mice displayed a significant increase in cardiac malonyl CoA content compared to wild-type (WT) mice. Despite augmented malonyl-CoA levels, cardiac glucose and palmitate oxidation rates were comparable between WT and ACC-DKI mice. Consistent with unchanged cardiac energy metabolism in ACC-DKI mice, baseline cardiac function assessed via transthoracic echocardiography and aerobic working heart perfusions were also similar between genotypes. Despite increased glucose oxidation in ACC-DKI mice following increased workload *ex vivo* myocardial function was similar between genotypes. Furthermore during pathological stress induced by *ex-vivo* I/R, ACC-DKI and WT hearts exhibited similar metabolic profile and functional recovery.

Conclusions: Collectively, our findings challenge the previously suggested role of AMPK-mediated ACC phosphorylation and inactivation as having a major role in the regulation of substrate metabolism and function in healthy and stressed myocardium.

Key Words: Cardiac Metabolism, Workload, Ischemia and Reperfusion, AMPK and ACC

Introduction

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor regulating numerous metabolic processes[1] including glucose and fatty acid (FA) uptake[2-4], oxidation[5, 6] and storage[7-10]. Cardiomyocyte AMPK activation augments glucose uptake, glycolysis [11] and FA β-oxidation[6] in an insulin-independent manner[12]. A notable change in myocardial FA oxidation (FAO) is reported to occur via the ability of AMPK to phosphorylate and inactivate its downstream target acetyl coenzyme A carboxylase (ACC) [13-15] . PKA and AMPK phosphorylate rat ACC265 isoform at Ser77 and Ser1200, and Ser79, Ser1200 and Ser1215, respectively. Inactivation appears to result from phosphorylation at Ser79 and Ser1200. The two isoforms of ACC (ACC 1 and 2) are phosphorylated by AMPK at two well-characterized serine residues (Ser79 and 221), leading to inhibition of ACC activity[16, 17]. AMPK-mediated phosphorylation of ACC1 at Ser79 inhibits catalytic activity in cell-free systems[18], however, the significance of ACC1 and/or ACC2 inhibition in the myocardium has yet to be tested.

ACC catalyzes the conversion of acetyl CoA to malonyl CoA an essential precursor for FA synthesis in some organs[19] and an endogenous inhibitor of carnitine palmitoyl CoA transferase (CPT) 1, which regulates long-chain FA import into the mitochondria for β-oxidation[20]. Prior studies have argued that AMPK accelerates FAO by inducing suppression of ACC activity and lowering malonyl CoA levels. However, whether changes in ACC activity by AMPK and its ensuing influence on myocardial substrate metabolism plays a major role in maintaining function during physiological and/or pathological stress is currently unclear. Based on this, understanding the importance of the AMPK-ACC-malonyl CoA axis in the control of myocardial energy metabolism will help define the mechanisms that control energy metabolism

in the heart as well as resolve controversies surrounding the role that AMPK activation plays in controlling myocardial injury in response to cardiac stress.

Methods

Animals

ACC double knock-in (ACC-DKI) mutant mice were generated as described in Chapter 2. Mice were housed on a 12-h light/12-h dark cycle with ad libitum access to chow diet (product number 5001 from Lab Diet, St. Louis, MO, with 13.5% kcal from fat) and water. For all experiments, littermate wild-type (WT) mice were used as controls. The University of Alberta Institutional Animal Care and Use Committee approved all protocols involving mice.

Echocardiography and Dobutamine Stress Echocardiography

Mice were mildly anesthetized using isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada). To assess *in vivo* heart function under high workload conditions, dobutamine was administered at a dose of 4mg/kg via intraperitoneal injections during echocardiography. Ten minutes after dobutamine injection, *in vivo* function was assessed, and hearts were harvested and frozen for subsequent biochemical analysis. Further details are described in Chapter 2.

Heart Perfusions

Hearts were perfused in the working heart mode at 11.5 mm Hg preload and 50 mm Hg afterload with Krebs-Henseleit buffer containing 1.2 mmol/L palmitate prebound to 3% delipidated bovine serum albumin (BSA), 5 mmol/L glucose, and 50 μU/mL insulin. To assess *ex vivo* heart function under high workload conditions, hearts were perfused for an initial period of 30 min at normal workload followed by a 30-min perfusion at 80 mmHg afterload with buffer containing an additional 300 nmol/liter isoproterenol. To assess *ex vivo* heart function during

ischemia and reperfusion, hearts were aerobically perfused for 30 minutes, or aerobically perfused for 30 minutes followed by 18 minutes of global no flow ischemia and 40 minutes of reperfusion. At the end of aerobic perfusion, high workload perfusions or reperfusion, hearts were immediately frozen in liquid N_2 with a Wollenberger clamp and stored at -80°C as described previously[21]. For metabolic measurements, palmitate and glucose were labeled using either a combination of [9,10-^{3}H]palmitate and [U-^{14}C]glucose (for determination of FAO and glucose oxidation) or [U-^{14}C]glucose and [5-^{3}H]glucose (for determination of glucose oxidation and glycolysis) as previously described in Chapter 2[21].

Tissue homogenization and Immunoblot analysis

Frozen hearts were ground using mortar and pestle, and tissue powder was homogenized in ice-cold lysis buffer containing 20 mmol/liter Tris-HCl (pH 7.4), 5 mmol/liter EDTA, 10 mmol/liter $Na_4P_2O_7$, 100 mmol/liter NaF, 1% Nonidet P-40, 2 mmol/liter Na_3VO_4, protease inhibitor (product number P8340, 10μl/ml; Sigma, St. Louis, MO), and phosphatase inhibitor (product number 524628, 20 μg/ml; Calbiochem, EMD Chemicals, Gibbstown, NJ) unless otherwise stated. Homogenates were centrifuged at 1,200 x g for 20 min at 4°C, and the supernatants were transferred to fresh tubes. Protein concentration in lysates was determined using the bicinchoninic acid (BCA) protein assay kit (product number 23255; Pierce, Thermo Fisher Scientific, Rockford, IL), and serum albumin was employed as the standard (product number 23210; Pierce). Lysates were aliquoted and stored at -80°C until further usage for immunoblot analysis. Tissue lysates were resolved by SDS-PAGE, and proteins were transferred onto a nylon membrane. Blotted proteins were reversibly visualized using MemCode stain (Pierce) and identified using the primary antibodies: anti-ACC (product number 07-303; EMD Millipore, Billerica, MA), anti-AMPKα2 (number 19131; Santa Cruz Biotechnology, Dallas, TX), anti-phospho ACC (product number 3661; Cell Signaling) and anti-phospho AMPK (product

number 2531; Cell Signaling) antibodies. Immunoblots were developed using the Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). All densitometric data were corrected against total protein loading visualized via Memcode (Pierce) staining as described in Chapter 2.

Nucleotide and Malonyl CoA Measurements

Malonyl CoA and nucleotides were extracted from 20 to 30 mg of frozen ventricular tissue by homogenization in ice-cold 6% (vol/vol) perchloric acid-1 mmol/liter dithiothreitol-0.5 mmol/liter EGTA. Homogenates were spun at 12,000 x g for 5 min at 4°C. Half of the homogenate was then used for quantification of malonyl CoA content by Ultra-high performance liquid chromatography (UPLC) and the other half of the homogenate was used for nucleotide extractions. The homogenate used for nucleotide measurements required neutralization of pH using K_2CO_3. Samples were kept on ice for an additional 30 minutes followed by centrifugation at 10,000 x g for 2 min at 4°C. Nucleotide concentrations in supernatants were determined by UPLC.

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were made by unpaired two-tailed Student's t-test. *P*-values of less than 0.05 were considered statistically significant.

Results

Cardiac malonyl CoA content is regulated by AMPK mediated ACC phosphorylation.

Hearts from double knock-in (ACC-DKI) mice but not WT mice exhibited a lack of ACC phosphorylation on Ser79 (ACC1) and Ser212 (ACC2; Fig. 5-1A and B), confirming the lack of inhibiting phosphorylation by AMPK. This lack of AMPK effect on ACC phosphorylation was not secondary to baseline changes in AMPK activity since the ratio of phosphorylated AMPK at Thr172 to total AMPKα2 protein expression were similar in hearts from either genotypes (Fig. 5-1C and D). As expected, the inability of AMPK to phosphorylate and inhibit ACC promoted the synthesis of malonyl CoA (Fig 5-1E and F) with hearts from ACC-DKI mice displaying a two-fold increase in cardiac malonyl CoA content compared to WT (Fig. 5-1F). These findings provide *in vivo* proof that cardiac malonyl CoA content can be mediated by AMPK phosphorylation and inhibition of ACC.

Alterations in inhibitory ACC phosphorylation do not influence baseline cardiac metabolism and function.

In the heart, metabolism of FA and glucose yields acetyl CoA that undergoes oxidative decarboxylation within the mitochondria to generate ATP. Given the regulatory role of malonyl CoA in controlling CPT-1 and ensuing FAO, we speculated that the observed increase in malonyl CoA in hearts from ACC DKI mice would correspondingly influence substrate oxidation. Interestingly, despite increases in malonyl-CoA content, glucose oxidation (Fig. 5-2A) and palmitate oxidation (Fig. 5-2B) rates were comparable between hearts from WT and ACC-DKI mice suggesting that the degree of increase in malonyl CoA content observed in hearts from ACC-DKI mice is not sufficient to alter baseline cardiac metabolism and function (Table 5-1).

Consistent with similar *ex vivo* metabolic and functional profile in hearts from either genotypes, hearts from ACC-DKI mice have comparable morphology and size compared to hearts from WT mice and displayed no difference in gross structure (Fig. 5-2C), ventricular weight to tibial length (Fig. 5-2D) and left ventricular posterior wall diameter (Fig. 5-2E). In agreement with unchanged cardiac structure and morphology, ejection fraction (Fig. 5-2H) and isovolumic relaxation time (Fig. 5-2I) were not altered in hearts from ACC-DKI mice hearts compared to WT (Table 5-2). Taken together, these data indicate that preventing ACC phosphorylation by AMPK in the non-stressed myocardium can increase myocardial malonyl CoA levels but that these changes are sufficient to influence cardiac oxidative metabolism, morphology or function.

Metabolic alterations in ACC-DKI hearts do not confer changes in myocardial performance during increased physiologic work.

Increased cardiac malonyl CoA and corresponding elevations in glucose oxidation are suggested to be necessary and sufficient to maintain optimal cardiac function during conditions of augmented workload [22, 23]. As such, we postulated that increased myocardial workload in ACC DKI mice would result in enhanced contractile function compared to WT mice. To address this hypothesis, we treated mice from both genotypes with dobutamine (2 mg/kg, i.p.) to induce a positive chronotropic and inotropic effect in the myocardium and subsequently assessed cardiac function by transthoracic echocardiography (Table 5-3). While dobutamine increased myocardial malonyl CoA content (Fig. 5-3A) in both genotypes compared to saline treatment (Fig. 5-1F), this effect was significantly potentiated in hearts from ACC-DKI mice compared to WT. The effect of *in vivo* dobutamine treatment on cardiac malonyl CoA content was not related to differences in heart rate (Fig. 5-3B), ejection fraction (Fig. 5-3C) or isovolumic relaxation time (Fig. 5-3D).

To eliminate the influence of non-cardiac tissue and other circulating systemic factors in regulating heart function and metabolism during increased workload, hearts from WT and ACC DKI mice were subjected to isoproterenol perfusions *ex vivo* to mimic higher workload conditions (Table 5-4). Contrary to what we expected, inhibiting AMPK-mediated phosphorylation of ACC did not result in changes in palmitate oxidation between genotypes (Fig 5-3E). However, glucose oxidation in hearts from ACC-DKI mice was significantly increased when compared to WT (Fig 5-3F). As well, the changes in oxidative phosphorylation seen in hearts from ACC-DKI mice during physiologic work did not induce functional changes *ex vivo* (Fig. 5-3G), which is consistent with the *in vivo* functional assessment via dobutamine stress echocardiography. Taken together, our data suggest that during increased myocardial workload, metabolic changes observed in hearts from ACC-DKI mice do not translate to changes in cardiac function compared to hearts from WT mice.

Functional recovery in the reperfused myocardium is similar between WT and ACC-DKI hearts.

Since physiological increases in workload failed to elicit a divergence in the functional profile between genotypes, we pursued a pathological model cardiac stress by subjecting hearts to *ex vivo* ischemia-reperfusion (I/R). While the effects of increasing intramyocardial malonyl CoA content via MCD ablation have shown alterations in metabolism and function in the reperfused myocardium [24] , the role that the AMPK-ACC-malonyl CoA axis plays during I/R and its effect on metabolism and function remain unknown. Therefore, to address this issue, *ex vivo* perfused working hearts from WT and ACC-DKI mice were subjected to 30 minutes of aerobic perfusion, followed by 18 minutes of global no-flow ischemia and 40 minutes of aerobic reperfusion as previously described (Table 5-5). Upon reperfusion following ischemia, ACC-DKI hearts displayed no change in malonyl CoA levels (Fig. 5-4A), palmitate oxidation (Fig. 5-4B) or glucose oxidation (Fig. 5-4C) following I/R, suggesting that lack of ACC inhibition by AMPK does

not induce metabolic alterations in the reperfused myocardium. Moreover, reperfused myocardium in both genotypes showed a significant decrease in ATP (Fig. 5-4D), indicating compromised high-energy phosphate energetics in the reperfused myocardium of both genotypes. Furthermore, in agreement with unchanged metabolism between genotypes during reperfusion, HR x PSP (Fig. 5-4E) and cardiac power (Fig. 5-4F) post reperfusion where also not significantly different demonstrating no change in myocardial function between genotypes. Taken together, our data suggest that ACC inhibition by AMPK is not sufficient to alter metabolism or function during acute I/R.

Table 5-1. Functional and Metabolic Parameters of *Ex Vivo* Perfused Working Hearts From WT and ACC DKI Mice

Parameters	WT	ACC DKI
Pressure		
Peak Systolic Pressure, mmHg	64 ± 1.0	63 ± 1.2
Diastolic Pressure, mmHg	50 ± 0.8	49 ± 1.1
Developed Pressure, mmHg	13.5 ± 0.5	13.6 ± 0.8
Mean Arterial Pressure, mmHg	55 ± 0.8	53 ± 1.1
Flow rates		
Cardiac Output, ml/min	7.8 ± 0.4	8.4 ± 0.5
Coronary Flow, ml/min	2.4 ± 0.2	2.6 ± 0.1
Mitochondrial Acetyl CoA Production		
Glucose	1956.4 ± 228	1932 ± 426
Palmitate	6056 ± 520	5995 ± 345
Total	8013 ± 627	7927 ± 541

Data are mean ± SEM of n = 12-16.

Table 5-2. Echocardiographic Parameters From WT and ACC DKI Mice

Parameters		WT	ACC DKI
Global Performance			
	Heart Rate, bpm	482 ± 8	465 ± 18
	Body Weight, g	24.1 ± 1.0	29.1 ± 1.0
	Tei Index	0.73 ± 0.02	0.71 ± 0.03
Systolic Function			
	Ejection Fraction, %	62.4 ± 2.0	61.2 ± 3.0
	Fractional Shortening, %	33.2 ± 1.4	32.6 ± 2.1
Diastolic Function			
	Isovolumic Relaxation Time, ms	16.9 ± 0.5	18.0 ± 0.7
	E/A ratio	1.05 ± 0.06	1.05 ± 0.08
	Mitral Valve Deceleration Time, ms	16.4 ± 1.2	17.4 ± 1.1
	E/E'	22.6 ± 1.9	25.7 ± 2.0
Wall Measurements			
	Interventricular Septal Thickness - Diastole, mm	0.75 ± 0.03	0.76 ± 0.02
	Interventricular Septal Thickness - Systole, mm	1.11 ± 0.04	1.15 ± 0.04
	Left Ventricular Internal Diameter - Diastole, mm	3.87 ± 0.10	3.92 ± 0.06
	Left Ventricular Internal Diameter - Systole, mm	2.58 ± 0.10	2.65 ± 0.11
	Left Ventricular Posterior Wall - Diastole, mm	0.75 ± 0.02	0.76 ± 0.03
	Left Ventricular Posterior Wall - Systole, mm	1.11 ± 0.04	1.18 ± 0.05

Data are mean ± SEM of n = 7-8.

Table 5-3. Dobutamine Stress Echocardiographic Parameters From WT and ACC DKI Mice

Parameters		WT	ACC DKI
Global Performance			
	Heart Rate, bpm	519 ± 13	538 ± 9
	Tei Index	0.69 ± 0.03	0.69 ± 0.03
Systolic Function			
	Ejection Fraction, %	79.9 ± 3.0	81.8 ± 3.7
	Fractional Shortening, %	48.8 ± 3.7	51.0 ± 4.0
Diastolic Function			
	Isovolumic Relaxation Time, ms	17.1 ± 1.2	16.5 ± 0.6
	E/A ratio	1.08 ± 0.08	1.02 ± 0.04
	Mitral Valve Deceleration Time, ms	13.1 ± 1.6	16.9 ± 2.1
	E/E'	19.8 ± 1.2	23.1 ± 1.3
Wall Measurements			
	Interventricular Septal Thickness - Diastole, mm	0.91 ± 0.07	0.90 ± 0.04
	Interventricular Septal Thickness - Systole, mm	1.43 ± 0.08	1.51 ± 0.10
	Left Ventricular Internal Diameter - Diastole, mm	3.56 ± 0.08	3.54 ± 0.05
	Left Ventricular Internal Diameter - Systole, mm	1.83 ± 0.2	1.74 ± 0.16
	Left Ventricular Posterior Wall - Diastole, mm	0.83 ± 0.05	0.87 ± 0.04
	Left Ventricular Posterior Wall - Systole, mm	1.42 ± 0.06	1.53 ± 0.08

Data are mean ± SEM of n = 7-8.

Table 5-4. Functional and Metabolic Parameters of *Ex Vivo* Perfused Working Hearts From WT and ACC DKI Mice

Parameters		WT	ACC DKI
Pressure			
	Peak Systolic Pressure, mmHg	84 ± 2.5	78 ± 2.2
	Diastolic Pressure, mmHg	53 ± 2.1	50 ± 1.4
	Developed Pressure, mmHg	13.7 ± 2.1	12.2 ± 1.2
	Mean Arterial Pressure, mmHg	56 ± 1.9	53 ± 1.4
Flow rates			
	Cardiac Output, ml/min	5.7 ± 1.5	4.8 ± 0.6
	Coronary Flow, ml/min	4.2 ± 0.7	3.5 ± 0.2
Mitochondrial Acetyl CoA Production			
	Glucose	1965 ± 503	3382 ± 381*
	Palmitate	12303 ± 6546	9041 ± 3336
	Total	14268 ± 6962	12423 ± 3538

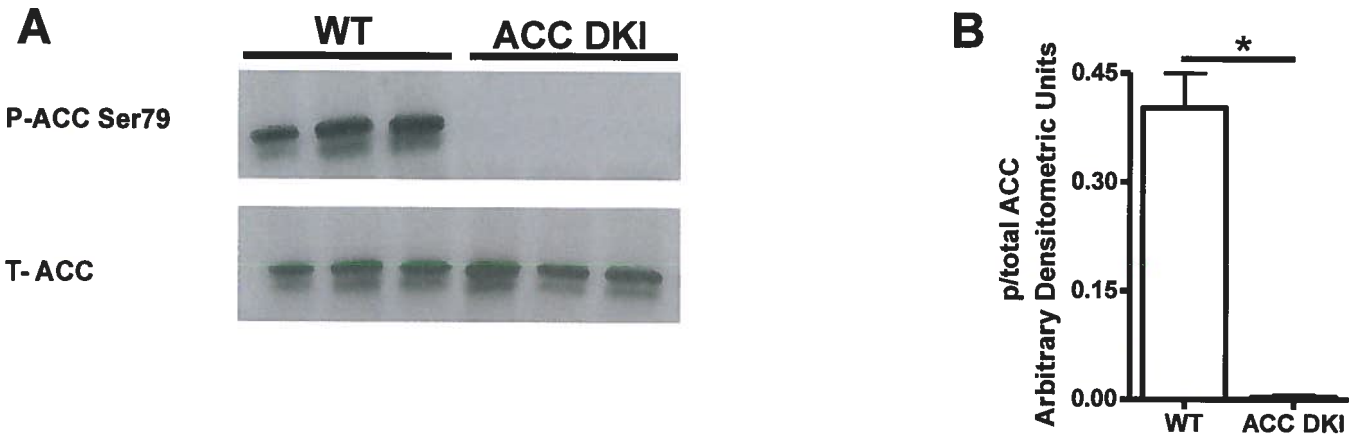
Data are mean ± SEM of n = 4-5.
*$p < 0.05$

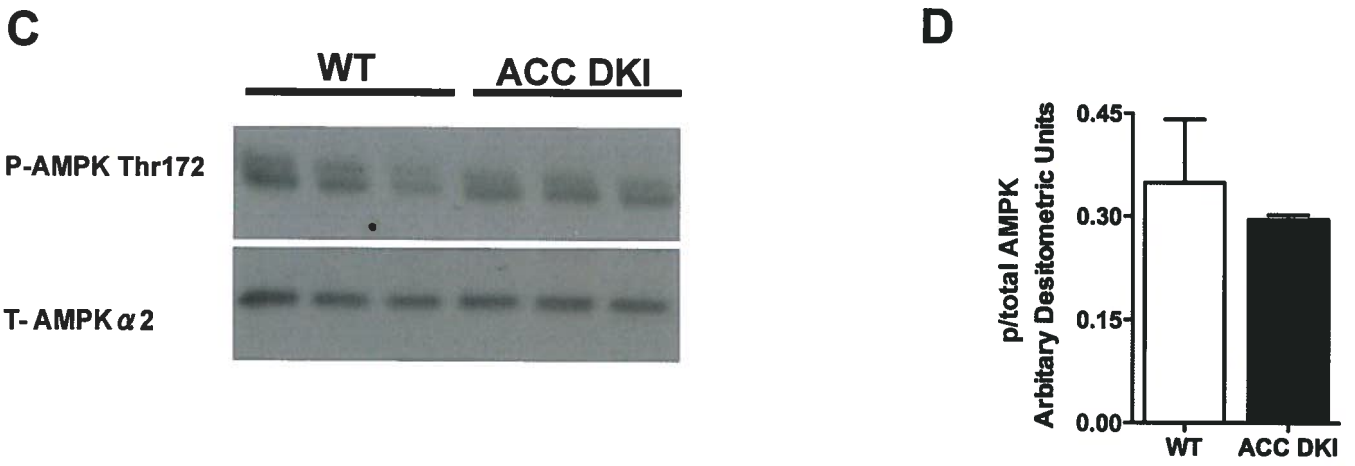
Table 5-5. Functional and metabolic Parameters of Ex-Vivo Reperfused Working Hearts From WT and ACC DKI Mice

Parameters		WT	ACC DKI
Pressure			
	Peak Systolic Pressure, mmHg	25 ± 10.5	36 ± 7.6
	Diastolic Pressure, mmHg	22 ± 10.6	32 ± 6.4
	Developed Pressure, mmHg	2.6 ± 0.8	3.9 ± 1.3
	Mean Arterial Pressure, mmHg	23 ± 10.5	33 ± 6.8
Flow rates			
	Cardiac Output, ml/min	1.2 ± 0.3	2.3 ± 1.0
	Coronary Flow, ml/min	1.2 ± 0.3	1.7 ± 0.5
Mitochondrial Acetyl CoA Production			
	Glucose	1270 ± 457	1308 ± 353
	Palmitate	6869 ± 1808	4946 ± 737
	Total	8139 ± 2217	6255 ± 940

Data are mean ± SEM of n = 4-5.
*$p < 0.05$

Figure 5-1. Characterization of the hearts from ACC-DKI mice. A. Immunoblot analysis was performed using ventricular homogenates. Immunoblot analysis of phospho-ACC and total ACC protein expression (n = 3). **B.** Levels of phospho-ACC were quantified by densitometry and normalized against total ACC (n = 3, *p < 0.05). **C.** Immunoblot analysis of phospho-AMPK Thr172 and total AMPK protein expression (n = 3). **D.** Levels of phospho-AMPK were quantified by densitometry and normalized against total AMPK (n = 3, *p < 0.05). **E.** Description of the effect of the ACC-DKI mutation on the AMPK-ACC-malonyl CoA axis. The ACC-DKI mouse inhibits AMPK mediated phosphorylation of ACC and thus renders ACC active. **F.** Intramyocardial malonyl CoA content (n = 4-5, *p < 0.05).


A
WT
ACC DKI
P-ACC Ser79
T- ACC
B
*
p/total ACC
Arbitrary Densitometric Units
0.45
0.30
0.15
0.00
WT
ACC DKI


C
WT
ACC DKI
P-AMPK Thr172
T- AMPK α2
D
p/total AMPK
Arbitary Desitometric Units
0.45
0.30
0.15
0.00
WT
ACC DKI

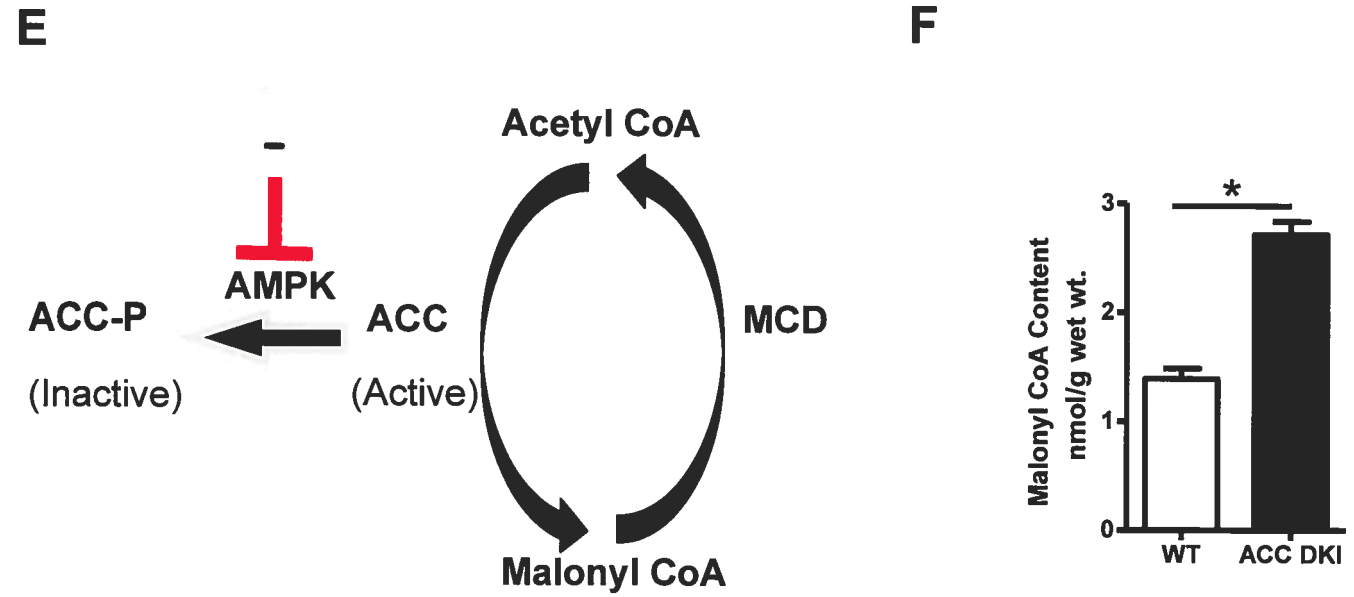

E
Acetyl CoA
AMPK
ACC-P
(Inactive)
ACC
(Active)
MCD
Malonyl CoA
F
*
Malonyl CoA Content
nmol/g wet wt.
3
2
1
0
WT
ACC DKI

Figure 5-2. *Ex vivo* energy metabolism and *in vivo* myocardial function. **A.** Glucose oxidation rates (*n* = 12 – 16). **B.** Palmitate oxidation rates (*n* = 12 – 16). **C.** Representative whole heart sections stained with Masson trichrome for visualizing structural morphology (*n* = 4). **D.** Ratio of ventricle weight to tibia length (*n* = 4 - 5). **E.** Left ventricular posterior wall thickness in systole (LVPWs; *n* = 7 - 8). **F.** Representative M-mode images. **G.** Heart rate (*n* = 15). **H.** Ejection fraction (*n* = 15) **I.** Isovolumic relaxation time (*n* = 15).

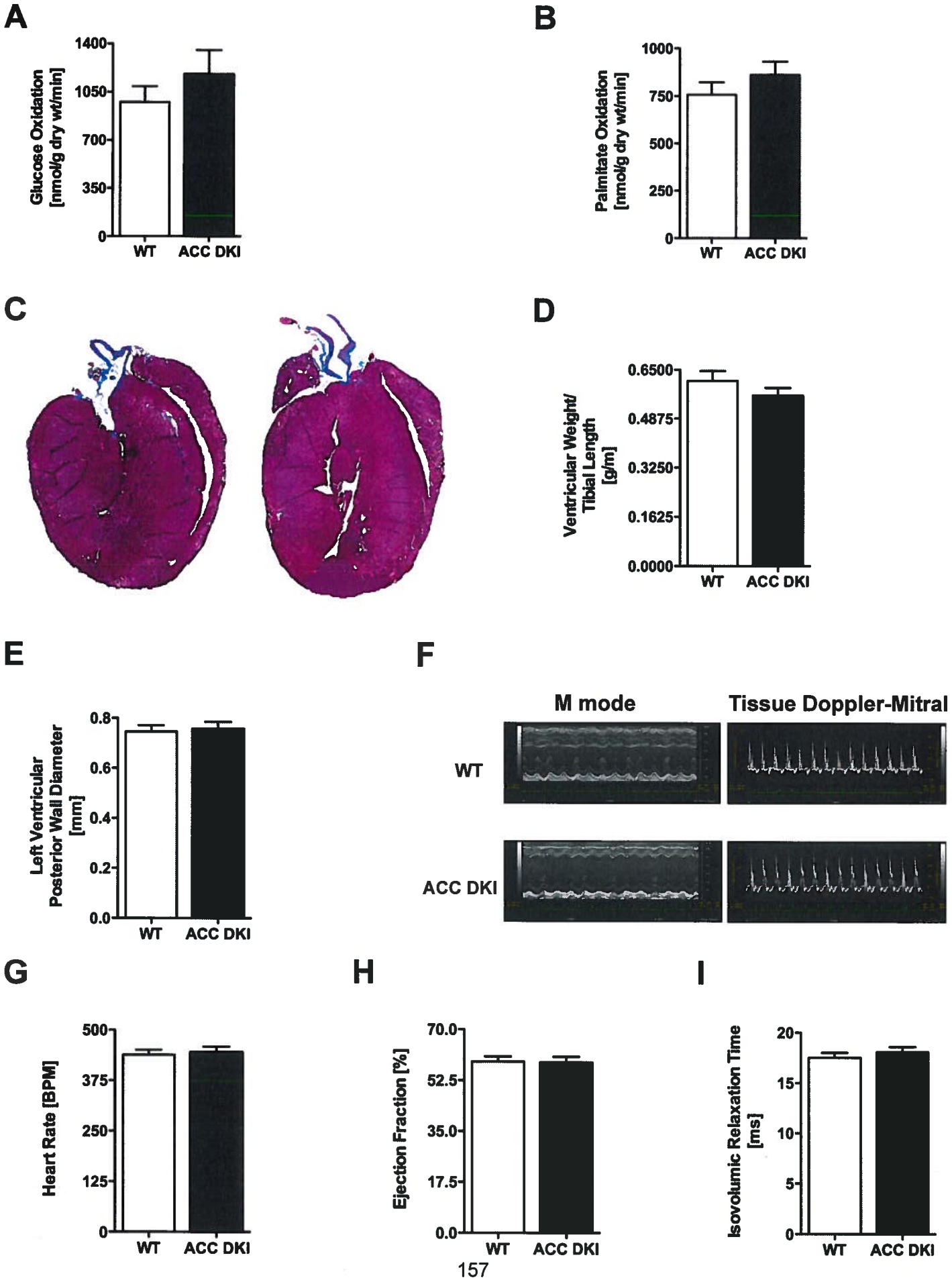

A
Glucose Oxidation [nmol/g dry wt/min]
1400
1050
700
350
0
WT
ACC DKI
B
Palmitate Oxidation [nmol/g dry wt/min]
1000
750
500
250
0
WT
ACC DKI
C
D
Ventricular Weight/ Tibial Length [g/m]
0.6500
0.4875
0.3250
0.1625
0.0000
WT
ACC DKI
E
Left Ventricular Posterior Wall Diameter [mm]
0.8
0.6
0.4
0.2
0.0
WT
ACC DKI
F
M mode
Tissue Doppler-Mitral
WT
ACC DKI
G
Heart Rate [BPM]
500
375
250
125
0
WT
ACC DKI
H
Ejection Fraction [%]
70.0
52.5
35.0
17.5
0.0
WT
ACC DKI
I
Isovolumic Relaxation Time [ms]
20
15
10
5
0
WT
ACC DKI

Figure 5-3. ***In Vivo*** **myocardial function and** ***ex vivo*** **energy metabolism during high workload.** *Dobutamine infusion* - **A.** Intramyocardial malonyl CoA content (*n* = 4 – 5). **B.** Heart rate (*n* = 4 – 5). **C.** Ejection fraction (*n* = 4 – 5). **D.** Isovolumic relaxation time (*n* = 4 – 5); *Isoproterenol working heart perfusions* - **E.** Palmitate oxidation rates (*n* = 4 – 5). **F.** Glucose oxidation rates (*n* = 4 – 5). **G.** Rate pressure product (*n* = 4 – 5).

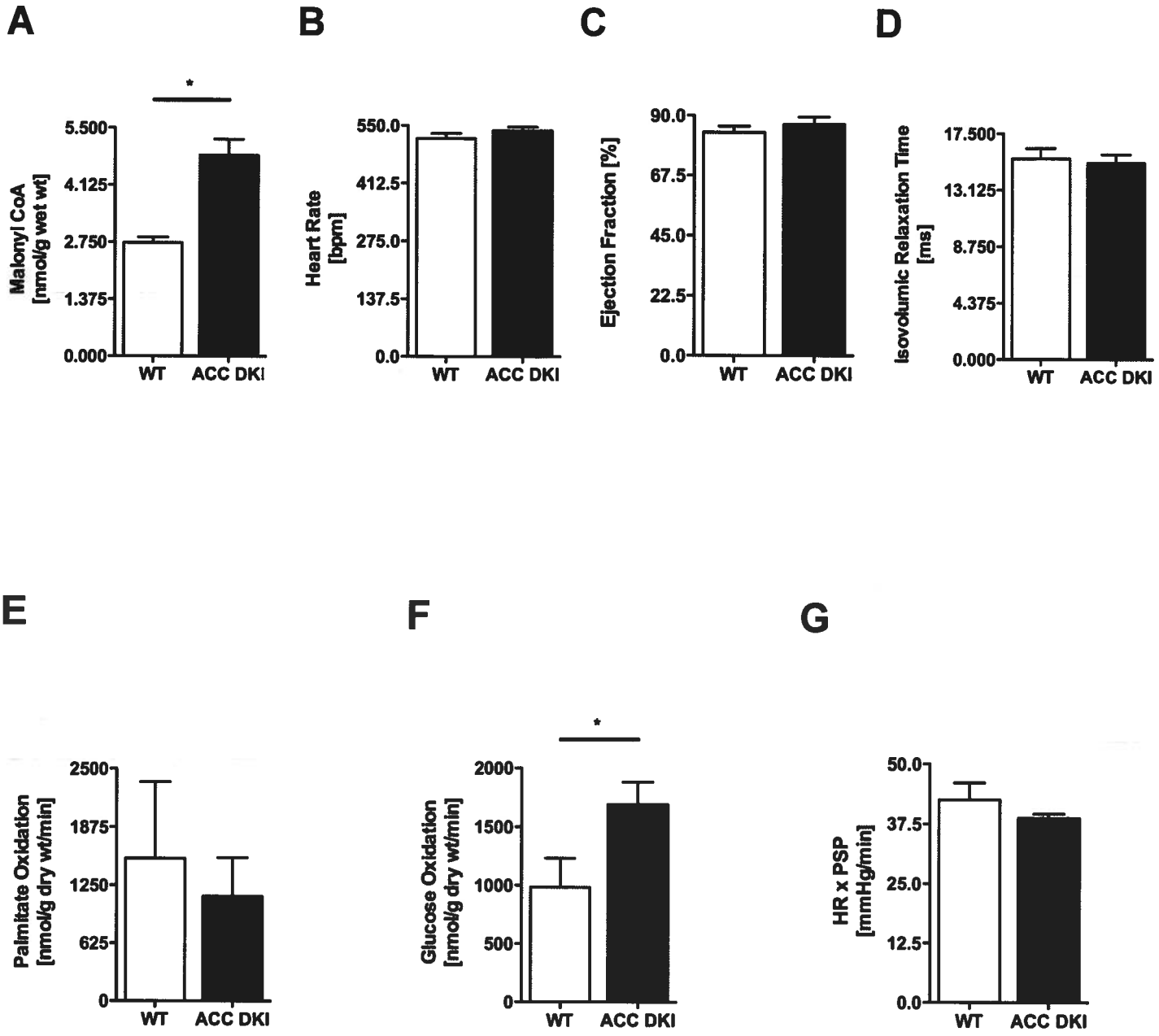
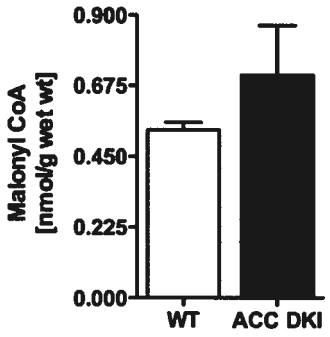
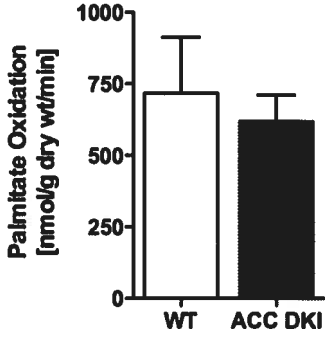

A
B
C
D
*
Malonyl CoA [nmol/g wet wt]
5.500
4.125
2.750
1.375
0.000
WT
ACC DKI
Heart Rate [bpm]
550.0
412.5
275.0
137.5
0.0
Ejection Fraction [%]
90.0
67.5
45.0
22.5
Isovolumic Relaxation Time [ms]
17.500
13.125
8.750
4.375
E
F
G
Palmitate Oxidation [nmol/g dry wt/min]
2500
1875
1250
625
Glucose Oxidation [nmol/g dry wt/min]
2000
1500
1000
500
HR x PSP [mmHg/min]
50.0
37.5
25.0
12.5

Figure 5-4. *Ex Vivo* myocardial energy metabolism and function during ischemia and reperfusion. A. Intramyocardial malonyl CoA content (*n* = 4 – 5). **B.** Palmitate oxidation rates (*n* = 4 – 5). **C.** Glucose oxidation rates (*n* = 4 – 5). **D.** Intramyocardial ATP content (*n* = 4 – 5) **E.** Rate pressure product (*n* = 4 – 5). **F.** Cardiac Power (*n* = 4 – 5).

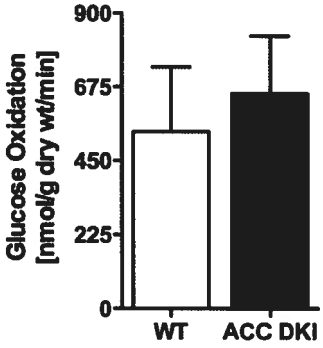
A


Malonyl CoA
[nmol/g wet wt]
0.900
0.675
0.450
0.225
0.000
WT
ACC DKI

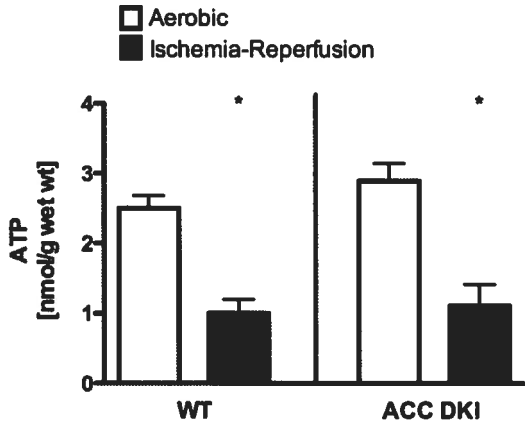
B


Palmitate Oxidation
[nmol/g dry wt/min]
1000
750
500
250
0
WT
ACC DKI

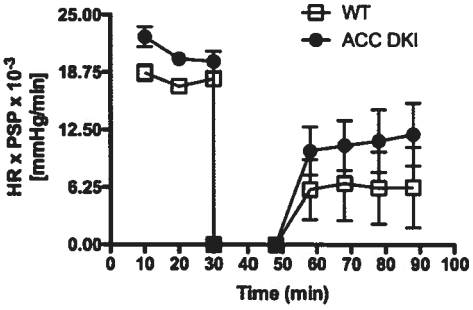
C


Glucose Oxidation
[nmol/g dry wt/min]
900
675
450
225
0
WT
ACC DKI

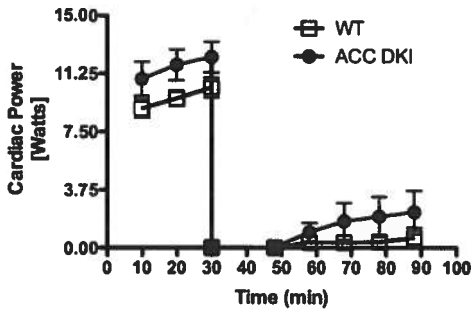
D


Aerobic
Ischemia-Reperfusion
ATP
[nmol/g wet wt]
4
3
2
1
0
*
*
WT
ACC DKI

E


HR x PSP x 10-3
[mmHg/min]
25.00
18.75
12.50
6.25
0.00
0 10 20 30 40 50 60 70 80 90 100
Time (min)
WT
ACC DKI

F


Cardiac Power
[Watts]
15.00
11.25
7.50
3.75
0.00
0 10 20 30 40 50 60 70 80 90 100
Time (min)
WT
ACC DKI

Discussion

Although the AMPK-ACC-malonyl CoA axis has been proposed to be a major regulator of cardiac metabolism [5, 6, 24, 25], the degree to which, AMPK control of ACC activity, dictates changes in cardiac metabolism and function in physiology and pathology has yet to be demonstrated. Therefore, to test the physiological importance of AMPK phosphorylation on ACC *in vivo* we generated ACC1-S79A and ACC2-S212A double knock in (DKI) mice. Herein we have shown that AMPK-mediated inactivating phosphorylation of ACC regulates cardiac malonyl CoA levels without altering baseline oxidative metabolism and function. Surprisingly, hearts from ACC-DKI mice did not exhibit functional alterations following physiological and pathological stress challenging the previously suggested role of AMPK-mediated inhibitory ACC phosphorylation in the regulation of metabolism and function in healthy and stressed myocardium.

Our results show that hearts from ACC-DKI mice show an almost two-fold significant increase in intramyocardial malonyl CoA content that did not lead to differences in cardiac energy substrate profile or function. Previous work by Kolwicz et al. demonstrated that mice with cardiomyocyte-specific ACC2 ablation exhibit an approximately 50% reduction in myocardial malonyl CoA content indicating lack of ACC activity [25]. In agreement, our study showed that increased ACC activity in ACC-DKI hearts resulted in a two fold increase in myocardial malonyl CoA content, demonstrating that baseline AMPK-ACC axis is sufficient to regulate malonyl CoA content in the heart. However, the finding that hearts from ACC-DKI mice show no difference in myocardial metabolism or function is in contrast to findings seen in the ACC2 KO mouse in which the ablation of ACC2 significantly increases FAO with subsequent decrease in glucose oxidation [25]. These findings suggest that although ACC itself is a key regulator of myocardial

substrate utilization, the phosphorylational control of ACC exerted by AMPK does not influence metabolism at baseline. Interestingly, the increase in myocardial malonyl CoA content observed in ACC-DKI model is significantly below the three-fold increase in malonyl CoA content seen in MCD knock out (*MCD*KO) mice [24] . This suggests that AMPK mediated ACC inhibition may be physiologically less significant compared to altered MCD activity in regulating myocardial malonyl CoA production.

Furthermore, our results are consistent with many previous studies examining the role of the AMPK-ACC axis in high workload. Hall et al. showed in a swine model of increased myocardial workload via dobutamine infusion that despite a decrease in malonyl CoA and an increase in myocardial FA uptake that both AMPK activity and ACC activity were unchanged [22]. As well, King et al. demonstrated in dobutamine infused pigs that increased myocardial work was associated with a decrease in malonyl CoA content, an increase in FAO and no changes to AMPK and ACC activity [23]. However, Zhou et al. showed in a swine model of increased cardiac work that despite increased FAO, myocardial malonyl CoA content was increased, with a decrease in AMPK activity and unchanging ACC activity when compared to heart performing normal work [27] . Taken together, we have shown that during increased cardiac work, malonyl CoA content is increased, and the inhibitory phosphorylation of ACC by AMPK does not affect the resultant increased rates of FAO or myocardial function.

Finally, in contrast to previous studies, our results show an insignificant role of AMPK inhibitory phosphorylation of ACC on myocardial metabolism and function during I/R. Previous work done by Kudo et al has shown in a rodent model of ex vivo I/R that the reperfused myocardium exhibits increased FAO, associated with increased AMPK activity, decreased ACC activity and decreased myocardial malonyl CoA content [5, 6]. Folmes et al. has demonstrated that FAO rates decrease after ex vivo I/R in a mouse model expressing a dominant-negative form of the AMPK α2 subunit, which was associated with decreased phosphorylation of ACC

[28] . Folmes et al. hypothesize that the difference in AMPK activity between the AMPK-DN mouse and a WT mouse may be the cause of differences in FAO rates during reperfusion likely due to the AMPK-ACC-malonyl CoA axis [28] . However, the authors did not measure malonyl CoA levels in this study. Taken together, our study is able to directly show the influence of the AMPK-ACC-malonyl CoA axis on oxidative phosphorylation and function during I/R. We show that inhibiting AMPK-mediated phosphorylation of ACC during I/R does not alter FAO or function in the reperfused myocardium.

In summary, the data presented herein show for the first time that AMPK phosphorylation control of ACC is not sufficient to alter myocardial function following physiologic and pathophysiologic stress. Collectively, these findings challenge the previously suggested role of AMPK-mediated inhibitory ACC phosphorylation in the regulation of FAO in healthy and stressed myocardium.

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CHAPTER 6.

Are users of sulfonylureas at the time of an acute coronary syndrome at risk of poorer outcomes?

My role in this work involved analysis and interpretation of the data as well as the writing of the manuscript.

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CHAPTER 6.

Are users of sulfonylureas at the time of an acute coronary syndrome at risk of poorer outcomes?

Abstract

Background: Adenosine triphosphate sensitive potassium (K_{ATP}) channel activity is cardioprotective during ischemia. One of the purported mechanisms for sulfonylurea adverse effects is through inhibition of these channels. The purpose of this study is to examine whether patients using K_{ATP} channel inhibitors at the time of an acute coronary syndrome are at greater risk of death or heart failure (HF) than those not exposed.

Materials and Methods: Using linked administrative databases we identified all adults who had an acute coronary syndrome between April 2002 and October 2006 (n= 21 023).

Results: Within 30 days of acute coronary syndrome, 5.3% of our cohort died and 15.6% were diagnosed with HF. Individuals with diabetes exhibited significantly higher risk of death (adjusted OR 1.20, 95% CI 1.03 to 1.40) and death or HF (aOR 1.73, 95% CI 1.59 to 1.89) than individuals without diabetes. However, there was no significantly increased risk of death (aOR 1.00, 95% CI 0.76-1.33) or death/HF (aOR 1.06, 95% CI 0.89 – 1.26) in patients exposed to K_{ATP} channel inhibitors versus patients not exposed to K_{ATP} channel inhibitors prior to their acute coronary syndrome.

Conclusions: Diabetes is associated with an increased risk of death or HF within 30 days of an acute coronary syndrome. However, we did not find any excess risk of death or HF associated with use of K_{ATP} channel inhibitors at the time of an acute coronary syndrome, raising doubts about the hypothesis that sulfonylureas inhibit the cardioprotective effects of myocardial K_{ATP} channels.

Introduction

Given a paucity of randomized trial evidence, there is substantial debate about the best management approach for glycemic control in those with diabetes and coronary disease[1]. A number of observational studies have suggested an excess risk of cardiovascular events in individuals treated with sulfonylureas compared to other glucose lowering drugs[2-5]. The sulfonylurea receptor functions as the regulatory subunit of the adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel. K_{ATP} channels are widely expressed in the heart, vascular smooth muscle cells, and pancreatic beta cells where they couple the metabolic status of the cells to their electrical properties[6, 7]. During myocardial ischemia, loss of intracellular ATP leads to an activation (opening) of myocardial K_{ATP} channels, and this is a key mediator of the ST segment elevation response to transmural ischemia[8] and ischemic cardioprotection[9]. Ischemic preconditioning describes the adaptation of the myocardium to ischemic stress as a result of preceding short periods of ischemia and reperfusion resulting in reduced infarct size after an acute coronary syndrome[10]. The opening of myocardial K_{ATP} channels is thought to be the mechanism of protection in early phase ischemic preconditioning[9].

Sulfonylureas bind to and close cardiovascular K_{ATP} channels. This has been demonstrated in human studies conducted during coronary angiography, but the clinical importance of these pathophysiologic findings remains uncertain[2-5]. Some authors have proposed that the closing of cardiovascular K_{ATP} channels impairs ischemic preconditioning[7] and that this accounts for the apparent adverse effects of sulfonylureas in patients with cardiac disease[7]. Other reports showing a neutral effect of K_{ATP} channel blocking drugs on ischemic events raise the possibility that cardiac K_{ATP} channels (as opposed to pancreatic beta cell K_{ATP}

channels) may be less sensitive to the pharmacological plasma levels of sulfonylureas used in patients with diabetes mellitus (DM) [11, 12].

To explore the hypothesis that sulfonylureas impair the cardioprotective response to ischemia mediated by K_{ATP} channels, we designed this study to determine whether DM patients who had an acute coronary syndrome (ACS) while taking K_{ATP} channel inhibitors were at higher risk of death or heart failure than patients who were not exposed to K_{ATP} channel inhibitors. We chose to study patients who were having an ACS as this is the group that should be at highest risk for exhibiting harm from K_{ATP} channel inhibitors if the mechanism of harm is impairment of the K_{ATP} channel mediated cardioprotective response to myocardial ischemia.

Methods

Study Design

All data were derived using linked administrative databases in the province of Alberta, Canada. A cohort of patients aged 20 years or older hospitalized with a most responsible diagnosis of ACS between April 1, 2002 and October 1, 2006 (Figure 6-1).

Data Sources

5 databases were linked using patients personal identification numbers: (1) the Alberta Inpatient Discharge Abstract Database, which records dates, most responsible diagnosis (as specified by their hospital attending physician) and up to 25 other diagnoses, and inpatient surgical procedures on all acute-care hospitalizations in Alberta; (2) the Physician claims database; (3) the Ambulatory Care Classification System which codes for all emergency department visits and minor outpatient surgical procedures and up to 10 primary/secondary diagnoses; (4) Vital statistics and (5) the Alberta Blue Cross Medication database, which includes outpatient medication dispensation data for all Albertans older than the age of 65 years.

Patient Population

The DM cohort was identified as patients who had at least one hospitalization for which DM (ICD-9 250.x or ICD-10 E10.x-E14.x) was the primary or secondary diagnosis at anytime between 1993 and the date of their index ACS event. In addition, we were also able to expand our identification of DM patients in our patients older than 65 years of age for which we had prescribing data. In this group of patients, we were able to identify additional DM patients by including those who filled a prescription for any glucose-lowering drug.

We defined drug exposure at the time of an ACS by linking data from the Alberta Blue Cross Medication database and examining prescriptions filled in the 100 days prior to index ACS hospitalization (Figure 6-2). We examined the association between glucose-lowering drug exposure and outcomes by comparing outcomes for those patients with any exposure to K_{ATP} channel inhibitors (sulfonylurea, meglitinide and repaglinide) in the 100 days prior to their ACS versus those patients without exposure to K_{ATP} channel inhibitors. We did not include post-ACS medication data, as we wanted to determine if there was any impact of pre-ACS exposure to K_{ATP} channel inhibitors at the time of the ACS.

Patient comorbidities were identified using ICD-9-CM and ICD-10 codes from the Discharge Abstract Database for the index hospitalization and all hospitalizations from 1993 until the index ACS date. The accuracy of these codes, disease case definitions, and databases have been previously validated in Alberta[13, 14].

Outcomes

The primary outcome was a composite of death from any cause or a physician-assigned diagnosis of heart failure (HF) within 30 days of the ACS date, and included events during the index ACS hospitalization or at presentation with the ACS.

Statistical Analysis

Categorical data were summarized as percentages and odds ratios, and differences were tested with the χ^2 test; continuous variables were summarized as medians and interquartile ranges, and differences were tested with the Wilcoxon rank-sum test. We used multivariable logistic regression to evaluate the association between DM and death/HF in the 30 days after ACS, adjusting for baseline differences and prognostically important covariates using a backward, stepwise selection method with $p \leq 0.10$ used as an exit criteria. We chose logistic regression over Cox proportional hazards analysis given the short timeline (30 days). All results

are presented as unadjusted and adjusted ORs with their respective 95% CIs. All tests were 2-sided, with the level of significance set at $p<0.05$, and all analyses were performed with SAS version 9.3(SAS Institute Inc, Cary, NC).

In the elderly subgroup (in whom we had prescribing data), we conducted a logistic regression analysis to examine the association between pre-ACS medication exposures and rate of mortality/HF after ACS, adjusting for differences in baseline covariates. In this analysis we specifically examined the association between K_{ATP} channel inhibitor exposure and outcomes by comparing those patients that used drugs that were K_{ATP} channel inhibitors with patients not using those drugs at the time of their ACS (which included individuals without diabetes as well as those with diabetes treated with diet or other glucose lowering medications) – as the model included diabetes this gave us an estimate of the effect of K_{ATP} channel inhibitor exposure over and above the effect of diabetes and all other covariates in the model.

Results

Between April 1, 2002 to October 1, 2006, 21 023 adult Albertans were hospitalized with a most responsible diagnosis of ACS. The 5761 individuals with DM were older, more likely to be female, and were more likely to have atherosclerotic comorbidities (Table 6-1). In addition, individuals with DM having an ACS were more likely to have prior history of ACS or heart failure, and were more likely to present with cardiogenic shock or pulmonary edema (Table 6-1). Individuals with diabetes were less likely to undergo cardiac catheterization or percutaneous coronary intervention in the first 7 days after their index ACS, but were more likely to undergo coronary bypass surgery and were more likely to have been treated with ACE inhibitors, angiotensin receptor blockers, beta-blockers, and statins in the 100 days prior to their ACS (all $p< 0.001$, Table 6-1).

The unadjusted rate of death or HF within 30 days for patients with a most responsible diagnosis of ACS was 30.9% in patients with DM (1778 events in 5761 patients) compared to 17.5% (2666 events in 15,262 patients) in those without DM (adjusted odds ratio [aOR] 1.81, 95% CI 1.67 to 1.96). The 7 day risk of death or HF was also elevated in those with DM even after adjustment for the imbalances in demographics and baseline comorbidities (aOR 1.70, 95% CI 1.56– 1.86). Other significant predictors of death or HF within 30 days of ACS included older age, chronic kidney disease, cancer, cerebrovascular disease, chronic obstructive pulmonary disease, and peripheral vascular disease (Table 6-2). The size of the hospital did not influence these outcomes but was included as a covariate in the multivariate analyses due to our belief that it was clinically relevant. A diagnosis of hypertension and cardiac catheterization with/without percutaneous coronary intervention in the first 7 days after the index ACS were all associated with a lower risk of death/HF at 30 days and each were thus included as covariates in our multivariate model. In sensitivity analyses by type of ACS, DM was a risk

factor for death/HF within 30 days in both those patients with NSTEMI (aOR 1.91, 95% CI 1.69–2.17) as well as those with STEMI (aOR 1.69, 95% CI 1.46 – 2.00).

Among ACS patients older than 65 years, the unadjusted rate of death or HF within 30 days was 39.4% (1362 events in 3456 patients) in patients with DM and 27.4% (2164 events in 7895 patients) in those without DM (aOR 1.35, 95% CI 1.18 – 1.54). In the over 65's, DM was a risk factor for death/HF within 30 days in both those patients with NSTEMI (aOR 1.41, 95% CI 1.16-1.71) and those with STEMI (aOR 1.26, 95% CI 1.00-1.60). Apropos to our primary research question, there was no significantly increased risk of death (aOR 1.00, 95% CI 0.76-1.33) or death/HF (aOR 1.06, 95% CI 0.89 – 1.26) in ACS patients who were users of K_{ATP} channel inhibitors at the time of their ACS compared to those not exposed to K_{ATP} channel inhibitors, even after adjustment for differences in baseline covariates and procedures within the first 7 days of the index ACS event. Of note, users of statins pre-ACS were at lower risk of death/HF at 30 days (aOR 0.77, 95% CI 0.68 – 0.86), suggesting that we did have sufficient sample size to detect clinically relevant differences in our composite end-point. It should be noted that 67% of the patients taking K_{ATP} channel inhibitors in our cohort were also taking other glucose lowering drugs, which were non-K_{ATP} channel inhibitors – a sensitivity analysis demonstrated similar lack of harm with K_{ATP} channel inhibitors when we excluded those taking non-K_{ATP} channel inhibitors concurrently (aOR for death/HF within 30 days 1.25, 95% CI 0.95-1.62). Of the K_{ATP} channel inhibitors used in our cohort, 88% were sulfonylureas (1063), and of these patients 61% (n=653) were using glyburide and 37% (n=389) were using gliclazide. There was no significant difference in risk of death ($p = 0.83$) or death/HF ($p = 0.43$) at 30 days when comparing glyburide users to non-glyburide users.

Independent Variables	no DM (n = 15262)	DM (n=5761)	p-value
Mean Age (SD)	66.20 (14.21)	68.41 (12.40)	<0.0001
Female	4963 (32.52)	2123 (36.85)	<0.0001
Diabetes with complications	0	2017 (35.01)	<0.0001
Hypertension	8460 (55.43)	4336 (75.26)	<0.0001
Cardiac arrythmia, including atrial fibrillation	2967 (19.44)	1299 (22.55)	<0.0001
Atrial fibrillation	1944 (12.74)	954 (16.56)	<0.0001
Peripheral arterial disease	967 (6.34)	704 (12.22)	<0.0001
Cerebrovascular disease	836 (5.48)	504 (8.75)	<0.0001
Acute renal failure	416 (2.73)	427 (7.41)	<0.0001
Chronic kidney disease	967 (6.34)	1009 (17.51)	<0.0001
COPD	2172 (14.23)	1086 (18.85)	<0.0001
Cancer	558 (3.66)	239 (4.15)	0.10
HF prior to index ACS hospitalization (1yr only)	1250 (8.19)	978 (16.98)	<0.0001
ACS prior to index ACS hospitalization (any time)	3344 (21.91)	1988 (34.51)	<0.0001
CABG/PCI prior to index ACS hospitalization (1 yr only)	154 (1.01)	95 (1.65)	0.0001
Shock at presentation (index episode)	348 (2.28)	218 (3.78)	<0.0001
Pulmonary edema at presentation (index episode)	106 (0.69)	77 (1.34)	<0.0001
Annual income categories	14807 (72.72)	5556 (27.28)	<0.0001
Lowest	4907 (33.14)	2025 (36.45)	
Low medium	4015 (27.12)	1531 (27.56)	
High medium	3201 (21.62)	1178 (21.20)	
Highest	2684 (18.13)	822 (14.79)	
Hospital Bed Size			0.07
small	1148 (7.56)	429 (7.48)	
medium	3645 (23.99)	1292 (22.52)	
Large	10400 (68.45)	4015 (70.00)	
Cardiac Procedures (on or within 7 days after index ACS date)			
Cardiac Catheterization	9251 (60.61)	3152 (54.71)	<0.0001
Percutaneous Coronary Intervention	5875 (38.49)	1761 (30.57)	<0.0001
Coronary Artery Bypass Graft Surgery	616 (4.04)	298 (5.17)	0.0003
Medications in 100 days prior to diagnosis of ACS (only available for patients older than 65 years)	n=7895	n=3456	
ACEi or ARB	3101 (39.28)	2029 (58.71)	<0.0001
Beta-blocker	2165 (27.42)	1220 (35.30)	<0.0001
Statin	1877 (23.77)	1122 (32.47)	<0.0001
K-ATP Channel Blockers	10 (0.13)	1064 (30.79)	<0.0001

Table 6-1. Baseline characteristics of Albertans with an acute coronary syndrome event

Independent Variables	Overall						In patients 65 years or older (in whom prescription data available)					
	Death (1111 events in 21 023 patients)			Death or HF (4444 events in 21 023 patients)			Death (955 events in 11 351 patients)			Death or HF (3526 events in 11 351 patients)		
	Point Estimate	95% Confidence Interval		Point Estimate	95% Confidence Interval		Point Estimate	95% Confidence Interval		Point Estimate	95% Confidence Interval	
		Lower Limit	Upper Limit		Lower Limit	Upper Limit		Lower Limit	Upper Limit		Lower Limit	Upper Limit
Age (per year)	1.06	1.05	1.06	1.05	1.04	1.05	1.06	1.04	1.07	1.05	1.04	1.06
Female	0.91	0.79	1.06	1.20	1.10	1.31	0.89	0.76	1.04	1.14	1.04	1.26
Diabetes Mellitus	1.20	1.03	1.40	1.73	1.59	1.89	1.25	1.02	1.53	1.35	1.19	1.54
Renal Disease	1.88	1.59	2.22	2.32	2.07	2.59	1.94	1.63	2.30	2.36	2.09	2.66
Cancer	1.26	0.96	1.65	1.11	0.93	1.33	1.27	0.97	1.67	1.19	0.98	1.43
Cerebrovascular Disease	1.74	1.42	2.13	1.20	1.04	1.38	1.69	1.37	2.08	1.22	1.05	1.42
Hypertension	0.66	0.57	0.76	0.96	0.88	1.05	0.66	0.56	0.78	0.87	0.78	0.97
COPD	1.15	0.97	1.36	1.64	1.48	1.81	1.12	0.94	1.34	1.56	1.40	1.74
Prior ACS	0.88	0.75	1.04	0.98	0.89	1.07	0.72	0.61	0.86	0.82	0.73	0.91
Peripheral Vascular Disease	1.42	1.16	1.74	1.41	1.24	1.60	1.31	1.06	1.62	1.38	1.20	1.60
Cardiac Catheterization (within 7 days of indexed ACS event)	0.32	0.26	0.39	0.82	0.74	0.90	0.36	0.29	0.45	0.87	0.77	0.97
PCI (within 7 days of indexed ACS event)	0.52	0.41	0.66	0.60	0.54	0.67	0.64	0.48	0.84	0.71	0.62	0.81
CABG (within 7 days of indexed ACS event)	0.60	0.41	0.89	1.17	0.97	1.41	0.61	0.39	0.97	1.17	0.92	1.47
Inpatient, Medium Hospital (vs Small)	1.45	1.07	1.97	1.16	0.97	1.39	1.75	1.29	2.37	1.53	1.27	1.85
Inpatient, Large Hospital (vs Small)	1.42	1.06	1.91	1.42	1.20	1.70	1.66	1.24	2.24	1.73	1.44	2.07
Statin use in 100 days prior to ACS	N/A	-	-	N/A	-	-	0.77	0.63	0.94	0.77	0.68	0.86
K-ATP Channel Blocker use in 100 days prior to ACS	N/A	-	-	N/A	-	-	1.00	0.76	1.33	1.06	0.89	1.26

Table 2. Mulitvariate Predictors of 30-day Death and Death/HF after an acute coronary syndrome event

Figure 6-1. Study flow diagram.

Figures

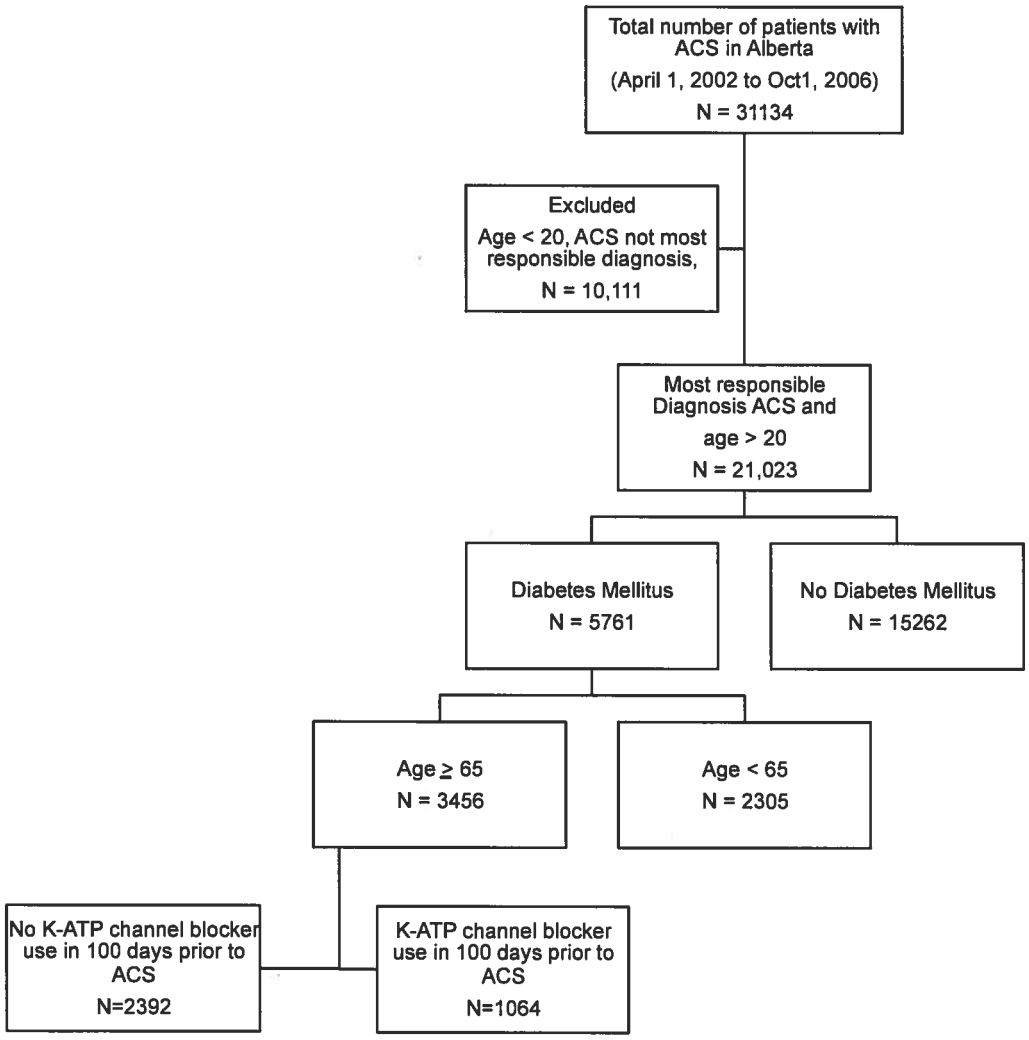
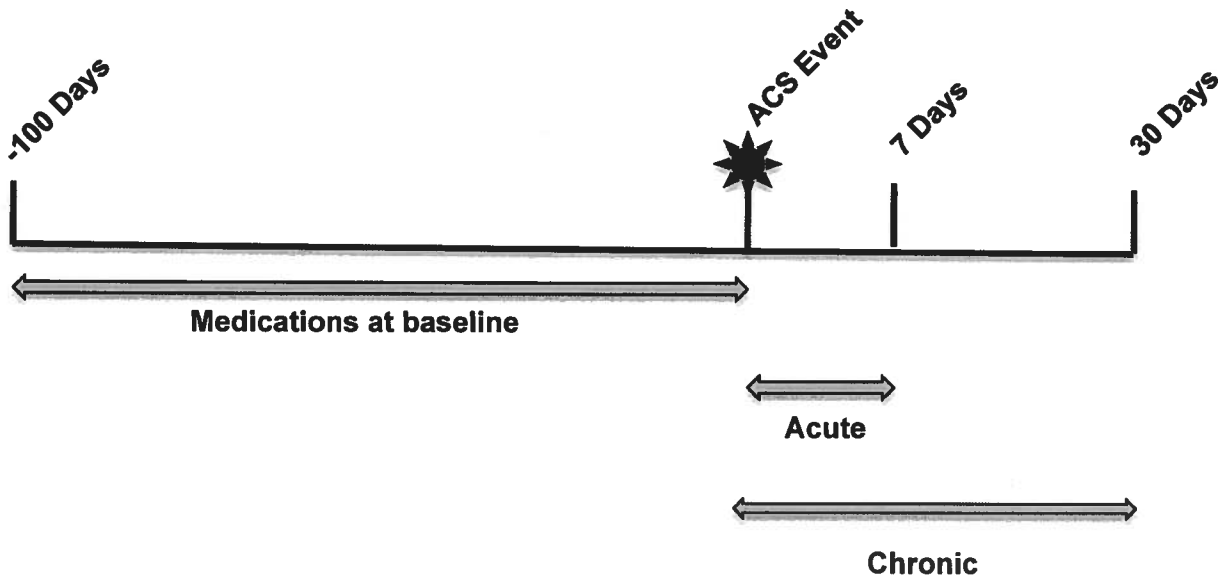

Total number of patients with ACS in Alberta
(April 1, 2002 to Oct1, 2006)
N = 31134
Excluded
Age < 20, ACS not most responsible diagnosis,
N = 10,111
Most responsible Diagnosis ACS and
age > 20
N = 21,023
Diabetes Mellitus
N = 5761
No Diabetes Mellitus
N = 15262
Age ≥ 65
N = 3456
Age < 65
N = 2305
No K-ATP channel blocker use in 100 days prior to ACS
N=2392
K-ATP channel blocker use in 100 days prior to ACS
N=1064

Figure 6-2. Study Design.


-100 Days
ACS Event
7 Days
30 Days
Medications at baseline
Acute
Chronic

Discussion

While previous studies have established that DM is an independent risk factor for mortality after an acute coronary syndrome[15-17], we have extended this evidence base in 3 ways. First, we have demonstrated that DM is a risk factor for not only mortality but also for HF. Second, we have demonstrated that DM exerts the same prognostic risk after both STEMI and NSTEMI. Third, we did not detect any increased risk for death or HF in individuals using K_{ATP} channel inhibitors at the time of their ACS and we believe this raises doubts about the putative impact of K_{ATP} channel inhibitors such as sulfonylureas on ischemic preconditioning (or at least the clinical implications of such an effect).

Our results are consistent with those of several observational studies in diabetic patients with an ACS event. Zeller and colleagues showed no worsening of outcomes associated with the use of sulfonylureas before MI in 1310 French individuals with DM[18]. Horsdal et al. also showed that the use of pre-admission sulfonylurea monotherapy did not alter prognosis after myocardial infarction when compared to other glucose lowering medications in 8494 patients[19]. Juurlink and colleagues also recently reported that there were no differences in the risk of death, HF, or repeat myocardial infarction over approximately one year of followup in a cohort of patients who had a myocardial infarction while taking glyburide vs. gliclazide.[20] Along with these studies showing that pre-ACS sulfonylurea use is not associated with changes in outcomes, the DIGAMI 2 randomized trial reported that among diabetic patients discharged after a myocardial infarction cardiovascular mortality at 3 years was not increased with sulfonylurea use compared to metformin use or insulin use (HR 1.15, 95% CI 0.80 – 1.64) [21].

Although we used data collected prospectively on all adults with an ACS within a Canadian province with universal health care access, there are several limitations to our study. First, we have data on all medications dispensed in the 100 days prior to an ACS but cannot tell

which medications were actually consumed within hours of the ACS. Moreover, any patient non-compliance with prescribed K_{ATP} channel inhibitors would have biased our study towards the null. However, if impairment of ischemic preconditioning is the mechanism of harm with K_{ATP} channel inhibitors then use in the months leading up to the ACS event should be when this effect will manifest, assuming that ischemic precondition has been triggered. Second, our identification of patients with DM was done based on physician chart diagnosis rather than actual lab values (hemoglobin A1c, glucose measurements). However, our method for the identification of patients with DM has been previously validated in Canadian administrative databases (sensitivity 84% and specificity 99% [22]). Third, some may feel that our study was too small to detect harm with K_{ATP} channel inhibitor exposure. However, it was large enough to demonstrate better outcomes in those diabetics using statins pre-ACS and we had 80% power to detect/rule out a 15% relative excess risk of death/HF with K_{ATP} channel inhibitor exposure. Fourth, we do not have data on left ventricular ejection fraction and, thus, cannot tell whether the cases of HF diagnosed after ACS were systolic or diastolic – however, all cases were diagnosed by a clinician before being coded into our administrative databases and thus were clinically validated. In the same vein, we were unable to stratify our analysis by infarct size as we only have physician-assigned diagnoses and do not have any data on troponin levels or clinical markers to judge size of infarcts. Finally, our study was observational and thus unmeasured confounders could have biased our study towards the null – a randomized trial such as the ongoing Cardiovacular Outcome Study of Linagliptin Versus Glimepiride in Patients with Type 2 Diabetes (the CAROLINA trial, NCT01243424) is required to definitively settle this issue.

While DM is an independent risk factor for death and/or HF after an ACS, our data suggests that users of K_{ATP} channel inhibitors are not at further increased risk in the first 30 days after an ACS event. As these would be the group we would most expect to exhibit harm if exposed to a drug that inhibits K_{ATP} channel-mediated cardioprotection, our findings raise

doubts about the putative mechanism for the adverse cardiovascular consequences of SU reported in earlier studies. Further research is required to elucidate the true mechanism for the excess cardiovascular hazards associated with chronic use of K_{ATP} channel inhibitors such as sulfonylureas in individuals with diabetes. In the meantime, for the short-term management of plasma glucose levels in a patient with an ACS, our data suggests that K_{ATP} channel inhibitors remain a viable option, which is consistent with current recommendations. [23]

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CHAPTER 7.

Discussion and Conclusions

CHAPTER 7

Discussion and Conclusions

Summary

The field of medicine revolves around patient care. With the underlying goal of continuously improving, research is the field of medicine that can have the single greatest impact on our patients. In no other medical specialty is this more evident than in disease of the heart. The heart is the most researched organ in the body and over the last 50 years we have seen enormous growth in the care of patients with cardiovascular issues. As our patient population continues to evolve so should the way we treat patients. We are now faced with this evolution as the world health organization has stated that in 2012 non-communicable disease has surpassed communicable disease as the world's major disease burden, with cardiovascular disease being responsible for almost half of all non-communicable diseases worldwide[1]. Today, cardiovascular disease is the world's leading cause of death and is projected around the world to cost the healthcare system over $7 trillion in the next 15 years[2].

A common paradigm in cardiovascular care is that mechanical problems require mechanical solutions. For example, the principle treatment for severe multi-vessel coronary artery disease is coronary artery bypass graft surgery. Another example is that

the only treatment for severe symptomatic aortic stenosis is aortic valve replacement. However, perhaps there are adjuncts to these 'mechanical solutions', such as optimizing myocardial energy metabolism that would further improve patient outcomes. Indeed, myocardial energy metabolism is an important field of research due to its contribution to cardiac function in health and disease[3-7]. As FAs are the principle substrate for acetyl CoA derived ATP in the cardiomyocyte[3-7], the study of myocardial FA uptake and utilization in the heart is a growing area of research. It is now evident that alterations in FA metabolism are related to numerous physiologic and pathologic cardiac conditions including, exercise physiology[8-11] and increased myocardial workload[12-14], ischemia and reperfusion[15-21], doxorubicin-induced cardiomyopathy[22-26] and diabetes[27-31]. Therefore, the study of cardiac energy metabolism may give insight into the pathophysiology of disease as well as identify novel pharmacologic targets that may eventually be used in therapy.

The overall aims of this thesis were to examine how manipulation of myocardial FA metabolism in health and disease could alter cardiac function and thus possibly alter the natural history of these conditions. This thesis has explored several aspects of cardiac FA metabolism, with an emphasis on: 1) determining whether decreased FA uptake could improve cardiac function following a brief period of ischemia; 2) characterizing the effects of doxorubicin-induced cardiac dysfunction on myocardial TAG metabolism and determining if manipulation of the intramyocardial TAG pool can be used as an approach to treatment; 3) determining the influence of AMPKs inhibitory phosphorylation of ACC on myocardial metabolism and function during increased cardiac work load and ischemia/reperfusion; 4) determine if diabetic patients taking K_{ATP} channel inhibiting medications are at increased risk of mortality after an ACS event than those patients that do not take K_{ATP} channel inhibitors. Together, the studies completed

in this thesis have shown that manipulation of FA metabolism in the heart may be used during pathologic stress to improve cardiac function along with a study examining the use of glucose lowering drugs in diabetic patients undergoing an ACS event. This chapter will provide additional detail about the major findings and clinical implications of the previous chapters, discuss limitations of the studies presented and most importantly, review possible future directions of study.

General Discussion

Cardiomyocyte-Specific Ablation of CD36 Improves Post-ischemic Functional Recovery

An inadequate circulation of blood to the myocardium is a phenomenon known as ischemia[32]. The results of ischemia can lead to infarction, which is necrosis due to ischemia. However, myocardial ischemia does not necessarily lead to infarction as the heart can tolerate brief ischemic episodes without leading to cell death. Reversible ischemic injury is a result of reperfusion. Many clinical scenarios result from myocardial ischemia and reperfusion including angina, acute coronary syndrome, coronary revascularization and cardiac transplantation. As well, the vast majority of patients undergoing open-heart surgery undergo a myocardial I/R injury, due to the fact that most patients undergoing cardiac surgery require cardiac arrest with cardiopulmonary bypass support. Numerous mechanisms have been implicated in I/R injury [18] and pre-clinical evidence has suggested that partial inhibition of FAO in the myocardium can prevent

cardiac injury and/or dysfunction in the setting of I/R[3, 18]. Despite early reports showing that FAO inhibitors were beneficial during I/R[33-37], more recent reports have suggested some of these FAO inhibitors may act via alternative pathways and subsequently exert their beneficial effects independent from partial inhibition of FAO[38-40]. As such, it is unclear as to whether partial inhibition of FAO is truly beneficial to the injured or diseased myocardium.

In addition to the uncertainty about the benefits of partial inhibition of myocardial FAO, there is a growing body of evidence suggesting that this approach may actually contribute to cardiac dysfunction[41, 42]. In these instances, partial inhibition of FAO may produce a mismatch between FA uptake into the cardiomyocyte and subsequent utilization leading to excessive lipid accumulation. Importantly, there is evidence in both animal models and humans that excessive lipid accumulation is associated with cardiac lipotoxicity[43]. Therefore, the potential detrimental effects of excessive lipid accumulation may negate the potential beneficial effects of partial inhibition of FAO. Based on this, we proposed that a combined approach of limiting FA uptake and subsequently imparting a partial inhibition of FAO might be a more beneficial therapeutic approach to treating the myocardium following I/R injury. In order to address this objective of determining whether limiting FA uptake and partially inhibiting FAO can be used to as a method of reducing or preventing I/R injury we generated and utilized an inducible cardiomyocyte-specific CD36 knock out mouse. As CD36 has been shown to be responsible for 50-60% of FA uptake into the myocardium[44, 45] this mouse model allowed us to answer specifically the role of short-term reduction in cardiomyocyte FA uptake on cardiac function and efficiency after I/R injury.

As described in Chapter 3, using histologic examination and transthoracic echocardiography, inducible cardiomyocyte-specific CD36 ablation did not alter cardiac morphology or function. Using ex vivo perfusions, hearts from ic*CD36*KO mice exhibited a significant decrease in FA uptake than hearts from control mice. As expected, this decrease in FA uptake in ic*CD36*KO hearts lead to these mice being much more reliant on glucose for energy than hearts from control mice. As well icCD36KO mice stored less FA in the form of TAG than control mice. This model achieved our goal of combining a reduction in FA uptake with a reduction in FAO. Therefore, we could address whether imparting a partial inhibition of FAO in the myocardium was beneficial to recovery following an I/R injury, without simultaneously imparting lipotoxicity as previous models have incurred. Indeed, we found that inducible cardiomyocyte-specific CD36 ablation does improve functional recovery and myocardial efficiency following I/R.

Although we utilized a sophisticated inducible, cardiomyocyte-specific mouse model to study our hypotheses, there are several limitations to our study. Limitations from general methodologies used in multiple chapters of this thesis will be described later in this chapter, as this discussion will focus on specific limitations to this study. First, the use of tamoxifen to induce the cardiomyocyte-specific CD36 ablation is a limitation since tamoxifen itself has effects on cardiac function[46]. That is, tamoxifen has been shown to cause cardiomyopathy[46]. To mitigate this issue, we chose to evaluate control and icCD36KO mice 4 weeks following tamoxifen administration. We chose this time point since previous work in our lab had shown that 4 weeks following tamoxifen administration, the detrimental effects of tamoxifen were absent in the heart (unpublished data). However, to truly show that tamoxifen administration was not a relevant variable to our results it would be necessary to perform studies on tamoxifen

administration specifically in our icCD36KO mouse colony. However, we have assumed that since our colony is on a 100% C57Bl/6 background that the ic*CD36*KO mouse line will behave in the same fashion as wild-type C57Bl/6 mice when administered tamoxifen.

Another limitation to our study is that we did not definitively show that Cre translocation in our mouse colony does not alter cardiac function 4 weeks after induction of the CD36 knock out. Although we have based our work on previous studies showing that myocardial function is not altered in C57Bl/6 mice undergoing cardiomyocyte-specific ablation with Cre translocation from the cytoplasm into the nucleus 4 weeks following Cre translocation[46], we did not specifically show this in our mouse colony. Third, although our assessment of FA uptake has been described previously[47] and used in multiple peer-reviewed publications[48, 49], it is still a calculated value based on *ex vivo* FAO rates and FA incorporation into the cardiomyocyte. However, this does not answer the question of whether *in vivo* FA uptake is altered in our model. As well, the final limitation of this study is that the I/R data presented is from the *ex vivo* model (limitations of *ex vivo* working heart perfusions will discussed later in this chapter) and extrapolation of the translational significance of this work is limited due to the lack of *in vivo* data.

Taken together, this study utilized a newly created mouse model that allowed us to demonstrate that limiting FA uptake and partially inhibiting FAO can be used to improve post-ischemic myocardial efficiency and recovery. This study suggests that inhibition of CD36 in the cardiomyocyte may be a potential strategy for mitigating I/R injury in the myocardium. Ultimately, this study has added knowledge and understanding to the cardiomyocyte-specific role of CD36.

Cardiomyocyte-Specific Adipose Triglyceride Lipase Over-Expression Prevents Doxorubicin-Induced Cardiac Dysfunction in Female Mice

Survival of all cancers has improved with advancement in screening and therapy over the last 4 decades[50-52]. Since chemotherapeutics continue to be central in the treatment of many types of cancer as well as the fact that patients are living longer after the diagnosis and treatment of their cancer, the short and long term effects of these therapies on the rest of the body is becoming a focus of modern research[50-52]. A well-characterized and potentially lethal side effect of a very common class of chemotherapy is drug-induced cardiotoxicity[24, 53-55]. The use of anthracycline-containing regimens (i.e., doxorubicin, epirubicin) is well recognized to trigger dose-dependent, cumulative, progressive left ventricular (LV) remodeling manifested as increased cavity size and reduced LV ejection fraction (LVEF) that can progress to congestive heart failure and death[24, 53-55]. The onset of cardiac toxicity is extremely important to the cancer treatment strategy since chemotherapy is withheld when LVEF falls below the lower limit of normal (i.e., 50%)[56]. Equally important is the fact that the anthracycline-induced cardiac injury may leave patients susceptible to late-occurring recurrent or progressive dysfunction and congestive heart failure[56]. At present, medical management of heart failure due to doxorubicin administration is similar to that of all patients with left ventricular dysfunction and heart failure and does not specifically target doxorubicin-induced cardiotoxicity[57]. This is largely due to our lack of understanding of the mechanisms that lead to doxorubicin-induced cardiac dysfunction. Due to the nature of their cardiomyopathy, patients with deteriorating doxorubicin-induced heart failure, who are already receiving optimal medical management, also have a paucity of surgical options available[58]. Furthermore, this patient population is often evaluated as potential

cardiac transplant recipients, which is also complicated by their history of previous cancer[59]. As well, recent studies have associated alterations in myocardial energy metabolism with the cardiotoxic effects of doxorubicin and that promotion of a hypolipidemic cardiomyocyte environment diminishes the cardiotoxic effects of doxorubicin-treated animals and patients[26, 60]. Based on these reported alterations in energy metabolism in doxorubicin-induced cardiomyopathy and the reported benefits of a hypolipidemic cardiomyocyte environment in diminishing the cardiac side effects of doxorubicin we wanted to examine the role of myocardial TAG metabolism in doxorubicin-induced cardiac dysfunction.

As described in chapter 4, our doxorubicin protocol resulted in systolic dysfunction in wild-type mice. We believe this protocol more closely mimics the clinical scenario since the incidence of overt heart failure in recent doxorubicin regimen trials is less than 2.1%[61] with the majority of cardiac related issues resulting in dysfunction. Furthermore, previous studies suggest that doxorubicin administration causes alterations in cardiac energy metabolism and myocardial substrate utilization[22]. However, our findings demonstrate that doxorubicin-induced cardiac dysfunction *in vivo* is independent of changes in glucose metabolism and FAO in WT mouse hearts. As well, our data suggest that alterations in energy metabolism may occur in consequence to impaired function, as opposed to causing cardiac dysfunction.

One reason for this discrepancy in our data as opposed to previous studies may be that we have measured cardiac energy metabolism in the perfused working heart, rather than in isolated cardiomyocytes. Interestingly, although doxorubicin-induced cardiac dysfunction did not alter myocardial substrate utilization, our data demonstrate that doxorubicin administration reduced myocardial TAG content in WT mice. Since doxorubicin-induced cardiomyopathy was associated with a significant decrease in

myocardial TAG content, we hypothesized that altering the intramyocardial TAG pool could influence the functional outcome of doxorubicin-induced cardiotoxicity. Indeed, utilizing mice with cardiomyocyte-specific over-expression of ATGL that results in chronically reduced TAG content, we demonstrated that ATGL over-expression protects from doxorubicin-induced cardiac dysfunction.

Although this study addressed the role of myocardial TAG metabolism and its contributions to doxorubicin-induced cardiotoxicity using a sophisticated cardiomyocyte-specific mouse model of ATGL over-expression, I do recognize that this study is not without limitations. Firstly, this study has not provided direct evidence or a mechanism linking the decrease in myocardial TAG with protection from doxorubicin-induced cardiac dysfunction. Moreover, we speculated in this study that the link between ATGL over-expression and protection of cardiac function during doxorubicin administration is due to the creation of a hypolipidemic cardiomyocyte environment. However, a full characterization of FA uptake, transport and utilization in WT and MHC-ATGL mice administered doxorubicin is required to substantiate this hypothesis. Also, as there is no specific pharmacologic agents that target cardiomyocyte TAG metabolism to date, the direct translational implications of this work may not be obvious. However, due to our current lack of understanding of the mechanisms that cause doxorubicin-induced cardiac dysfunction it is imperative that we search for novel pharmacologic targets that may aid in treatment of doxorubicin-induced cardiac dysfunction.

This study provided considerable insight into how myocardial TAG metabolism is able to prevent doxorubicin-induced cardiac injury. Specifically, this work allowed us to better understand how a chronically low TAG cardiomyocyte environment contributed to the beneficial effects of ATGL over-expression in preventing doxorubicin-induced cardiac

injury and heart failure. As doxorubicin-induced cardiac dysfunction and heart failure are significant concerns in oncology, cardiology, and cardiac surgery, understanding the mechanisms responsible for regulating these pathophysiological processes as well as the mechanisms behind the beneficial effects of ATGL over-expression may allow us to use myocardial TAG content manipulation clinically for this purpose or develop more effective strategies to prevent this condition.

Myocardial function during physiological and pathological stress is independent of metabolic changes induced by AMPK-mediated inhibitory phosphorylation of ACC

The cell's ability to survive situations involving energetic stress relies, in part, on the responses of intracellular stress kinases[62, 63]. These kinases modulate cellular function through various effector pathways that assist in lessening energetic stress and/or allowing the cell to adapt to it. One well-described energy sensing kinases is AMPK. When activated, AMPK restores energy balance by switching on catabolic pathways (glucose uptake and FAO) that produce ATP and switching off anabolic pathways (glycogen synthesis and protein synthesis) that consume ATP[64]. Through these adjustments to ATP production and consumption, AMPK assists in the re-establishment of cellular ATP levels and can promote cell survival and/or recovery of cellular function during and following energetic stress[62]. In the cardiomyocyte, numerous AMPK-mediated mechanisms have evolved to lessen disturbances in ATP levels that would otherwise disrupt the ability of the heart to maintain continuous contraction[65]. One suggested mechanism by which AMPK exerts its influence on

overall myocardial energetic homeostasis is through changes in myocardial FAO via the ability of AMPK to phosphorylate and inactivate its downstream target acetyl coenzyme A carboxylase (ACC)[66, 67]. Furthermore, previous work has shown that manipulation of ACC, via loss of function studies[68], significantly alter myocardial FAO. However, the precise role that the AMPK-ACC axis plays in myocardial metabolism has not been directly assessed in previous work. This is of importance since numerous previous studies suggest a key role of the AMPK-ACC axis in overall energy metabolism during myocardial stress without definitively showing this relationship[20, 21, 69-71]. Therefore in this thesis, our purpose was to investigate AMPK's influence on myocardial metabolism via ACC.

To address this question, we used knock-in mice to the AMPK phosphorylation sites ACC1 Ser 79 and ACC2 Ser 212. This knock-in mutation of a single amino acid residue in ACC1 and ACC2 resulted in replacement of the serine phosphorylation sites with alanine. Therefore, in the knock-in mutation ACC cannot be phosphorylated by AMPK. Since the heart expresses both ACC1 and ACC2 isoforms after the generation of ACC1 and ACC2 single KI mice, these mice were crossed to generate the ACC double-knock-in mouse line used in the current study. From the examination of these ACC-DKI mice we were able to show that cardiac malonyl CoA content is regulated by AMPK mediated ACC phosphorylation. The rate-limiting step of FAO is the import of long chain FAs across the mitochondrial membrane through carnitine palmitoyl transferase I (CPT1)[72]. This action is strongly inhibited by malonyl CoA, which is formed by the carboxylation of acetyl CoA via ACC[72]. Therefore, from this initial observation we expected that the ACC-DKI mutation would result in decreases in myocardial FAO rates and via the Randle cycle[73, 74] increases in glucose oxidation

rates. Interestingly, our data demonstrate that the ACC-DKI mutation do not influence cardiac metabolism and subsequent function.

Next, in order to understand if this ACC-DKI mutation was capable of altering metabolism and function of the heart during stress, we decided to examine the ACC-DKI mouse in comparison to WT during times when AMPK is known to be activated. This approach would cause the largest differences between ACC-DKI and WT mice. We employed two conditions in which AMPK is known to be upregulated, one physiologic condition of high myocardial workload and one pathophysiologic condition of I/R. Interestingly, the ACC-DKI mouse showed no significant differences in myocardial function in either physiologic or pathophysiologic stress. As well, previous work with the ACC-knock out mouse model has shown that ACC ablation causes alterations in myocardial metabolism and function at baseline and during stress[68]. As well, previous work done on the MCD-knock out mouse model has shown that MCD ablation results in improved myocardial performance after stress[17]. Therefore, placing our data in the context of previous work, we believe that indeed malonyl CoA is a potent inhibitor of the CPT-1, the rate-limiting step in FAO. However, collectively our data challenge the previously suggested role that the AMPK-ACC-Malonyl CoA-CPT-1 axis plays in regulating myocardial FAO rates.

Although we utilized a sophisticated double knock-in mouse model to study our hypotheses, there are several limitations to our study. First, this study was done completely on female mice since that it all that our collaborators had available for us to use. In order to fully elucidate the role of the AMPK-ACC-malonyl CoA axis on cardiac function, both genders will need to be examined to form conclusions. Another limitation of this study is that the ACC-DKI mutation is a whole body mutation. Whole body

mutations may incur differences in circulating substrate levels, as well as intra-organ communications. As well, since these mutations are chronic, developmental adaptations may occur. However, our attempt to mitigate whole body responses were to study the heart in the *ex vivo* working heart mode in order to minimize the issues inherent with all whole body mutations. Another limitation of this study is the lack of data presented on CPT-1 involvement in the ACC-DKI mutation. In order to definitively answer our questions about the role of the AMPK-ACC axis, CPT-1 activity is needed as alterations in this enzyme at baseline or during stress may alter the results presented.

In summary, using the ACC-DKI mutant mouse model, we demonstrated that AMPK mediated ACC phosphorylation regulates cardiac malonyl CoA levels. However, this baseline increase in malonyl CoA content did not alter myocardial exogenous substrate utilization or cardiac function at baseline or during physiologic and pathologic stress. Therefore, these findings challenge the previously suggested role of the AMPK-ACC axis in regulating FAO in healthy and stressed myocardium.

Are users of sulfonylureas at the time of an acute coronary syndrome at risk of poorer outcomes?

The sulphonylurea receptor (SUR) functions as the regulatory subunit of the ATP-sensitive potassium (K_{ATP}) channel[75-78]. K_{ATP} channels are widely expressed including the heart, vascular smooth muscle cells and pancreatic beta cells where they couple the metabolic status of the cells to their electrical properties[79-82]. For example, during myocardial ischemia, loss of intracellular ATP leads to an activation (opening) of

myocardial K_{ATP} channels. The activation of the myocardial K_{ATP} channels is a key mediator of the ST segment elevation response to transmural ischemia[83]. In addition, myocardial K_{ATP} channels also play a key role in ischemic cardioprotection. Indeed, intravenous nicorandil, a K_{ATP} channel opener, leads to beneficial clinical outcomes and prevents both early and late cardiovascular events in patients with STEMI who received percutaneous coronary intervention[84, 85]. Insulin secretagogues such as sulfonylureas and glinides bind to cardiovascular K_{ATP} channels leading to closure of the channels and potential impairment of ischemic preconditioning[86]. This has been demonstrated in human studies conducted during coronary angiography, but the clinical import of these pathophysiologic findings remains uncertain[87]. Given the critical protective role of the K_{ATP} channels, we designed this study to explore whether diabetic patients who are taking K_{ATP} channel blockers may have an adverse clinical outcome in the setting of ACS.

As described in Chapter 6, this study demonstrates that DM is an independent risk factor for mortality after an ACS event, recapitulating previous findings from others. A novel finding from this work is that we establish that DM is an independent risk factor for HF after an ACS event. As well, we have shown that DM is a risk factor for death/HF within 30 days in both patients with NSTEMI and STEMI. While this result may appear intuitive, this data does demonstrate that DM poses similar prognostic risk on a patient undergoing an ACS event regardless of the severity of that event. There was no significantly increased risk of death or death/HF in ACS patients who were users of K_{ATP} channel inhibitors at the time of their ACS compared to those not exposed to K_{ATP} channel inhibitors, even after adjustment for differences in baseline covariates and procedures within the first 7 days of the index ACS event. This result was certainly surprising and raised concerns about the power of our study. To mitigate these

concerns we explored the effect of statins, a class of drug that has been proven in many other studies to be beneficial to prognosis of death and HF after an ACS event. We found that users of statins were indeed at lower risk of death/HF at 30 days, suggesting that our study did have sufficient sample size to detect medication differences in our composite end-point. Interestingly, since K_{ATP} channel inhibitors did not result in an increase in death or death/HF at 30 days after an ACS event, we believe this raises doubts about the putative impact of K_{ATP} channel inhibitors on ischemic preconditioning.

Although we used data collected prospectively on all adults with an ACS within a Canadian province with universal health care access, there are several limitations to our study. First, we have data on all medications dispensed in the 100 days prior to an ACS but cannot tell which medications were actually consumed within hours of the ACS. Moreover, any patient non-compliance with prescribed K_{ATP} channel inhibitors would have biased our study towards the null. However, if impairment of ischemic preconditioning is the mechanism of harm with K_{ATP} channel inhibitors then use in the months leading up to the ACS event should be when this effect will manifest, assuming that ischemic precondition has been triggered. Second, our identification of patients with DM was done based on physician chart diagnosis rather than actual lab values (hemoglobin A1c, glucose measurements). However, our method for the identification of patients with DM has been previously validated in Canadian administrative databases (sensitivity 84% and specificity 99%[88]). Third, some may feel that our study was too small to detect harm with K_{ATP} channel inhibitor exposure. However, it was large enough to demonstrate better outcomes in those diabetics using statins pre-ACS and we had 80% power to detect/rule out a 15% relative excess risk of death/HF with K_{ATP} channel inhibitor exposure. Fourth, we do not have data on left ventricular ejection fraction and, thus, cannot tell whether the cases of HF diagnosed after ACS were systolic or diastolic

– however, all cases were diagnosed by a clinician before being coded into our administrative databases and thus were clinically validated. In the same vein, we were unable to stratify our analysis by infarct size as we only have physician-assigned diagnoses and do not have any data on troponin levels or clinical markers to judge size of infarcts. Finally, our study was observational and thus unmeasured confounders could have biased our study towards the null – a randomized trial such as the ongoing Cardiovascular Outcome Study of Linagliptin Versus Glimepiride in Patients with Type 2 Diabetes (the CAROLINA trial, NCT01243424) is required to definitively settle this issue.

Therefore, it is our recommendation to the primary physician that K_{ATP} channel inhibitors remain a viable option for the management of plasma glucose levels in patients with coronary artery disease or who have had an ACS event, which is consistent with current ACC/AHA guidelines for the management of an ACS event.

Justification of Methodology and Experimental Limitations

Isolated Working Mouse Heart Perfusions

In order for the heart to supply blood to the end organs of the rest of the body and match basal whole body metabolic demand, the heart is continuously contracting. Due to this constant state of work, the heart is the king of basal energy demand in the body. One method to measure energy metabolism in the heart is the isolated working heart perfusion model. There are several advantages to the use of this model. The

isolated working mouse heart perfusion system allows direct steady state measurements of flux through different pathways. As well, activity through the TCA cycle can be measured by acetyl CoA production. Furthermore, with the ability to control for loading conditions and drug administration, this model allows for measurement of physiological, biochemical, pharmacologic and metabolic parameters in a highly reproducible fashion[89]. The most important advantage of this model is that it measures cardiac parameters while the heart is beating against a mechanical load. Furthermore, this method of assessing cardiac metabolism is relatively inexpensive compared to other methods used.

While isolated working mouse heart perfusions have several advantages as stated above, this method is not without its limitations. One limitation is that the substrate solution used to perfuse the heart during working heart perfusions is not a precise solution of what the heart would be perfused with *in vivo*. That is, this crystalloid based buffer solution lacks blood elements (hemoglobin, white blood cells, platelets), lactate, protein and other substances that are contained *in vivo*. Due to this lack of inflammatory components, that have been shown to play an especially integral role in I/R injury, it is important to perform *in vivo* studies to support or refute claims made by *ex vivo* models. Another limitation of this model deals with assumptions made with the use of ^{14}C labeled glucose. In this model, we assume that $^{14}CO_2$ is the only fate of [^{14}C]-glucose, however, [^{14}C]-glucose can also be sent through the pentose phosphate pathway and others. As well, although it possible with the isolated working heart model to measure rate of flux in endogenous TAG and glycogen stores, we have not completed these studies in this thesis. Therefore, endogenous substrate stores of FAs and glucose may influence our assumption of steady state flux through FAO and glucose oxidation. Finally, in the ex vivo model, our studies are not able to preserve neuro-hormonal effects

on cardiac function which further highlights the need to correlate our *ex vivo* findings with *in vivo* studies.

Doxorubicin-induced Cardiac Dysfunction

Over the past several decades, a variety of murine models of doxorubicin-induced cardiotoxicity have been employed to attempt to mimic the human condition. However, the most relevant model to the patient with doxorubicin-induced cardiomyopathy should possess a number of key characteristics. We believe, in order to mimic the clinical scenario as closely as possible a doxorubicin administration should include a multiple dose regimen of doxorubicin given over a period of several weeks. As well, the final result of the doxorubicin administration should lead to cardiac dysfunction and not overt heart failure since only a very small percentage of patients with cardiotoxicity as a result of doxorubicin exhibit heart failure[61]. That is, the vast majority (>97%) of patients with doxorubicin-induced cardiomyopathy display dysfunction (35%<LVEF<60%). A limitation with this model is due to our focus on metabolism. Our doxorubicin-induced cardiac dysfunction protocol causes mice to lose approximately 10% of their initial body weight over the course of the doxorubicin administration. This is likely due to a decrease in food consumption and as such, the effect on cardiac energy metabolism may be masked by a general fasted state of the mice. However, although clinical work on patients treated with doxorubicin and food consumption has not been done, we believe that this may mimic the clinical scenario and is a relevant model for the study of this pathophysiologic process.

Genetically Modified Mice

A limitation of this thesis was the reliance on genetically modified mouse strains (ic*CD36*KO, MHC-ATGL, ACC-DKI) to generate data. This is considered a limitation due to the changes in baseline phenotype as a result of genetic manipulation. For example, in the MHC-ATGL mouse strain we see improved systolic function at baseline when compared to WT mice. However, in the ACC-DKI and the icCD36KO mouse models we see no differences in baseline ex vivo or in vivo function. As well, an advantage of two of our mouse models is the fact that they are cardiomyocyte-specific which leads to less whole body compensatory changes. As well, our ic*CD36*KO mouse model is an inducible knock out which eliminates developmental compensatory changes as well. Nonetheless, pharmacologic data should be considered in order to support findings and conclusions we have made with genetically altered mice. However, we believe with the use of these highly sophisticated mouse models that we have been able to answer important questions about myocardial FA uptake and utilization and that this work provides a basis for future studies on the manipulation of myocardial energy metabolism as a means to improve cardiac function in health and disease.

Absence of Cell Culture Experiments

One of the oldest most widely used methods to study myocardial energy metabolism in the heart is with cell culture studies. These studies have been a powerful tool in the assessment of biochemical intermediates involved in energy metabolism, as

well as measurements of mitochondrial function. Although, the importance of contractile work in measurements of energy metabolism is lost in cell culture studies, the combination of cell culture work, *ex vivo* working heart perfusions and *in vivo* myocardial assessments, give the greatest yield when exploring new mechanisms. Therefore, future work in cell culture will be necessary to support the findings shown in this thesis.

Future Directions

Cardiomyocyte-Specific Ablation of CD36 Improves Post-ischemic Functional Recovery

Further studies are required to determine the mechanism(s) behind the differences in functional recovery between control and ic*CD36*KO mice. For example, whether mitochondrial morphology, abundance and function are altered between the two genotypes. Although calculated FA uptake was significantly decreased in ic*CD36*KO mice when compared to controls *ex vivo*, it is necessary to measure myocardial substrate uptake *in vivo* using combined positron emission tomography and computerized tomography (PET/CT) as well as single-photon emission CT to confirm that cardiomyocyte-specific ablation of CD36 results in a significant decrease in FA uptake when compared to controls. As well, another necessary future experiment will be to validate our ex vivo I/R findings in an *in vivo* I/R model of temporary LAD ligation. The temporary LAD ligation model for *in vivo* I/R in mice is the best tool to assess for protection from I/R *in vivo*. This study will not only allow for assessment of differences in

infarct size between genotypes, it will also allow for recovery experiments in which LV remodeling may be assessed. These experiments will certainly advance our understanding of the role of cardiomyocyte-specific CD36 deficiency and will bring us one step closer towards translation of this work.

As previously stated, the advantages of our inducible cardiomyocyte-specific CD36 ablation mouse model, is that it is a combined approach of limiting FA uptake and subsequently imparting a partial inhibition of FAO on the cardiomyocyte. Therefore, it would be appealing to assess this mouse in a diet-induced obesity (DIO) model of insulin resistance. That is, in the DIO model the ability of CD36 to prevent a lipotoxic cardiomyocyte environment and alter insulin sensitivity could prove to be an interesting finding due to the fact that the obesity epidemic is only increasing in Canada. Therefore, if cardiomyocyte-specific CD36 ablation could be shown to improve I/R recovery as well as cardiac function during obesity and insulin resistance it may have a high translational value to patient care in the future.

Cardiomyocyte-Specific Adipose Triglyceride Lipase Over-Expression Prevents Doxorubicin-Induced Cardiac Dysfunction in Female Mice

Although we generated a sophisticated mouse model that specifically decreases cardiac TAG levels, it is possible that other mechanisms are contributing to the protective effects from doxorubicin-induced cardiac dysfunction. As such, further studies are required to elucidate these potential mechanisms. In order to find direct evidence that the decrease in myocardial TAG content mediates the protection of doxorubicin-

induced cardiac dysfunction isolated cell culture studies could be performed. This work on isolated cardiomyocytes would allow for direct visualization of lipid droplets in the cardiomyocyte as well as biochemical analysis of markers of apoptosis, and reactive oxygen species, which have both been implicated as possible mechanisms that cause cardiac dysfunction following doxorubicin administration.

Furthermore, Chapter 4 has hypothesized that a possible mechanism for the effects of forced ATGL over-expression in the cardiomyocyte on doxorubicin-induced cardiac dysfunction may be the promotion of a hypolipidemic environment. To further test this hypothesis, utilization of a genetically altered mouse model with a reduction in FA uptake may add evidence to support this hypothesis. Therefore, the inducible cardiomyocyte-specific CD36 knock out mouse model may be an ideal model to delineate the importance of maintaining a decrease in FA uptake in terms of doxorubicin-induced cardiac dysfunction. Based on the hypotheses of this thesis, an experiment in which the knock out of CD36 before and after doxorubicin administration should results in drastically different outcomes. That is, if doxorubicin indeed requires the uptake of FAs as a co-transporter to enter the cardiomyocyte then one should be able to confer protection from doxorubicin-induced cardiotoxicity by inducing ablation of CD36 prior to the administration of the first dose of doxorubicin. As well, if the creation of a hypolipidemic cardiomyocyte environment is the key factor causing protection against doxorubicin-induced cardiotoxicity then the ablation of CD36 from the cardiomyocyte after the administration of the last dose of doxorubicin should not alter the natural history of the doxorubicin-induced cardiotoxicity as the doxorubicin administered would already have gained access to the cardiomyocyte in a similar manner to that of a WT mouse. This set of experiments will give more insight into the claim that a hypolipidemic

cardiomyocyte environment is beneficial in protection from doxorubicin-induced cardiotoxicity, a concept that is novel to this thesis.

Myocardial function during physiological and pathological stress is independent of metabolic changes induced by AMPK-mediated inhibitory phosphorylation of ACC

To determine whether AMPK inhibitory phosphorylation of ACC influences myocardial metabolism and function during physiologic and pathophysiologic stress, chronic stress conditions should be examined. The studies done in this thesis focus on acute increased myocardial workload and acute I/R injury. Our conclusions about the influence of the AMPK-ACC axis on myocardial metabolism and function only apply to the acute scenario. It is still unclear if inhibiting AMPKs ability to phosphorylate and thus inhibit ACC in the chronic scenario would result in metabolic and functional differences when compared to WT mice. Furthermore as stated in the limitations section of this discussion chapter, further work with male and female mice, instead of with female mice only will help elucidate whether the data obtained in this thesis are due to gender differences or not. Finally another interesting study that would aid in our understanding of the AMPK-ACC-malonyl CoA axis would be to pharmacologically treat ACC-DKI and WT mice with MCD inhibitors and examine if there are differences in metabolism and function during physiologic and pathophysiologic stress. This would further enhance our understanding of the relationship between ACC and MCD in terms of their ability to regulate malonyl CoA levels in the myocardium.

Are users of sulfonylureas at the time of an acute coronary syndrome at risk of poorer outcomes?

Although no difference in the death or death/HF was observed in ACS patients over 65 years of age who were users of K_{ATP} channel inhibitors at the time of their ACS compared to those not exposed to K_{ATP} channel inhibitors at 30 days, it may be likely that the difference in mortality between groups may not be seen early. Therefore, future study into the long-term outcomes of ACS patients with DM using K_{ATP} channel inhibitors or not would be necessary to investigate the safety of K_{ATP} channel inhibitors in ACS patients with DM.

In regards to any observational cohort study, the next step in future directions directly related to this study would be to perform a randomized controlled trial. Although it would be difficult to recapitulate a population of patients that will undergo an ACS event, the patient cohort could include those patients with diagnosed CAD along with DM. This could be a double-blind controlled trial evaluating the effect of K_{ATP} channel inhibitors on survival in patients with known CAD and DM. Patients can be randomly assigned to a K_{ATP} channel inhibitor or non-K_{ATP} channel inhibitor.

Final Conclusions

In conclusion, the studies in this thesis have addressed questions regarding the manipulation of FA uptake and utilization as an approach to the treatment of myocardial dysfunction in cardiovascular health and disease. The findings of this thesis highlight possible novel targets for pharmacologic therapy of I/R injury, and doxorubicin-induced cardiomyopathy. As well, this thesis provides data that refutes the previous held notion that the AMPK-ACC axis may be an important drug target for the control of metabolism and function of the heart during stress, as well as refutes the suggested harm that K_{ATP} channel inhibitors cause to patients undergoing an ACS event. Individually, these studies demonstrate that:

1) Cardiomyocyte-specific CD36 ablation can significantly reduce FA uptake, FAO, and TAG storage in healthy hearts as well as improve myocardial efficiency and functional recovery following an ischemic injury. Our findings are consistent with recent studies that have suggested that contractile dysfunction during and after myocardial ischemia can be reduced by stimulating glucose oxidation either directly or secondary to inhibition of FAO[17, 90]. Based on our findings, it is tempting to speculate that pharmacotherapy designed to specifically inhibit cardiomyocyte CD36 may represent a promising new approach to lessen myocardial I/R injury in patients.

2) Doxorubicin-induced cardiac dysfunction occurs in the absence of alterations in myocardial glucose and FA oxidation. More importantly, our results show that manipulation of intramyocardial TAG metabolism leading to a chronically

decreased myocardial TAG pool protects from doxorubicin-induced cardiomyopathy. Moreover, these findings suggest that the reduction in myocardial TAG accumulation in WT hearts is an adaptive, albeit insufficient response to doxorubicin treatment. Therefore, novel pharmacological targets aiming at reducing myocardial TAG concentrations may provide new avenues for the treatment of cardiomyopathy in cancer patients undergoing anthracycline chemotherapy by producing a hypolipid environment within cardiomyocytes. Although this has implications for many patient populations, this may be extremely relevant in obese/diabetic patients who are being treated with doxorubicin-containing chemotherapy as these conditions are associated with elevated intramyocardial TAG levels[91] and previous rodent work has shown that high-fat fed obese rats are more sensitive to doxorubicin-induced cardiotoxicity than their chow fed non-obese counterparts[91]. Whether or not myocardial TAGs play a role in this increased sensitivity to doxorubicin-induced cardiotoxicity in obese/diabetic patients has yet to be explored.

3) An ACC-DKI mutation is unable to alter myocardial function following physiologic and pathophysiologic stress. Collectively, these findings challenge the previously suggested role of AMPK-mediated inhibitory ACC phosphorylation in the regulation of FAO in healthy and stressed myocardium.

4) Users of KATP channel inhibitors are not at further increased risk in the first 30 days after an ACS event. As these would be the group we would most expect to exhibit harm if exposed to a drug that inhibits KATP channel-mediated cardioprotection, our findings raise doubts about the putative mechanism for the adverse cardiovascular consequences of SU reported in earlier studies. Further

research is required to elucidate the true mechanism for the excess cardiovascular hazards associated with chronic use of KATP channel inhibitors such as sulfonylureas in individuals with diabetes. In the meantime, for the short-term management of plasma glucose levels in a patient with an ACS, our data suggests that KATP channel inhibitors remain a viable option, which is consistent with current recommendations.

Taken together, these studies suggest that manipulation of FA uptake and utilization in the cardiomyocyte represent potential therapeutic targets for ischemia reperfusion and doxorubicin-induced cardiac dysfunction. This optimization of myocardial energy metabolism in health and disease may prove to be an important adjunct to current therapy of cardiovascular disease.

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