Investigations into the regulation of skeletal muscle mitochondrial metabolism

by

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ABSTRACT

INVESTIGATIONS INTO THE REGULATION OF SKELETAL MUSCLE MITOCHONDRIAL METABOLISM

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This thesis is a series of investigations into the regulation of skeletal muscle mitochondrial metabolism. Novel regulatory mechanisms regarding mitochondrial fatty acid oxidation are continually being identified and alterations in skeletal muscle mitochondrial metabolism have been implicated in the pathogenesis of type II diabetes (T2DM). Therefore, advancing our basic understanding of mitochondrial regulatory processes is required to provide insight into the progression of T2DM.

In study one, with the utilization of knockout mice for the putative mitochondrial fatty acid transport protein FAT/CD36, it was observed that mitochondrial FAT/CD36 played a functional role in mitochondrial long chain fatty acid (LCFA) oxidation. Specifically, FAT/CD36 was found to be located on the outer mitochondrial membrane (OMM) upstream of acyl-CoA synthetase.

In study two, it was observed that in rat muscle, malonyl-CoA (M-CoA) inhibition kinetics of carnitine palmitoyltransferase I (CPT-I) displayed a more physiological IC$_{50}$ in permeabilized muscle fibre bundles (PmFB) compared to isolated mitochondria. These data suggest that the cytoskeleton may have a role in regulating M-CoA inhibition. Additionally, a significant effect of LCFA-CoA on M-CoA inhibition kinetics was observed. These data indicate that M-CoA content does not need to decrease to promote an increase in CPT-I flux.
Finally, in a model of T2DM (ZDF rat), submaximal ADP-stimulated respiration rates and the content of adenine nucleotide translocase 2 (ANT2) content were lower compared to lean control animals. Resveratrol treatment in ZDF rats recovered these declines concomitantly with improving insulin-stimulated skeletal muscle glucose uptake and the cellular redox state.

A number of novel findings are presented, specifically, 1) a functional role for mitochondrial FAT/CD36 in mitochondrial LCFA oxidation was confirmed and the topology of this protein along the OMM was expanded upon, 2) M-CoA inhibition kinetics of CPT-I were re-evaluated in PmFB and a regulatory role of LCFA-CoA on M-CoA inhibition kinetics was established, and 3) submaximal ADP-stimulated respiration rates and ANT2 content were lower in the ZDF rat and resveratrol supplementation prevents these decrements.
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To my parents, Larry and Lynn Smith, I love you both and I sincerely thank you for all of your love and support over the years. And finally, to my lovely girlfriend Jessica, thank you for your patience, your love and the immense amount of support.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminooimidazole-4-carboxamide-1-beta-4-ribofuranoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>βHAD</td>
<td>β-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>CACT</td>
<td>Carnitine acyl-carnitine translocase</td>
</tr>
<tr>
<td>COXIV</td>
<td>Cytochrome c oxidase IV</td>
</tr>
<tr>
<td>CPT-I</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CPT-II</td>
<td>Carnitine palmitoyltransferase II</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CrAT</td>
<td>Carnitine acetyl transferase</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transfer chain</td>
</tr>
<tr>
<td>ETF</td>
<td>Electron transfer flavoprotein</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FABPc</td>
<td>Cytosolic fatty acid-binding protein</td>
</tr>
<tr>
<td>FABPpm</td>
<td>Plasma membrane associated fatty acid-binding protein</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>Fatty acid translocase/Cluster of Differentiation 36</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>IMF</td>
<td>Intermyofibrillar</td>
</tr>
<tr>
<td>IMTG</td>
<td>Intramuscular triacylglycerol</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
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<tr>
<td>LCFA-CoA</td>
<td>Long chain fatty acyl coenzyme A</td>
</tr>
<tr>
<td>MCD</td>
<td>Malonyl-CoA decarboxylase</td>
</tr>
<tr>
<td>M-CoA</td>
<td>Malonyl-CoA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1 alpha</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RCR</td>
<td>Respiratory control ratios</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSO</td>
<td>Sulfo-N-succinimidyl oleate</td>
</tr>
<tr>
<td>SS</td>
<td>Subsarcolemmal</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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Type II diabetes mellitus  T2DM
Wild-type  WT
Zucker diabetic fatty  ZDF
CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW
1.1 Introduction and overview

Mitochondria are key organelles within the human body and have a well-defined role in the maintenance of energy (ATP) homeostasis. The production of ATP through mitochondrial oxidative phosphorylation is classically viewed as a product of 3 basic components; 1) the energy demand and subsequent supply of ADP, 2) the supply of reducing equivalents (NADH and FADH₂ carrying pairs of electrons), and 3) intracellular oxygen pressure.

\[
2\text{NADH} + 2 \text{H}^+ + 5\text{ADP} + 5\text{Pi} + \text{O}_2 \rightarrow 2\text{NAD}^+ + 5\text{ATP} + 7\text{H}_2\text{O}
\]

The rate at which the mitochondria consume oxygen and utilize the reducing power of NADH and FADH₂ to generate ATP is usually determined by the cellular demand for ATP, and therefore is proportional to mitochondrial ADP supply (during aerobic respiration) [1].

More recently, it has become apparent that the systems designed to regulate the provision of the three aforementioned components are more heavily coordinated and complicated than we previously imagined. The complexities surrounding local control of blood flow to regulate oxygen delivery, the intricate regulation of carbohydrate and fatty acid catabolism for the supply of reducing equivalents, and the kinetics of how ADP can move from being a by-product of contraction to a substrate for re-synthesis of ATP are just snapshots of the wealth of topics to pursue regarding the field of energy metabolism. Within skeletal muscle, it is important to elucidate mechanisms of control as alterations in skeletal muscle mitochondrial metabolism have been implicated in the pathogenesis of many diseases including type II diabetes (T2DM). Thus, a better understanding of mitochondrial regulatory processes and mitochondrial bioenergetics may provide insight into the progression of this disease.

Therefore, this thesis has focused on two of the broad components within mitochondrial metabolism, including; 1) elucidating the basic regulation of fatty acid transport into
mitochondria, and 2) determining the potential relationship between a dysregulation of mitochondrial ADP supply and insulin resistance.

1.2 Fatty acid metabolism

Fatty acids serve a number of essential structural, metabolic and molecular functions within muscle, including, 1) structural components of the cellular membranes, 2) mediating signal transduction, 3) influencing protein modifications, 4) transcriptional regulation, and 5) are an essential substrate for energy production. Given that this thesis is focused on mitochondrial bioenergetics, fatty acid metabolism will be discussed in detail.

Fatty acids contribute a large portion of the required energy at rest, and although they are a less efficient means of energy production per unit of oxygen compared to carbohydrates, they provide more energy per gram wet weight. In addition, the human body contains ~35 times the amount of energy stored as fatty acids compared to carbohydrate, making fatty acid metabolism a vital component of overall energy homeostasis. Within the literature, the regulation of carbohydrate metabolism is better characterized than the regulation of fatty acid metabolism. For example, the regulation of the rate-limiting enzymes in carbohydrate catabolism including, glycogen phosphorylase, phosphofructokinase and pyruvate dehydrogenase (PDH) have been investigated and described [2-4]. In contrast, the regulation of skeletal muscle fatty acid metabolism has remained poorly understood and we are only now becoming aware of the many different regulatory points within this system. Much of the previous regulatory information stems from exercise and/or nutritional interventions, as these challenges are powerful stimuli to invoke a regulatory change. Therefore, throughout this thesis, an attempt has been made to discuss both of these metabolic challenges within the context of the research presented.
A few of the regulatory points surrounding how fatty acids can be liberated from adipose tissue and delivered to the working muscle during exercise are highlighted in Figure 1.1.

**Figure 1.1: The potential regulatory points for fatty acid oxidation.** A cooperative arrangement between many different tissues aims to provide the skeletal muscle with free fatty acids (FFA) to generate adenosine triphosphate (ATP). Many different regulatory points therefore exist which may include, but are not restricted to; 1) The liberation of FFA from adipose tissue, 2) FFA transport across endothelium onto albumin in blood 3) liberation of FFA from low density lipoproteins (LDL) via lipoprotein lipase (LPL) 4) FFA transport from capillary endothelium into interstitium, 5) transport across the sarcolemma, 6) liberation of FFA from intramuscular triacylglycerol (IMTG) stores, 7) activation of FFA to FFA-CoA via acyl-CoA synthetase (ACS) 8) mitochondrial membrane transport and, 9) electron transport chain regulation. FABPc; cytosolic fatty acid binding protein. CPTI; carnitine palmitoyltransferase I. ETC; electron transport chain. TAG; triacylglycerol. [5]
Although these various regulatory points have been identified, the intricate control of these mechanisms has yet to be fully characterized. Therefore, a need to further delineate the regulation of fatty acid oxidation is apparent. Previous work has primarily focused on plasma membrane [6] and mitochondrial transport of fatty acids [7,8] as regulatory points for fatty acid oxidation, and these concepts are reviewed below.

1.3 Delivery to the muscle

Upon liberation from adipose tissue, fatty acids must be transported to the skeletal muscle to be oxidized. However, due to the hydrophobic nature of the hydrocarbon chain on the fatty acid, they are insoluble in an aqueous solution. As a result, fatty acids must be bound to circulating proteins such as albumin in order to be transported through the circulation, and therefore only a small percentage of fatty acids exist in a free solution and are metabolically available (free fatty acids; FFA). The concentration of FFA is dependent on the ratio of fatty acid to albumin, and albumin is thought to contain as many as seven sites capable of binding fatty acids [9]. Following the circulatory delivery of FFA to the muscle, FFA cross the endothelium to enter the interstitial space and are then presented to the sarcolemma for transport into the cell.

1.4 Fatty acid transport across sarcolemma

Historically, fatty acid transport across the sarcolemma was thought to be solely regulated by passive diffusion due to the lipid soluble nature of fatty acids [10]. It was speculated that the hydrophobic acyl chain of a fatty acid could insert into the outer leaflet of the phospholipid bilayer. While the amphipathic nature of the fatty acids would seem prohibitory for traversing the phospholipid bilayer (pKa ~4.5), upon insertion into the membrane, the pKa of the carboxyl group shifts upward to 7~7.6, allowing for protonation and temporarily abolishing its negative charge [10]. This relieves electrochemical constraints and allows the fatty acid to 'flip-
flop' from the outer to the inner leaflet of the bilayer. Once flip-flop has occurred, the proton is lost to the interior of the cell and charge is restored to the carboxyl terminus. Desorption of the fatty acid from the inner leaflet into the cytosol is then facilitated by the soluble cytosolic carrier proteins such as FABPc, which exist in excess within the cytosol [11].

More recently, a considerable body of literature has accumulated to highlight the importance of integral membrane proteins in facilitating the transport of long chain fatty acids (LCFA) across the sarcolemma. The original evidence for a protein mediated process came from experiments reporting that the rate of fatty acid transport into muscle obeyed saturation kinetics similar to those observed in well-studied protein mediated transport systems [12]. Initially, the conclusion that sarcolemmal fatty acid transport may be protein mediated was contested and it was suggested that the saturation was a reflection of the limits of intracellular metabolism rather than protein-mediated transportation events at the plasma membrane. However, subsequent studies have provide strong evidence that fatty acid uptake across the plasma membrane is indeed a saturable process and occurs independent of intracellular metabolism [12-14]. In particular, studies using a variety of inhibitors including proteases such as trypsin and protonase suggested that fatty acid transport was not just a passive, mass action process, but alternatively, involved membrane proteins. It is now recognized that a number of fatty acid transport proteins exist within muscle including; plasma membrane fatty acid binding protein (FABPpm), the fatty acid translocase protein (FATP) family and FAT/CD36 [15].

Independent overexpression of these various fatty acid transport proteins into mature rodent skeletal muscle has suggested that FAT/CD36, along with FATP4, possess the highest sarcolemmal fatty acid transport capacity [16]. While less is currently known concerning the regulation of FATP4, considerable additional evidence indicates that FAT/CD36 is a key
sarcolemmal fatty acid transporter as mechanistic studies utilizing either an inhibitor of FAT/CD36 (sulfo-N-succinimidy oleate (SSO)) or ablation of FAT/CD36 have reported significant inhibition of fatty acid transport [17,18]. Despite these observations, the method by which FAT/CD36 facilitates the transport of LCFA across the sarcolemmal membrane is unknown, as FAT/CD36 does not create a pore akin to traditional transport proteins [19]. Therefore, based on the amino acid sequence of FAT/CD36, it has been proposed that FAT/CD36 contains a hairpin loop that projects into the interstitial space to interact with LCFA transported by albumin. FAT/CD36 then facilitates LCFA delivery and insertion into the outer leaflet of the sarcolemma. This process has been proposed to increase rates of ‘flip-flop’ across the membrane and ultimately rates of fatty acid transport [19]. Given this proposed mechanism, the functional effect of FAT/CD36 only occurs when FAT/CD36 is present on the sarcolemma, indicating that the expression of FAT/CD36 at the sarcolemma is paramount for an increase in the rate of fatty acid transport. Comparisons between red and white skeletal muscle highlight the finding that increased sarcolemmal FAT/CD36 increases the maximal transport (Vmax) of palmitate without altering the Km (concentration of substrate that elicits 1/2 Vmax). These data suggest that an increase in sarcolemmal FAT/CD36 will increase the rate at which LCFA are transported into the muscle, an effect that would be particularly advantageous during exercise [13], but may have negative consequences in the absence of metabolic demand and promote intramuscular lipid accumulation [20,21].

In skeletal muscle, FAT/CD36 exists within intracellular pools and can be induced to translocate to the plasma membrane to regulate LCFA uptake [17]. The principle of dynamic regulation of FAT/CD36 to promote LCFA uptake parallels many facets of GLUT4 translocation to promote glucose uptake. The concept of cellular dynamics to facilitate substrate uptake is
governed by signalling events related to a given stimulus. In a metabolic challenge such as feeding, the insulin signalling cascade has been implicated in the translocation of FAT/CD36 to the sarcolemma as treatments with inhibitors of the insulin signalling cascade (PI3K inhibitors wortmannin and LY294002) ablate insulin-induced sarcolemmal FAT/CD36 translocation [17,22]. Cell culture studies have also indicated a required role for atypical PKC-ζ regarding insulin-stimulated FA transport [23]. In addition to a feeding stimulus, in an energy demanding situation such as exercise, exercise signalling cascades have been implicated in promoting FAT/CD36 translocation to the sarcolemma including AMPK [24], calcium-calmodulin kinase kinase (CaMKK) [25] and extra cellular signalling receptor kinase (ERK1/2) [26]. Therefore, sarcolemmal LCFA transport appears to be a regulated step for LCFA oxidation [15]; however, LCFA transport across the mitochondrial membranes has also been identified as a key regulator of LCFA oxidation.

1.5 Classical regulation of mitochondrial fatty acid transport: Carnitine palmitoyltransferase I

In addition to fatty acid transport across the sarcolemma, the transport of fatty acids into the mitochondria represents a regulated step for LCFA oxidation (Figure 1.1). Classically, this mitochondrial membrane transport process involves i) the activation of a LCFA to an acyl-CoA moiety by acyl-CoA synthetase (ACS) ii) the conversion of acyl-CoA to fatty acylcarnitine via carnitine palmitoyltransferase I (CPT-I), iii) transport of acylcarnitine across the outer and inner mitochondrial membrane via carnitine acylcarnitine translocase (CACT) and iv) the re-conversion of acyl-CoA from acylcarnitine within the mitochondrial matrix via CPT-II [8,27] (Figure 1.2).
**Figure 1.2: Carnitine mediated transport of long chain fatty acids into the mitochondrial matrix.** Mitochondrial membrane transport involves: 1) the activation of a LCFA to an acyl-CoA moiety by acyl-CoA synthetase (ACS), 2) the conversion of acyl-CoA to fatty acyl-carnitine via carnitine palmitoyltransferase I (CPT-I), 3) transport of palmitoylcarnitine into the mitochondrial matrix by carnitine acylcarnitine translocase (CACT), and, iv) the re-conversion of palmitoyl-CoA from palmitoylcarnitine within the mitochondrial matrix via CPT-II. AMPK; adenosine monophosphate-activated protein kinase; LCFA; long chain fatty acid, OMM; outer mitochondrial membrane, IMM; inner mitochondrial membrane, ACC; acetyl-CoA carboxylase, MCD; malonyl-CoA decarboxylase

As far back as 1966 [28], the CPT-I reaction has been viewed as the rate-limiting step for mitochondrial LCFA oxidation:

\[
CPT-I \\
\text{LCFA-CoA} + \text{L-carnitine} \rightarrow \text{LCFAcarnitine} + \text{CoA}
\]

Over the past ~50-60 years a body of literature investigating the remarkable features of CPT-I has been produced, and a few of the salient points are briefly reviewed below.
There are two isoforms of CPT-I, a liver isoform (CPT-IA) and a muscle isoform (CPT-IB) which are 60% homologous [29]. The classical work has primarily been done with liver mitochondria as it is much easier and less time-consuming to isolate. However, marked differences between these two isoforms exist. Importantly, for the purpose of this thesis which is focused on muscle (i.e. CPT-IB), the muscle CPT-I isoform is more sensitive to the inhibitory effects of malonyl-CoA (M-CoA) (rat skeletal muscle M-CoA content ~0.7-1.6 μM, M-CoA IC$_{50}$ = 0.034 μM) compared to liver tissue (rat liver M-CoA content ~1.7-7.5 μM, M-CoA IC$_{50}$ = 2.7 μM) [30].

The primary role of M-CoA within skeletal muscle has been linked to its ability to inhibit CPT-I activity and therefore regulate fuel selection. The mechanism by which M-CoA inhibits CPT-I is related to its ability to inhibit L-carnitine binding to CPT-I as the Km for L-carnitine increases in the presence of M-CoA [31]. Current models of CPT-I propose a hairpin structure, with both N and C termini located in the cytosol [32] and the interaction of the N and C termini is essential for modulating and preserving M-CoA sensitivity [33,34]. Additionally, the N-terminal regulatory domain of the liver isoform of CPT-I has been shown to switch between inhibitory and noninhibitory structural states [35] and structure-based site-directed mutagenesis of CPT-IA suggest that this ratio between structural states dictates M-CoA sensitivity.

M-CoA is formed by carboxylating acetyl-CoA via acetyl-CoA carboxylase (ACC) and can be broken down by malonyl-CoA decarboxylase (MCD). The regulation of M-CoA content, and therefore the inhibition of CPT-I activity, has been traditionally related to the interactions of M-CoA, ACC and AMPK [36] (Figure 1.2). More specifically, CPT-I activity can be inhibited by the presence of M-CoA and metabolic stimuli that increase mitochondrial LCFA oxidation (eg. exercise), activate signalling pathways (AMPK) to phosphorylate and inhibit the enzyme.
responsible for the production of M-CoA (ACC). In other words, metabolic challenges that activate AMPK are thought to decrease M-CoA levels and “release the brake” on CPT-I to promote mitochondrial LCFA transport and oxidation. Importantly, this mechanism of action indicates that a reduction in M-CoA content must be present to observe and change in LCFA oxidation. In support of this working model, following 30 minutes of exercise in rat skeletal muscle, M-CoA content decreased from 1.66 ±0.17 to 0.60±0.05 nmol/g [37]. The decrease in M-CoA with exercise (potentially through AMPK activation) has been repeated numerous times in animal models [36,38-40]. However, upon examination of human skeletal muscle following 70 minutes of low-moderate intensity exercise, no significant decrease in M-CoA was observed (rest = 1.53±0.18 μmol/kg dm, exercise = 1.22±0.15 μmol/kg dm) despite a robust increase in fatty acid oxidation [40]. In a subsequent study in humans, following 10 minutes of exercise at 35%, 65% and 90% VO₂max, M-CoA content was unchanged despite large variations in rates of fatty acid oxidation [41]. The take home point of these findings has been repeated by two independent groups [42,43] suggesting that during exercise in human skeletal muscle, there is more complexity surrounding CPT-I regulation than simply M-CoA content. In an attempt to explain the disparity within the literature surrounding human skeletal muscle and exercise-induced increases in fatty acid oxidation, Dr. Lawrence Spriet’s group examined the impact of classical exercise regulators of exercise metabolism (i.e. calcium, free AMP, ADP, and Pi) and found that none of these conventional exercise regulators had an effect on M-CoA inhibition of CPT-I or CPT-I activity [44]. Clearly, additional research is warranted regarding exercise-induced increases in fatty acid oxidation and skeletal muscle M-CoA content.
1.6 Non malonyl-CoA content regulatory mechanisms for CPT-I

In addition to the disparity within the exercise studies described above, a number of other discrepancies exist regarding M-CoA content and CPT-I activity. Firstly, in skeletal muscle, the reported M-CoA IC$_{50}$ values in isolated mitochondria display a vast (70 fold) range (~0.025-0.49 μM) [30,45,46]. These IC$_{50}$ values are also appreciably lower than reported resting M-CoA content (0.7-2 μM) [30,47-50] which would suggest that CPT-I activity and rates of LCFA oxidation should be negligible at rest. As such, the published IC$_{50}$ values are inconsistent with the well characterized reliance on LCFA oxidation at rest in vivo [51,52]. Furthermore, in T2DM, the role of M-CoA in regulating mitochondrial LCFA entry has shown disparate findings as M-CoA levels are elevated in the skeletal muscle of type II diabetic humans [53] and rats [54,55], yet LCFA entry into the mitochondria is increased in both species as the acylcarnitine profile indicates mitochondrial LCFA overload [56,57]. These conflicting data do not necessarily indicate that the AMPK/ACC/M-CoA/CPT-I axis is unimportant, but more likely highlights our lack of knowledge regarding the elegant workings of the cell and that other regulatory mechanism are at play.

To explain these discrepancies, investigations regarding non M-CoA content mechanisms of mitochondrial LCFA oxidation have been proposed and include: 1) free L-carnitine content (section 1.6.1), 2) change in sensitivity of CPT-I to M-CoA (section 1.6.2), 3) direct modulation of CPT-I activity (section 1.6.3) and 4) accessory proteins to CPT-I (section 1.7).

1.6.1 L-carnitine in the regulation of fatty acid oxidation

L-carnitine is a required substrate for the CPT-I reaction and as a result it has been hypothesized that L-carnitine supplementation will lead to an increase in skeletal muscle free carnitine and a subsequently promote increased LCFA oxidation [43].
Acetyl-CoA is a product of both the PDH reaction and beta oxidation and has been shown to inhibit the PDH complex at rest [58,59]. In times of increased substrate flux through carbohydrate and fat catabolism (e.g., exercise and overnutrition), acetyl-CoA production can outstrip the rate at which the tricarboxylic acid (TCA) cycle can utilize acetyl-CoA. The enzyme carnitine acetyl transferase (CrAT) catalyzes a reaction whereby carnitine is added and CoA is removed from the acetyl moiety to buffer the acetyl-CoA oversupply in these instances [2,60,61]:

\[
\text{CrAT} \\
\text{Acetyl-CoA + carnitine} \rightarrow \text{acetylcarnitine} + \text{CoA}
\]

In effect, this reaction utilizes the free carnitine pool within the mitochondrial matrix to remove the inhibitory acetyl-CoA. However, the ability of acetyl-CoA to inhibit PDH does not appear to be a powerful player during exercise as acetyl-CoA increases with exercise at the same time that flux through PDH increases. Nonetheless, it has been suggested that the resulting decrease in available carnitine due to the CrAT reaction decreases the rate at which CPT-I can promote fatty acid entry into the mitochondrial matrix. Therefore, under special circumstances, free carnitine may limit mitochondrial LCFA oxidation [62-64]. In the past, it has been difficult to study this concept directly as it is difficult to alter the free carnitine content within muscle due to the low bioavailability of orally supplemented L-carnitine [65], and the tight regulation of L-carnitine uptake at the level of the sarcolemma [66]. However, following infusion of L-carnitine (with insulin) [67] or high dose oral supplementation (4g/day for 24 weeks [68]), levels of muscle free carnitine have been successfully elevated (15-21%). In a recent L-carnitine supplementation trial [68] (in combination with 160g of carbohydrate), the L-carnitine supplemented group showed 55% less muscle glycogen use and 31% less PDH activation after 30 minutes of exercise at 50% VO₂max. However, there were no RER measurements taken to show alterations in whole body...
fuel selection. Nonetheless, these data suggest that increasing skeletal muscle free carnitine promotes LCFA oxidation and subsequent glycogen sparing, adding credence to the hypothesis that L-carnitine availability can regulate CPT-I flux.

In the context of T2DM, the physiological impact of L-carnitine supplementation has also been examined in human type II diabetic subjects [69]. In support of a mechanistic role for L-carnitine, it was observed that L-carnitine supplementation improved the type II diabetic condition (improved HOMA-IR index) and it was hypothesized that this beneficial effect was due to the diversion of fatty acid derived acetyl-CoA out of the mitochondria through the CrAT reaction, which permitted carbohydrate oxidation and subsequent metabolic flexibility [69]. In support of this hypothesis, both acetylcarnitine levels and pyruvate oxidation were elevated in the muscle following L-carnitine supplementation [69]. However, muscle content of free carnitine was not reported.

Two mechanisms therefore are postulated to exist regarding the impact of L-carnitine supplementation on mitochondrial LCFA oxidation independent of M-CoA content; 1) promote LCFA oxidation and spare CHO during exercise and 2) divert excess substrate away from mitochondria in the overfed state.

1.6.2 Changing CPT-I sensitivity to M-CoA

In 1984 Dr. Denis McGarry’s group showed that increasing the pH of the incubation medium decreased the effectiveness of M-CoA inhibition of CPT-I in rat liver mitochondria [70]. More recently in humans, following 30 minutes and 2 hours of exercise, the sensitivity of CPT-I to M-CoA inhibition decreased when compared to resting values [46]. This exercise study in humans suggests that exercise signalling cascades may play a role in M-CoA inhibition kinetics. Indeed, incubation of skeletal muscle with protein kinase A (PKA) causes phosphorylation of
CPT-IB and decreased M-CoA sensitivity (matching [46]) (69). In contrast, incubation with CAMKII caused phosphorylation of CPT-IB but increased the sensitivity of CPT-I to M-CoA [71]. Regardless of these divergent findings, these data suggest that exercise signalling cascades can induce a change in the M-CoA inhibition kinetics of CPT-I.

Additionally, previous work from both McGarry’s group [72] and Dr. Victor Zammit’s group [73], observed that increasing palmitoyl-CoA (P-CoA) concentrations suppresses the inhibitory effect of M-CoA on CPT-I activity. These investigations concluded that P-CoA can decrease the binding affinity of M-CoA to CPT-I and lower the sensitivity of CPT-I to M-CoA inhibition. However, these studies were completed with isolated mitochondria which may contain membrane topology disruption [74], and this effect of P-CoA may be a by-product of the isolation procedure.

1.6.3 Directly altering CPT-I activity

In addition to altering pH to change M-CoA inhibition kinetics, it has been found that lowering human skeletal muscle pH from 7 to 6.8 decreases CPT-I activity directly (~40%) [44,45]. This effect of pH appears to be physiologically significant as during high intensity exercise, fatty acid oxidation is reduced concomitantly with a lower muscle pH (~6.7) [75]. Moreover, it has been shown that post-translational modifications through peroxynitrate-induced cellular stress can promote glutathiolation of CPT-I and decrease CPT-I activity [71]. Therefore, both pH and post-translational modifications can directly alter CPT-I activity independent of a change in M-CoA content.

1.7 Accessory proteins to CPT-I: VDAC, FATP1 and FAT/CD36

Recently, two other pioneers of the CPT-I literature, Dr. Janos Kerner and Dr. Charles Hoppel, have shown evidence that CPT-I exists in a hetero-oligomeric outer mitochondrial
membrane (OMM) complex with ACS and voltage dependant anion channel (VDAC) [76]. This complex is designed to facilitate the movement of fatty acids through the OMM to produce acylcarnitine in the intermembrane space. Including ACS as a player in mitochondrial LCFA oxidation goes against some of the classical work as the ACS reaction rate is ~2 fold higher than that of CPT-I [28]. However, in a muscle cell culture model (L6E9) over-expressing fatty acid transport protein 1 (FATP1), mitochondrial ACS activity and mitochondrial LCFA oxidation rates were both increased ~70%, highlighting the potential impact of altering ACS function with respect to mitochondrial LCFA oxidation rates [77].

Interestingly, in addition to its role at the sarcolemma, FAT/CD36 has also been found on the mitochondrial membranes of heart, liver and skeletal muscle [78,79], where it co-immunoprecipitates with CPT-I [78,80,81]. Therefore, FAT/CD36 has been hypothesized to influence mitochondrial fatty acid transport and oxidation [20,77,80-84].

Akin to exercise inducing FAT/CD36 translocation to the sarcolemma, FAT/CD36 has been shown to accumulate on mitochondrial membranes in response to exercise, potentially creating another level of regulation for LCFA oxidation. Specifically, in rodents, following 30 minutes of contraction [78,84], and also in human skeletal muscle following 2 hours of moderate intensity cycling exercise [46], FAT/CD36 has been observed to translocate to the mitochondria (Figure 1.3). The observation that FAT/CD36 can be acutely redistributed to the mitochondria during exercise suggests that this protein may be involved in the upregulation of mitochondrial LCFA oxidation. In addition, 6 weeks of exercise training in humans increases mitochondrial FAT/CD36 content [85].
Figure 1.3: Rest to exercise changes in fatty acid translocase (FAT/CD36) localization. Exercise-induced signalling cascades (potentially including calcium, ERK1/2, PKC and AMPK) promote the translocation of intracellular stores of FAT/CD36 to both the sarcolemma and the mitochondria. AMPK; adenosine monophosphate activating protein kinase. ERK; extra-cellular signalling receptor kinase. PKC; protein kinase C. FAO; fatty acid oxidation. [5].

1.7.1 Mitochondrial FAT/CD36 in LCFA oxidation

In FAT/CD36 KO mice, mitochondrial LCFA oxidation rates are reduced ~20% in resting muscle [84]. Considering the majority of mitochondrial LCFA oxidation is still present under resting conditions within FAT/CD36 KO mice, these data indicate that FAT/CD36 is not “essential” for mitochondrial LCFA oxidation but rather plays an accessory role to CPT-I (which is required). Interestingly, the attenuation of mitochondrial LCFA oxidation in FAT/CD36 KO mice is significantly amplified during exercise, highlighting the potential importance of this protein in the normal exercise response [84]. Specifically, while exercise increases rates of mitochondrial LCFA oxidation in WT mice, this response is negated in FAT/CD36 KO mice, exacerbating the difference between genotypes [84]. Additionally, in L6E9 myotubes, transfection of FAT/CD36 significantly increased mitochondrial palmitate oxidation supporting the KO mouse data [77].
In contrast, a separate investigation in FAT/CD36 KO mice did not observe differences in P-CoA (20 μM) and palmitoyl carnitine (40 μM) state III respiration rates in isolated mitochondria [86]. These divergent results [77,84,86] may reflect the substrates selected as it is currently unknown where FAT/CD36 resides on mitochondrial membranes, and selective substrates (including palmitoyl-CoA and palmitoylcarnitine) may have bypassed the regulatory step (if present) exerted by FAT/CD36.

1.7.2 Mitochondrial membrane topology of FAT/CD36

Mitochondrial membranes are organized in such a way that 3 main locales exist. There is the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM) and the contact sites. Contact sites of mitochondrial membranes are the fusions points of the OMM and the IMM. It is has been suggested that CPT-I is prevalent in contact sites [87,88], and as such, contact sites are likely important for facilitating fatty acid transport into the mitochondria for subsequent oxidation [8]. Therefore, if FAT/CD36 is important for mitochondrial LCFA oxidation we would hypothesize that it is located within the contact sites. However, several conflicting reports exist regarding the placement of FAT/CD36 along the mitochondrial membranes. One report depicts FAT/CD36 residing within the contact sites distal to CPT-I [89], and this conclusion was based on the observation that SSO treatment of isolated mitochondria inhibited palmitoylcarnitine oxidation by 92%. Considering palmitoylcarnitine is a product of the CPT-I reaction and palmitoylcarnitine oxidation was inhibited by inhibiting FAT/CD36, the authors concluded that FAT/CD36 must be influencing mitochondrial fatty acid transport downstream of CPT-I, potentially still within the contact sites of the mitochondria. However, SSO has now been shown to inhibit ETC function indicating that this inhibitor is not specific to FAT/CD36 [84,90]. Therefore, the decrease in palmitoylcarnitine oxidation observed in this
study was likely due to the inhibitory effect of SSO on the ETC and not due to inhibition of mitochondrial FAT/CD36 function. Another report which utilized an outer mitochondrial membrane isolation procedure identified FAT/CD36 within the isolated OMM fractions but not within mitochondrial contact sites [79]. Therefore, controversy currently exists concerning where FAT/CD36 is located within the mitochondrial membranes.

1.8 Regulation of mitochondrial fatty acid oxidation downstream of CPT-I

In 1966, it was proposed that CPT-I activity was the rate-limiting step for mitochondrial LCFA oxidation [28]. Interestingly, in the following year, a manuscript was published pointing out that tissue acylcarnitine levels are elevated under conditions known to involve increased rates of fatty acid oxidation (in their example, Alloxan-induced diabetes [91]). The increase in acylcarnitine concentration suggests that the rate-limiting step must be downstream of CPT-I because if CPT-I was rate-limiting there would be no excess substrate to undergo the carnitine acyltransferase (CAT) reaction to produce the acylcarnitines. In support of this hypothesis, during moderate-intensity exercise (55% VO2max) when fatty acid oxidation is elevated, acylcarnitines were increased [92]. Both of these examples indicate that the catabolism of substrate within the metabolic pathways is outpacing the rate at which the mitochondria can utilize the resultant products supplied. Therefore, assuming there are no problems with oxygen supply or within the metabolic pathways, the rate-limiting step appears to be within the mitochondria. As described in section 1.1, the rate of which the mitochondria utilize the energy supply is proposed to be directly proportional to the energy demand, i.e. ATP utilization:ADP supply is 1:1. More recently, additional regulation within mitochondria has been identified.
1.9 Mitochondrial bioenergetics

In 1961, Dr. Peter Mitchell published his ‘chemiosmotic theory of oxidative phosphorylation’ and was awarded the Nobel Prize in chemistry in 1978 [93]. When oxygen is available, cellular metabolic pathways catabolize fatty acids and carbohydrate to provide reducing equivalents (NADH, FADH\textsubscript{2}) carrying pairs of electrons. These electrons are transferred through a series of electron carriers in the electron transport chain (ETC) (complexes I-IV) with O\textsubscript{2} serving as the final electron acceptor at complex IV, ultimately reducing O\textsubscript{2} to 2H\textsubscript{2}O. In three of these electron carrier complexes (I, III and IV), the difference in reducing potential generates enough free energy to drive the translocation of protons (“pump”) from the matrix to the intermembrane space. This creates a proton gradient (deemed “membrane potential”) across the inner membrane, which in effect contains potential energy. Dr. Mitchell’s theory concludes that this potential energy created by the generation of membrane potential is sufficient to drive the synthesis of ATP from ADP and Pi as protons flow back through the ATP synthase complex into the matrix (Figure 1.4). This movement of protons also lowers membrane potential indicating that, in addition to promoting ATP synthesis, the supply of ADP may have significant consequences regarding mitochondrial ROS production, as membrane potential is intricately linked to ROS production [94].
Reducing equivalents (NADH, FADH$_2$) derived from the catabolism of fatty acids (FAT) and carbohydrate (CHO) carry pairs of electrons. The reducing equivalents are reduced and the electrons are transferred through a series of electron carriers in the ETC (complexes I-IV). Molecular oxygen ($O_2$) serves as the final electron acceptor at complex IV, and reduces $O_2$ to $2H_2O$. In complexes I, III and IV, the difference in reduction potential generates enough free energy to drive the translocation of protons from the matrix to the intermembrane space. This creates a proton gradient across the inner membrane which can then be utilized to drive ATP synthesis. NADH; nicotinamide adenine dinucleotide, FADH$_2$; flavin adenine dinucleotide, TCA; tricarboxylic acid cycle, A-CoA; acetyl-CoA, PDH; pyruvate dehydrogenase, ETF; electron transfer flavoprotein, Cyt c; cytochrome c.

1.10 Mitochondrial reactive oxygen species (ROS) production

In the absence of ATP demand (and therefore ADP supply), providing excess reducing equivalents will increase membrane potential and as membrane potential builds, a “back pressure” is created which slows the movement of the electrons through the ETC [95]. Slowing the rate of electron movement through the ETC via high membrane potential is related to an
increased propensity for superoxide production (parent molecule of all ROS) [94]. To explain this increase in risk, it should be noted that at a number of the electron transfer steps within the ETC, electrons are transferred one at a time. Therefore, by slowing down the rate at which the system is moving an unpaired electron, the risk for this unpaired electron to “slip” onto an oxygen molecule to generate superoxide is substantially increased. Superoxide has previously been shown to inhibit aconitase and this effect has been linked to the metabolic syndrome [96]. Additionally, superoxide reacts with metals to produce highly toxic compounds suggesting that preventing the formation of large quantities of superoxide or quenching this reactive compound is imperative [96-98]. We have a number of defence mechanisms to suppress and/or combat an increase in mitochondrial ROS. Firstly, the mitochondria can lower the risk of superoxide production by dissipating membrane potential via “uncoupling.” Uncoupling describes the movement of protons back through the IMM “not coupled” with ATP formation. In other words, rather than supplying ADP to promote proton movement through ATP synthase, the mitochondria simply allow the protons to move through the membrane. A family of uncoupling proteins (UCP1-3) have been identified and literature reviews describing UCP isoforms in detail exist [99,100]. One of the more recent findings surrounding the regulation of UCP is the concept that ROS promotes the activation of the uncoupling function through de-glutathiolation [101,102]. This represents a well designed feedback system to dissipate membrane potential when ROS is elevated. Post-translational modifications induced by an increase in ROS production have been shown to impact not only uncoupling function, but also ETC function. For instance, ROS-induced glutathiolyation of complex I has been observed to lower complex I activity [103], which again may represent a feedback loop to lower membrane potential.
As a defence mechanism to quench superoxide, manganese superoxide dismutase (MnSOD) catalyzes a reaction whereby superoxide is converted into a less toxic hydrogen peroxide (H$_2$O$_2$) and oxygen [104].

\[
\textit{MnSOD} \\
\text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

Due to the highly active nature of MnSOD, we can view H$_2$O$_2$ as a by-product of high membrane potential, which in turn is a barometer of the balance between energy supply and demand. Considering H$_2$O$_2$ can diffuse out of the mitochondria to influence cellular processes (while superoxide cannot), it has been suggested that H$_2$O$_2$ is the signalling mediator of this barometer system [95]. Importantly, mitochondrial H$_2$O$_2$ can be reduced to H$_2$O and O$_2$ by various systems within the mitochondria and the cytosol which include; catalase [105], thioredoxin [106], peroxiredoxin [107] and glutathione [108].

1.11 Skeletal muscle insulin resistance

The prevalence of insulin resistance and T2DM has dramatically increased over the past 50 years and it is now estimated that ~24% of the Canadian population is living with T2DM (Canadian Diabetes Association). T2DM has traditionally developed in adulthood but sadly, an increasing number of children are being diagnosed signifying that this is a very important issue to address.

Fuel oversupply in the absence of energetic demand (overnutrition, overeating, high fat diets, etc) has long been linked to T2DM. As far back as the Franco-Prussian war (1871), a French physician (Dr. Apollinaire Bouchardat) noticed the disappearance of glycosuria in his diabetic patients during the Siege of Paris as food supplies had been cut off (Canadian Diabetes Association). In 1916 Boston pathologist Dr. Elliott Joslin observed that the mortality of his
patients was ~20% lower when simply introducing fasting and an emphasis on regular exercise (Joslin Diabetes Centre). Insulin was not discovered until 1921 however medical professionals had already identified “metabolic balance” as a critical factor within the context of T2DM.

In a healthy individual, blood glucose concentrations are very tightly regulated. Following the ingestion of a meal (containing carbohydrate), insulin is released from the β cells of the pancreas to promote the uptake of glucose into peripheral tissues, including skeletal muscle. Insulin signalling is initiated by insulin binding to its receptor and activating inherent tyrosine kinase activity. Tyrosine phosphorylation of insulin receptor substrate (IRS) leads to recruitment and activation of phosphoinositol 3-kinase (PI3K) to the plasma membrane. The major substrate for PI3K is the membrane lipid phosphatidylinositol-4,5-bisphosphate which is phosphorylated to produce phosphatidylinositol-3,4,5-trisphosphate. An increase in phosphatidylinositol-3,4,5-trisphosphate attracts phosphoinositol-dependent protein kinase-1 (PDK1) which can phosphorylate Akt. Akt can subsequently phosphorylate Akt substrate of 160 kDa (AS160) which relieves the inhibition on GLUT4 translocation to the plasma membrane [109,110] (Figure 1.5). For full review please see [111].
Figure 1.5: Insulin signalling pathway to promote GLUT4 translocation to sarcolemma. Insulin binding to its receptor activates tyrosine phosphorylation activity resulting in phosphorylation of insulin receptor substrate (IRS). This phosphorylation event leads to recruitment and activation of of phosphoinositide 3-kinase (PI3K). Activation of PI3K results in the attraction of phosphoinositide-dependent protein kinase-1 (PDK1) which can phosphorylate Akt/PKB. Akt/PKB subsequently phosphorylates Akt substrate of 160 kDa (AS160) allowing GLUT4 translocation to the sarcolemma. LCFA; long chain fatty acid, IMTG; intramuscular triacylglycerol.

It is estimated that skeletal muscle is responsible for 70-80% of insulin-stimulated glucose uptake [112] and early within the pathogenesis of T2DM, the ability of skeletal muscle to promote glucose uptake in response to insulin is impaired. These points highlight that skeletal muscle represents an important tissue within the context of T2DM [113]. It has also long been known that oversupply of food, especially heavy intake of fatty acids, can induce insulin resistance in skeletal muscle and numerous hypotheses as to the mechanism have been proposed.
[114,115]. Originally, diet-induced insulin resistance was linked to increased intramuscular triacylglyceride (IMTG) content [116], however more recent evidence demonstrates that IMTG content can be elevated in the face of heightened insulin sensitivity [117,118]. Other culprits related to lipid oversupply including diacylglycerol (DAG) and ceramide (deemed reactive lipids) have now been identified as inhibitors of insulin signalling [119-121] (Figure 1.6). Elevated levels of intramuscular DAG have been shown to activate PKC isoforms and subsequently threonine-serine phosphorylate IRS-1 and reduce the efficacy of the insulin signalling cascade [122]. Ceramides can inhibit insulin signalling via two mechanisms; 1) activation of protein phosphatase 2 (PP2) which can dephosphorylate Akt [123,124], and 2) activation of PKCζ which prevents Akt recruitment [125].

Although the data surrounding DAG and ceramides as inhibitors of insulin signalling is relatively strong, controversy still exists surrounding this “reactive lipid hypothesis.” For example, etomoxir blocks the entry of fatty acids into the mitochondria by inhibiting CPT-I and treatment of humans with etomoxir results in an increase in DAG but improved insulin sensitivity, thus divorcing DAG content from insulin resistance [126]. In addition, this hypothesis suggests that reactive lipids are increased in the presence of insulin resistance due to mitochondrial dysfunction and decreased mitochondrial fatty acid oxidation. However, the role of mitochondrial function/dysfunction/content in insulin resistance is one of the more polarizing scientific topics of today. Indeed a number of studies have reported an association between insulin resistance and mitochondrial dysfunction [127-131] while other reports have not observed this association [132-136] indicating that there is considerable controversy surrounding this topic. For example, in BMI-matched human subjects, Mogensen et al (2007) found that state III pyruvate respiration in isolated mitochondria was significantly lower in patients with T2DM.
following normalization to citrate synthase activity [130]. In addition, when examining state III glutamate respiration in permeabilized muscle fibres normalized to citrate synthase activity (or mtDNA), mitochondrial function was found to be repressed in human diabetic subjects compared to BMI-matched controls [129]. However, in direct contrast, Boushel et al (2007) observed that state III glutamate respiration in permeabilized muscle fibres was unchanged between lean control (non-BMI matched) and diabetic human subjects following normalization of respiratory flux to mtDNA content [132]. Differences in methodologies and subject characteristics may explain some of this disparity however, it is quite clear that there is considerable controversy surrounding the mechanisms causing insulin resistance, including those hypotheses related to mitochondrial dysfunction.

1.12 Skeletal muscle insulin resistance and reactive oxygen species

More recently, a hypothesis involving mitochondrial ROS as a causal factor for skeletal muscle insulin resistance has garnered attention. Direct treatment of muscle cells in vitro with H₂O₂ results in reduced insulin-stimulated glucose transport [137]. The mechanism surrounding ROS-induced insulin resistance is not fully understood but is hypothesized to be related to the inactivation of IRS-1 (Figure 1.6). An increase in ROS leads to the activation of serine/threonine kinase signalling cascades and these activated kinases can inhibit IRS activation [138,139]. Specific kinases induced by ROS production include JNK, p38 MAPK and IKKβ [140-142]. Notably, blocking the NfκB pathway has been shown to protect against diet-induced obesity [143]. However, the IRS-1 centric view of ROS-induced insulin resistance is still contentious [144]. Regardless of the potential inherent mechanism, the link between mitochondrial ROS and insulin resistance has been examined using a variety of approaches and support for this proposed relationship is amplifying.
Figure 1.6: Proposed mechanisms of fuel overload to cause insulin resistance.

Fuel overload increases the reactive lipids (diacylglycerol (DAG) and ceramides) and mitochondrial reactive oxygen species (ROS) production. DAG activates PKC isoforms which can threonine/serine phosphorylate insulin receptor substrate 1 (IRS-1) and inhibit IRS-1 activation. An increase in ROS can also inhibit IRS activation by increasing NfκB activation (or other stress kinases) whereas ceramides may inhibit Akt activation. As a result, insulin signalling is repressed in the overfed state and GLUT4 translocation to the sarcolemma is blunted. PI3K; phosphoinositide 3-kinase, PDK1; phosphoinositide-dependent protein kinase-1, NfκB; nuclear factor kappa B, AS160; PKCθ; protein kinase C, LCFA substrate of 160 kDa, LCFA; long chain fatty acid, IMTG; intramuscular triacylglycerol.

1.12.1 Mitochondrial-targeted antioxidants and insulin resistance

Mitochondrial-targeted antioxidants have been designed to scavenge excess superoxide and/or H₂O₂ within the mitochondria to subdue the potential for ROS to negatively affect normal cell function. Two primary strategies have been employed to target small molecule antioxidants
to mitochondria; 1) incorporation of antioxidant moieties into mitochondrial-targeted peptides [145] and 2) conjugation of antioxidant compounds to lipophilic cations [146]. Anderson et al (2009) employed a mitochondrial-targeted peptide with antioxidant function known as SS31 which had been previously shown to localize to the mitochondrial inner membrane [147,148]. In high fat fed rats, following 6 weeks of SS31 supplementation, mitochondrial H$_2$O$_2$ emission rates were 50-60% lower compared to control high fat diet rats and the development of insulin resistance was prevented. In addition, the authors utilized a transgenic mouse model expressing mitochondrial-targeted catalase (MCAT) to isolate the role of H$_2$O$_2$ in diet-induced insulin resistance [147,149]. Following a high fat diet, MCAT mice maintained whole-body and muscle-specific insulin sensitivity comparable to the WT mice on a stock diet suggesting that H$_2$O$_2$ emission from the mitochondria is the primary driver of ROS-induced insulin resistance. In agreement with Anderson et al (2009) a number of other investigations have reported a link between ROS and insulin sensitivity [150-155].

In contrast to these studies, following 16 weeks of high fat feeding in C57Bl/6 mice, supplementation with the a lipophilic cation with antioxidant function improved the redox status of the muscle cell without preventing high fat diet-induced insulin resistance [156]. This report divorces the relationship between the prevention of oxidative stress and the occurrence of skeletal muscle insulin resistance. The difference in the murine model (rat vs. mouse), the difference in the high fat feeding timeline (6 weeks vs. 16 weeks) and/or the difference in the design of the mitochondrial antioxidant could all play a role in this disparity.

1.12.2 Mitochondrial uncoupling and insulin resistance

In 1933, the idea of dissipating membrane potential and “wasting” fuel was examined in humans utilizing a non-selective mitochondrial uncoupler known as 2,4dinitrophenol (DNP). In
humans, 3–5 mg/kg DNP supplementation led to a 20–30% increase in energy expenditure and a pronounced improvement in health [157]. Unfortunately, a by-product of uncoupling is heat generation and by 1938 DNP was removed from the shelves by federal regulation due to large increases in the risk for cardiac events and various other dangerous side effects. More recently, a reemergence of interest in uncouplers to fight diet-induced insulin resistance has surfaced. This may be a result of a rousing agreement that the balance between energy supply and demand is paramount in the governance of insulin resistance and artificially increasing demand clearly holds potential for treatment [158]. Indeed, utilization of uncouplers in cell lines and animal models [153,158], in addition to genetic overexpression of UCP3 [153,158-160] have supported the idea that energy balance is key.

1.13 ADP transport, fatty acid oversupply and mitochondrial ROS

Adenine nucleotide translocase (ANT) is a key component of a supercomplex of proteins known as the “mitochondrial interactosome” [161]. The mitochondrial interactosome is comprised of ATP synthase, phosphate carriers, mitochondrial creatine kinase, VDAC with bound cytoskeleton proteins (specifically β-tubulin) and importantly, ANT. The interaction of these components is responsible for the coupling of cytosolic ATP turnover with ADP supply to the mitochondrial matrix.

Many of the elegant details surrounding ADP transport were characterized by Dr. Martin Klingenberg’s group [162]. In a number of landmark studies back in the 1960s, it was shown that ADP transport, not ATP synthase, drove respiration [163-166], helping initiate an extensive research pursuit regarding mitochondrial to cytosolic ADP-ATP exchange.

ANT is an integral membrane protein of 30 kDa and has an obligatory role in counter-transporting ADP and ATP across the inner mitochondrial membrane [167]. It is the most
abundant protein in the mitochondria and comprises up to 10-15% of mitochondrial membrane protein [168]. Four highly homologous (68-88%) isoforms of ANT have been described in humans (and rats, ANT1-4), which show tissue-specific expression patterns [162,169-171]. ANT1 is mainly expressed in heart and skeletal muscle, ANT2 is expressed in tissues able to undergo proliferation including skeletal muscle whereas ANT3 and 4 are expressed ubiquitously [171-173]. While skeletal muscle expresses both ANT1 and ANT2, ANT2 possesses a higher transport efficiency and has a higher $V_{\text{max}}$ [174,175]. Therefore any change in ANT2 content appears to have significant physiological impacts as previously highlighted in dilated cardiomyopathy [175,176] and in mice which express low levels of ANT2 [177].

With respect to the regulation of ANT activity, numerous studies have observed extensive post-translational regulation of ANT function. Evidence now exists that ANT1 can be phosphorylated on tyrosine 194 which increases ANT activity [178], whereas exposure to oxidative stress causes glutathionylation and decreases ANT activity [179,180]. Interestingly for the purposes of this thesis, LCFA-CoA moieties can directly suppress ANT function [181]. The inhibition of ANT by LCFA-CoA appears to be competitive for ADP as demonstrated by binding studies in isolated mitochondria [182,183]. From a physiological stand point, an increase in LCFA-CoA as seen in the skeletal muscle of type II diabetes [184,185] would result in a depressed ability to transport ADP into the matrix. As highlighted previously in section 1.9, ADP can mitigate ROS formation [186] such that a decrease in ADP transport due to fatty acid oversupply would elevate mitochondrial ROS formation and potentiate the issues regarding the overfed state and promote insulin resistance.
1.14 Summary

Traditional regulatory systems describing mitochondrial fatty acid transport and oxidative phosphorylation have been more recently expanded upon. This expansion is a result of many factors including; the quality of available methods and the general principle of evaluating mechanistic data and placing these data within a physiological context. Although extensive strides have been made, future research is warranted to better characterize mitochondrial fatty acid transport and ADP supply within the context of insulin resistance.
CHAPTER 2:

AIMS OF THESIS
2.1 Specific Objectives

The purpose of this thesis was to examine a selection of regulatory points with skeletal muscle mitochondrial metabolism. This thesis includes two projects surrounding the regulation of skeletal muscle mitochondrial fatty acid transport/oxidation and one project examining skeletal muscle mitochondrial responsiveness to a change in ADP supply within the context of T2DM. A main stake of these projects was the evaluation of mitochondrial respiration to which end the permeabilized muscle fibre technique was utilized.

Study 1:

Utilizing mitochondrial membrane subfractionation techniques and FAT/CD36 KO mice, I aimed to determine if and where FAT/CD36 resided on skeletal muscle mitochondrial membranes and investigate the functional role of mitochondrial FAT/CD36 in this tissue. It was hypothesized that FAT/CD36 would reside on mitochondrial membranes upstream of CPT-I and play a functional role in mitochondrial fatty acid oxidation.

Study 2:

Discrepancies exist pertaining to the role of malonyl-CoA inhibition of CPT-I activity. As such, I re-examined M-CoA inhibition kinetics of CPT-I utilizing permeabilized muscle fibres in the presence of varying concentrations of LCFA-CoA levels. I hypothesized that LCFA-CoA would attenuate M-CoA inhibition kinetics.
Study 3:

Mitochondrial derived reactive oxygen species and mitochondrial function have been implicated in the etiology of T2DM. Therefore, I examined these two parameters in the ZDF rat model of T2DM and hypothesized that these two parameters would be intrinsically associated.
CHAPTER 3:

FAT/CD36 IS LOCATED ON THE OUTER MITOCHONDRIAL MEMBRANE, UPSTREAM OF LONG CHAIN ACYL-COA SYNTHETASE, AND REGULATES PALMITATE OXIDATION

Presented as published:

3.1 Abstract

FAT/CD36, a plasma membrane fatty-acid transport protein, has been found on mitochondrial membranes, however it remains unclear where FAT/CD36 resides on this organelle or its functional role within mitochondria. We demonstrate, using several different approaches, that in skeletal muscle FAT/CD36 resides on the outer mitochondrial membrane (OMM). To determine the functional role of mitochondrial FAT/CD36 in this tissue we determined oxygen consumption rates in permeabilized muscle fibers in wild-type and FAT/CD36 KO mice using a variety of substrates. Despite comparable muscle mitochondrial content, as assessed by unaltered mtDNA, citrate synthase, β-hydroxyacyl-CoA dehydrogenase, cytochrome c oxidase complex IV and respiratory capacities (maximal OXPHOS respiration) in WT and KO mice, palmitate supported respiration was 34% lower in KO animals. In contrast, palmitoyl-CoA supported respiration was unchanged. These data indicate that FAT/CD36 is key for palmitate-supported respiration. Therefore, we propose a working model of mitochondrial fatty-acid transport, in which FAT/CD36 is positioned on the OMM, upstream of long chain acyl-CoA synthetase, thereby contributing to the regulation of mitochondrial fatty acid transport. We further support this model by providing evidence that FAT/CD36 is not located in mitochondrial contact sites, and therefore does not directly interact with carnitine palmitoyltransferase-I as original proposed.
3.2 Introduction

Fatty acid translocase, (FAT)/CD36 is a class B scavenger receptor with strong homology to human CD36 (glycoprotein IV) and has been shown to function as a plasma membrane long chain fatty acid (LCFA) transporter in various tissues including skeletal muscle, heart, liver, adipose tissue and the small intestine [13,80,187-191]. Additionally, in heart and skeletal muscle, intracellular pools of FAT/CD36 exist which can be induced to translocate to the plasma membrane to regulate LCFA uptake through the activation of several signalling cascades, including AMP activated protein kinase (AMPK) [24], calcium-calmodulin kinase kinase (CaMKK) [25], extra cellular signalling receptor kinase (ERK1/2) [26], and insulin [17].

FAT/CD36 has also been found on the mitochondrial membranes of heart, liver and skeletal muscle [78,79], where it co-immunoprecipitates with carnitine palmitoyltransferase I (CPT-I) [78,80,81] and has been hypothesized to influence mitochondrial fatty acid transport and oxidation [20,77,80-84]. However, the functional role of FAT/CD36 in mitochondrial fatty acid oxidation has remained controversial. For example, studies examining the function of mitochondria isolated from FAT/CD36 KO mice have yielded conflicting results with reductions in palmitate oxidation reported in one study [84], and in contrast, unaltered palmitoyl-CoA (P-CoA; 20μM) and palmitoyl carnitine (40μM) state III respiration rates in another [86]. These divergent results may reflect the substrates selected, as it is currently unknown where FAT/CD36 resides on mitochondrial membranes, and selective substrates (including palmitoyl-CoA and palmitoylcarnitine) may have bypassed the regulatory step exerted by FAT/CD36.

In addition to the equivocal reports examining mitochondrial function in FAT/CD36 KO mice, a recent investigation has also failed to detect FAT/CD36 on mitochondrial membranes utilizing both immunoblotting and fluorescence immunocytochemistry techniques [192]. Indeed,
contamination of other cell fractions has been an issue when isolating skeletal muscle mitochondria. These recent data in combination with the ill-defined functional role of FAT/CD36 on mitochondrial membranes raises the possibility that FAT/CD36 does not reside on mitochondrial membranes, and previous reports may have observed contamination from regions within the cell that contain FAT/CD36, in particular, the plasma membrane.

Since there is controversy as to whether FAT/CD36 is a) located on mitochondrial membranes and b) whether this protein plays a functional role in contributing to the regulation of mitochondrial fatty acid oxidation, we have 1) isolated mitochondria, purified them, and further sub-fractionated the mitochondria to determine the location of FAT/CD36 within this organelle. In addition, we have 2) determined respiration rates of mitochondria in the presence of various substrates (including palmitate) in permeabilized muscle fibres from wild type (WT) and FAT/CD36 KO mice. These studies provide evidence that i) FAT/CD36 is located on the outer mitochondrial membrane (OMM) upstream of long chain acyl-CoA synthetase (ACS) and ii) that FAT/CD36 is involved in regulating mitochondrial fatty acid oxidation.

3.3 Experimental

3.3.1 Animals

Female rats, (8-10wks of age and weighing ~175g) were housed in a climate- and temperature-controlled room, on a 12:12-h light-dark cycle, with standard chow and water provided ad libitum. FAT/CD36 KO mice were obtained from Dr. Maria Febbraio (Cleveland Clinic, Cleveland OH) [189]. Breeding of WT and KO mice was conducted on site at the University of Guelph. Age matched (8-10 wks) female WT (20.6±0.8g) and KO (21.2±1.1g) mice were used in this study. This study was approved by the University of Guelph Animal Care Committee.
3.3.2 Isolation of highly purified mitochondria, cytosolic compartment, plasma membrane and homogenate from skeletal muscle

The plasma membrane and muscle homogenate samples were prepared as previously described [135,193]. Differential centrifugation was used to obtain intermyofibrillar mitochondrial fractions and all procedures were identical to those that we have previously published [84]. The cytosolic fraction was obtained by generating plasma membrane vesicles followed by 3 freeze-thaw cycles (to fractionate the plasma membrane from the cytosol) and then high speed centrifugation to separate out the respective fractions.

3.3.3 Outer mitochondrial membrane digestion

To determine the subcellular location of FAT/CD36 on mitochondrial membranes, the OMM of mitochondria was selectively digested by incubating with, or without, digitonin. Mitochondria were exposed to varying concentrations of digitonin for 15 minutes (0, 25, 50, 100, 200, 400 μg/mg mitochondria), and thereafter centrifuged at 10,000g, resuspended, and markers of inner mitochondrial membrane (IMM) and OMM via Western blotting (described below) were quantified. We compared the dose response results as the ratio of FAT/CD36:PDH to ensure that the digitonin treatment was selectively removing the OMM and not degrading the IMM. The FAT/CD36:PDH ratio for the respective digitonin concentrations used were as follows; 1.00, 0.82, 0.64, 0.54, 0.45, 0.35. These results indicated that as we increased the concentration of digitonin we concentrated the inner mitochondrial membrane (IMM) proteins within the sample, and therefore the ratio between FAT/CD36 and PDH decreased, as expected. The digitonin concentration of 100μg/mg mitochondria was used for subsequent experiments and is identical to that previously employed elsewhere [194]. In addition, we determined if proteinase K exposure altered either OMM or IMM proteins. For these purposes, mitochondria were incubated for 15
minutes at varying concentrations of proteinase K (ranging from 3.125ng/ml up to 50ng/ml), followed by the addition of 2mM phenylmethylsulfonyl fluoride as described previously [195]. In contrast to digitonin, across all concentrations examined, proteinase K did not alter either FAT/CD36 or PDH contents, and therefore the ratio of FAT/CD36:PDH was constant. We therefore did not perform subsequent experiments in the presence of proteinase K.

3.3.4 Outer mitochondrial membrane and contact site isolation

Following isolation of mitochondria (as described above), separation of OMM and contact sites was achieved via a swell/shrink method followed by further differential centrifugation [196]. Separation of OMM and contact sites was performed via discontinuous sucrose gradient centrifugation. The sucrose gradient was 1.2 ml of 51.3%, 37.7%, and 25.2% sucrose in 20mM potassium phosphate. Addition of 1ml of crude membrane fraction was loaded and centrifuged for 60 minutes at 4°C and 121,000g. The membranous material at the interface between the 25.2%/37.7% (purified OMM) and the 37.7%/51.3% (contact site fraction) phases were collected and diluted by adding 10ml 20mM potassium phosphate. The diluted fractions were then centrifuged for 1hr at 184,000g and resuspended in 100µl 20 mM potassium phosphate for subsequent analysis. To obtain sufficient mitochondria, skeletal muscle from 10 animals was pooled for each independent experiment.

3.3.5 Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using the Pierce Classic IP Kit (Thermo Scientific, Nepean, Ontario, Canada) according to manufacturer’s directions. Briefly, 200 µg of intact or fractionated mitochondrial protein was incubated with 4 µg of FAT/CD36 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight, followed by a 1 hour incubation at 4°C in the presence of protein L agarose beads (Santa Cruz Biotechnology).
Following centrifugation at 1000g for 1 min, the elutant was kept as a control for subsequent Western blotting analyses. The immune complex was then recovered by the addition of SDS loading buffer and boiling for 5 minutes.

3.3.6 Mitochondrial enzymatic activities

Muscle samples (~10 mg) were homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer and used for the measurements of citrate synthase (CS) and β–hydroxyacyl-CoA dehydrogenase (β-HAD) [197,198]. Total CS activity was assayed spectrophotometrically at 37°C at 412 nm, and β-HAD activity was measured at 340 nm (37°C).

3.3.7 Transmission electron microscopy

TEM of muscle was performed as we have previously reported [135]. Briefly, samples were rapidly immersed in a fixing buffer, incubated overnight, and thereafter sections (100 nm) were cut and laid onto 200 mesh formvar/carbon copper grids and then stained with 2% uranyl acetate and Reynold's lead citrate. Samples were viewed on a Philips CM 10 transmission electron microscope (TEM) at 80 kV, and images were obtained with an Olympus/SIS Morada CCD camera using the Olympus/SIS iTEM software.

3.3.8 Mitochondrial DNA

mtDNA copy number was determined using real-time PCR, as previously reported [135] using the following primers: NADH dehydrogenase subunit 5 forward, 5’-GCAGCCACAGGAAAATCCG-3’ and reverse, 5’-GTAGGGCAGAGACGGGAGTTG-3’; and the solute carrier family 16 member 1 forward, 5’-TAGCTGGATCCCTGATGCGA-3’ and reverse, 5’-GCATCAGACTTCCAGCTTCC-3’.
3.3.9 Western Blotting

Mitochondria and mitochondrial membrane protein samples were separated using SDS-PAGE as previously described [84]. The monoclonal antibody MO25 [199] was used to detect FAT/CD36. Commercially available antibodies were used to detect Bcl-2 (Santa Cruz Biotechnology), cytochrome c oxidase complex IV (COXIV; Invitrogen), complexes I, II, IV, and V of the electron transport chain (Mitosciences), CPT-I (Alpha Diagnostic), transferrin (Chemicon), calnexin (Sigma), GLUT-4 (Chemicon), Cav-3 (BD Biosciences), PDHE1α (Invitrogen), βHAD (Abcam) and SERCA2 (Sigma). MCT1, and 4 antibodies were gifts from Dr. Hideo Hatta (University of Tokyo, Tokyo, Japan). Signals were detected using enhanced chemiluminesence (Perkin Elmer Life Science, Boston, MA, USA) and were subsequently quantified by densitometry by Gene Tool as per the manufacturer's instructions (SynGene, ChemiGenius2, Perkin-Elmer).

3.3.10 Preparation of permeabilized fibres

The preparation of saponin permeabilized fibres was based on previously published methods [147]. Following dissection of red gastrocnemius, fibre bundles (~2mg) were separated with fine forceps under a binocular dissecting microscope in BIOPS buffer containing, CaK₂EGTA (2.77mM), K₂EGTA (7.23mM), Na₂ATP (5.77mM), MgCl₂·6H₂O (6.56mM), Taurine (20mM), Na₃Phosphocreatine (15mM), Imidazole (20mM), Dithiothreitol (0.5mM) and MES (50mM). Following separation, fibre bundles were placed in BIOPS containing 50µg/ml saponin, agitated for 30min and then washed in respiration buffer (MIRO5) containing EGTA (0.5mM), MgCl₂·6H₂O (3mM), K-lactobionate (60mM), Taurine (20mM), KH₂PO₄ (10mM), HEPES (20mM), Sucrose (110mM) and fatty acid free BSA (1g/L). Fibres were left in cold MIRO5 until respiration analysis.
3.3.11 Mitochondrial respiration

Mitochondrial respiration was measured in permeabilized fibers from WT and FAT/CD36 by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria) at 30° and room air saturation. Separate fibres from the same animal were used to determine (in duplicate); 1) palmitate supported respiration, 2) P-CoA supported respiration, or 3) ETC function using a modified substrate-uncoupler-inhibitor titration (SUIT) protocol as recommend by Oroboros. To measure palmitate supported respiration, MIRO5 + 1mM ATP + 5mM ADP, 2mM malate, 2mM L-carnitine + 1mM CoA was used as the respiration medium. Palmitate was then titrated automatically (Tip2K) every 5 minutes to generate a kinetic curve. To measure P-CoA supported respiration, MIRO5 + 1mM ATP + 5mM ADP, 2mM malate, and 2mM L-carnitine was used as the respiration medium. Pilot experiments titrating P-CoA (10µM) every 5 minutes determined 70µM was optimal and was the concentration used for subsequent analysis. To measure complex I and II supported respirations, a modified SUIT method was utilized. MIRO5 was used as the respiration medium. Malate (2mM) + glutamate (10mM) were added to chamber and complex I supported respiration was determined following the addition of 5mM ADP. Succinate (10mM) was then added to determine Complex I and II supported respiration. Subsequent titration of 1µM carbonylcyanide-p-trifluoromethoxy phenylhydrazone (FCCP) was then performed to estimate maximal ETC activity. Antimycin A (2.5µM) was added to inhibit complex III for measurement of residual oxygen consumption. Cytochrome c was added following all experiments (Palmitate, P-CoA and SUIT) to ensure OMM integrity.
3.4 Results

3.4.1 Presence of FAT/CD36 on mitochondria in resting skeletal muscle

A recent report has suggested that the presence of FAT/CD36 on mitochondrial membranes is due to contamination [192]. Given the current controversy regarding the presence of FAT/CD36 on mitochondrial membranes, we first compared selected marker proteins of different subcellular compartments to our highly purified mitochondria (Figure 3.1).

Figure 3.1: Characterization of isolated mitochondria. Mitochondria were isolated from Sprague-Dawley rats and were characterized via Western blotting. 7.5 μg of mitochondrial protein were loaded, while 40 μg of muscle homogenate, 10μg of plasma membrane (PM) and 10μg of the cytosolic fraction was loaded for comparison. The enrichment of mitochondrial proteins (PDHE1α, subunits I and II, ATP synthase) and the absence of non-mitochondrial proteins (Caveolin 3, MCT-1, MCT-4, SERCA 2, calnexin, GLUT-4 and transferrin) suggest FAT/CD36 resides on mitochondrial membranes.
Comparison of plasma membrane and highly purified mitochondrial fractions revealed the expected subcellular localization of sarcolemmal proteins to the plasma membrane and mitochondrial proteins to the isolated mitochondrial samples, validating our fractionation procedures (Figure 3.1 left panel). Specifically, in highly purified mitochondrial samples, the absence of caveolin 3 (Cav-3), monocarboxylate transporters (MCT) 1 and 4 and GLUT 4 suggest a lack of sarcolemmal contamination, while the absence of SERCA-2, transferrin and calnexin suggest a lack of sarcoplasmic and endoplasmic reticulum contaminations, respectively (Figure 3.1 right panel). In contrast, the mitochondrial proteins; PDHE1α, subunits of complex II and IV of the electron transport chains, as well as ATP synthase subunit alpha, were all highly expressed and enriched in our isolated mitochondria despite using a much lower protein concentration (homogenate - 40 μg; mitochondria - 7.5 μg). Collectively, these data suggest our isolation yielded highly purified mitochondria devoid of contamination by other subcellular components. FAT/CD36 was highly expressed in these mitochondrial samples, indicating that FAT/CD36 does reside on mitochondrial membranes in skeletal muscle.

3.4.2 Location of FAT/CD36 on mitochondrial membrane

Since FAT/CD36 was present in the mitochondrial fraction we investigated the location of FAT/CD36 on rat mitochondrial membranes via two separate experiments. Following OMM specific digestion by digitonin, FAT/CD36 and Bcl-2 (protein located on the OMM [200]) were both reduced (~20%, p<0.05) (Figure 3.2A and B). In contrast, PDHE1α (IMM specific protein) and β-HAD (mitochondrial matrix protein) were increased (~20 and 30%, respectively) following digitonin digestion (Figure 3.2C and D).
Figure 3.2: Effect of outer mitochondrial membrane (OMM) digestion on FAT/CD36, Bcl-2, PDHE1α and β-HAD content. Equal protein amounts (7.5 μg) of A) FAT/CD36, B) Bcl-2, C) PDHE1α and D) β-HAD were loaded. Bcl-2 is known to reside on the OMM, while PDHE1α is known to reside on the inner mitochondrial membrane and β-HAD within the mitochondrial matrix. FAT/CD36 and Bcl-2 were significantly lower while PDHE1α and β-HAD were significantly higher following digitonin (Dig.) treatment. A Ponceau stain of the membrane was performed as a loading control. Altogether, these data suggest that FAT/CD36 resides on the OMM N=4 for all independent experiments, and data is expressed as mean ± SEM, *p<0.05 Con. vs. Dig.

To ensure constant loading, FAT/CD36 and PDHE1α were run on the same gel and the PVDF membrane was subsequently cut, enabling the detection of two proteins from one gel. In addition, Ponceau staining revealed equal loading of sample allowing us to conclude that protein loading is not a potential confounding error. Collectively, these results indicate that the digitonin digestion was successful in concentrating the IMM by selectively removing a portion of the OMM, therefore suggesting that FAT/CD36 is located on the OMM.

This was also confirmed following an extensive sub-fractionation procedure to isolate the OMM (see Experimental). Specifically, FAT/CD36 and Bcl-2 were both enriched in the OMM fraction (~50%, p<0.05) (Figure 3.3).
Figure 3.3: Expression of outer (OMM) and inner (IMM) mitochondrial membrane proteins following OMM isolation. Equal protein amounts (7.5 μg) were loaded and isolated mitochondria (Mito) was used as a standard. A) Characteristic blots of FAT/CD36, Bcl-2, complex I, II, III, IV subunits following OMM isolation. B) Quantification of blots illustrated that FAT/CD36 and Bcl-2 were enriched and complex I, II, III, IV subunits were reduced in the OMM fraction. N=3 for all independent experiments, and data is expressed as mean ± S.E.M., *p<0.05 isolated mitochondria vs. isolated OMM.

In contrast, IMM proteins, subunits of complex I, II, III, IV were all reduced (~75%, p<0.05) in the OMM fraction suggesting that the OMM isolation protocol was successful (Figure 3.3). These two approaches, (digitonin digestion and extensive mitochondrial sub-fractionation), both indicate that FAT/CD36 resides on mitochondrial membranes, and more specifically, the OMM.
3.4.3 Palmitate respiration, mitochondrial content and mitochondrial function in FAT/CD36 KO mice

Given that FAT/CD36 was found on the OMM, we attempted to discern a potential function for this protein in regulating mitochondrial fatty acid oxidation by examining respiration rates in permeabilized skeletal muscle fibres from WT and FAT/CD36 KO mice. The typical substrate utilized to study fatty acid supported bioenergetics is palmitoyl carnitine. However, palmitoyl carnitine bypasses all known potential regulation by proteins located on the OMM, and therefore, while this substrate is ideal within the context of studying electron transport chain (ETC) function, it is not appropriate for examining the regulation of mitochondrial LCFA transport. Therefore, in WT and FAT/CD36 KO mice, we measured palmitate supported respiration. This approach revealed that maximal palmitate supported respiration was 34% lower (p<0.05) in FAT/CD36 KO mice in the presence of a variety of concentrations of palmitate (between 75 μM and 525 μM; Figure 3.4B). These data suggest that FAT/CD36 has a direct regulatory role in mitochondrial fatty acid oxidation.
Figure 3.4: Palmitate supported respiration in WT and FAT/CD36 KO mice. A) Palmitate supported respiration was determined in the presence of malate (2mM) and ADP (5mM). Palmitate respiration was lower in the FAT/CD36 KO mice at all palmitate concentrations ≥75μM. N=6-8 for all independent experiments, and data is expressed as mean ± S.E.M. *p<0.05 WT vs. FAT/CD36 KO.

A decrease in mitochondrial content, independent of FAT/CD36, can also result in decreased rates of fatty acid oxidation [201]. Therefore, to verify that FAT/CD36 KO mice do not have lower mitochondrial content we determined markers of mitochondrial content and mtDNA in WT and KO mice. Analyses of CS, β-HAD, COXIV, and mtDNA demonstrated that mitochondrial content was not different between in WT and KO mice (Figure 3.5A).

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Furthermore we show that the morphology of SS and IMF mitochondria are not different in WT and KO mice (Figure 3.5B).

Figure 3.5: Mitochondrial morphology (B) and content (A) in WT and FAT/CD36 KO mice. Markers of whole muscle mitochondrial content, including CS (absolute values; 80±8 vs. 85±6, mmol/kg ww/min), β-HAD (absolute values; 25±2 vs. 26±2, mmol/kg ww/min), COXIV and mtDNA, indicate mitochondrial content is not altered in FAT/CD36 KO mice. In addition, TEM images of muscle taken at 25,000 x magnification suggest the morphology of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria are not different between WT and FAT/CD36 KO mice. The bar = 2μm.

To confirm that reductions in palmitate supported respiration in FAT/CD36 KO mice were not a result of mitochondrial dysfunction in FAT/CD36 KO mice, we determined
respiration rates induced by a variety of substrates. WT and FAT/CD36 KO animals displayed similar rates of complex I (glutamate+malate) and II (glutamate+succinate+malate) supported respiration (Figure 3.6A). Maximal ETC activity as measured by the uncoupler FCCP (further confirming equivalent mitochondrial content), and basal respiration as estimated by the inhibition of complex III via Antimycin A (Figure 3.6A) also demonstrated that mitochondrial content and function did not differ between WT and KO mice. In addition, the respiratory control ratios (RCR) were not different between WT and FAT/CD36 KO mice (RCR=8.0±0.7 and 8.3±0.6, respectively). Collectively, these data suggest that FAT/CD36 has a direct role in regulating mitochondrial fatty acid oxidation.

Figure 3.6: Mitochondrial respiration in WT and FAT/CD36 KO mice. A) The conditions; malate (2mM) + glutamate (10mM), malate (2mM) + glutamate (10mM) + ADP (5mM), malate (2mM) + glutamate (10mM) + ADP (5mM) + succinate (10mM), FCCP titrations and Antimycin A (2.5µM) were not different between strains. B) Palmitoyl-CoA respiration rates were not different whereas palmitate respiration rates were different between WT and FAT/CD36 KO. N=6-8 for all independent experiments, and data is expressed as mean ± S.E.M. *p<0.05 WT vs. FAT/CD36 KO.
3.4.4 Palmitoyl-CoA respiration in FAT/CD36 KO mice

The foregoing experiments provide evidence that FAT/CD36 resides on the OMM and regulates palmitate supported respiration. However, where along the OMM FAT/CD36 exerts this role is unclear. Therefore, to determine if FAT/CD36 is located proximal or distal to long chain ACS (depicted in summary Figure 3.8), we next examined P-CoA supported respiration in WT and FAT/CD36 KO mice. In contrast to palmitate supported respiration, P-CoA respiration rates were not different in WT and FAT/CD36 KO animals (Figure 3.6B). The markedly divergent result in FAT/CD36 KO animals with respect to palmitate and P-CoA respiration rates strongly implies that FAT/CD36 influences fatty acid oxidation proximal to ACS.

3.4.5 Contact sites and immunoprecipitation

Contact sites of mitochondrial membranes are the fusions points of the OMM and the IMM. It is known that CPT-I is prevalent in contact sites [87,88], and as such, it has been suggested that contact sites are important for facilitating fatty acid transport into the mitochondria for subsequent oxidation [8]. Considering that FAT/CD36 has been observed to co-immunoprecipitate with CPT-I [77,78,80,81] it was somewhat unexpected that FAT/CD36 did not influence P-CoA respiration (substrate for CPT-I). Therefore, we performed additional sub-fractionation experiments in rat skeletal muscle to isolate both the OMM and the contact site fractions. These additional experiments revealed that CPT-I is located within contact sites, but not the OMM fraction (Figure 3.7A). In contrast, FAT/CD36 and Bcl-2 (which has previously been shown to be located on the OMM [200]) were enriched in the OMM, while their contents were reduced in the contact site fraction (Figure 3.7A). To further supplement these findings, we performed co-immunoprecipitation experiments in intact and fractionated mitochondria. While FAT/CD36 and CPT-I co-immunoprecipitated in intact mitochondria (Figure 3.7B), these
proteins did not co-immunoprecipitate in fractionated mitochondria (Figure 3.7), suggesting these proteins do not reside in the same region of the OMM. The observation that Bcl-2 co-immunoprecipitates with FAT/CD36 (Figure 3.7C) indicates that regions of the OMM remained connected to FAT/CD36, further confirming that FAT/CD36 resides on the OMM.

**Figure 3.7: Distribution of FAT/CD36, Bcl-2 and CPT-I on mitochondrial membranes.** A) Equal protein amounts (7.5 μg) of isolated mitochondria, outer mitochondrial membrane (OMM) and contact site fraction were loaded. FAT/CD36 and Bcl-2 were enriched in the OMM fraction and reduced in the contact site fraction. In contrast, CPT-I was enriched in the contact site fraction and reduced in the OMM fraction. B) Within intact mitochondria, FAT/CD36 and CPT-I co-immunoprecipitate together. C) When mitochondria were fractionated, FAT/CD36 and CPT-I failed to co-immunoprecipitate. These data indicate FAT/CD36 and CPT-I do not reside in the same region of the mitochondrial membranes.
3.5 Discussion

In the current studies, we investigated the presence of FAT/CD36 on skeletal muscle mitochondrial membranes and the functional role of this protein in mitochondrial fatty acid oxidation. We provide evidence that FAT/CD36 a) resides on mitochondrial membranes, specifically the OMM, and at this location b) influences mitochondrial fatty acid oxidation proximal to ACS. Collectively, these two major findings establish that FAT/CD36 contributes to the regulation mitochondrial fatty acid oxidation.

3.5.1 FAT/CD36 on mitochondria

Our mitochondrial purification technique and contamination checks, in combination with the data from our previous work [78,84,89] and from four independent laboratories [77,80,81,86], indicate that FAT/CD36 is located on the mitochondrial membranes in several tissues including skeletal muscle [19,46,77,78,80,82,84,86,89,201], liver [79] and heart [78] in a variety of species (rat, mouse and humans). It is unclear why one group [192] has been unable to replicate these findings. Nevertheless, there is now considerable evidence to support the presence of FAT/CD36 on mitochondria. This is further supported by our experimental approaches which suggest FAT/CD36 is localized to the OMM in skeletal muscle. Moreover, previously in liver tissue, FAT/CD36 was identified on the OMM via mass spectrometry [79]. The congruence in these reports provides evidence that FAT/CD36 is present on OMM.

3.5.2 Functional role of FAT/CD36

When comparing fatty acid stimulated respiration rates in FAT/CD36 KO to WT mice, we observed 1) lower palmitate supported respiration rates in the KO mice and 2) no difference in P-CoA supported respiration rates between WT and KO mice. Our novel comparison between these two substrates (palmitate vs. P-CoA) combined with the use of an in situ model
(permeabilized fibres) highlights the importance of selecting a substrate which is subject to the native regulation of a physiological system. Our observations are in agreement with two previous reports comparing isolated mitochondrial fatty acid oxidation rates between WT and FAT/CD36 KO mice [84,86]. Holloway et al (2009a) observed that basal levels of mitochondrial palmitate oxidation rates were lower in KO mice and King et al (2007) observed that P-CoA state III respiration rates were not significantly different between strains. Indeed, FAT/CD36 does appear to have a regulatory role in mitochondrial fatty acid oxidation as it is now apparent that ablation of FAT/CD36 reduces palmitate-supported respiration, but not P-CoA supported respiration. As a result, we conclude that FAT/CD36 is influencing mitochondrial fatty acid oxidation upstream of ACS.

3.5.3 CPT-I and FAT/CD36

CPT-I has long been viewed as the sole regulator of mitochondrial fatty acid oxidation, due to the integral role of this protein in LCFA movement into the mitochondrial matrix [27,91,202,203]. However, several studies have suggested that mitochondrial fatty acid oxidation can be altered independent of CPT-I activity [82,84,204], and the current data supports this notion as palmitate oxidation was reduced in FAT/CD36 KO mice, a model known to have unaltered CPT-I activity [84]. Other mechanisms can/may also influence mitochondrial fatty acid oxidation including, putative fatty acid transport proteins such as the FATP family [16,77], the acetylation status of mitochondrial proteins [205,206], complex I glutathionylation [103], and/or phosphorylation of ETC complexes [207]. Clearly more work on the complexities surrounding the role of ‘non-CPT-I’ proteins and processes is necessary to unravel the complete regulation of mitochondrial fatty acid metabolism, although evidence is mounting to suggest FAT/CD36 contributes at this level.
Originally CPT-I and FAT/CD36 were hypothesized to interact directly due to positive co-immunoprecipitation results from a number of laboratories [78,80,81]. However, the current data do not support the belief that CPT-I and FAT/CD36 directly interact, as CPT-I but not FAT/CD36 was found within contact sites. Furthermore, when the mitochondrial membranes were sufficiently fractionated, FAT/CD36 and CPT-I did not co-immunoprecipitate, suggesting that these proteins are not in close proximity and do not directly interact. Consequently, we interpret the previous co-immunoprecipitation data, in addition with our own, as providing further support that FAT/CD36 is located on the OMM, rather than directly interacting with CPT-I within the contact sites.

3.5.4 Proposed working model

Proteins involved in the movement of fatty acids across cell membranes (such as FAT/CD36) have been hypothesized to act as LCFA acceptors to promote binding to downstream proteins as opposed to typical transport proteins which create a pore in the membrane (i.e. GLUT-4 [15,19]). At the sarcolemma, FAT/CD36 has a large hairpin loop projecting into the interstitial space, which may function to accept LCFA from albumin and facilitate LCFA delivery and insertion into the outer leaflet of the plasma membrane. This process has been proposed to increase rates of ‘flip-flop’ across the membrane and ultimately rates of fatty acid transport [6,208]. At the mitochondria, FAT/CD36 may function in a similar fashion, and we hypothesize a working model for fatty acid entry into the mitochondria (Figure 3.8).
Figure 3.8: Proposed mechanism of action regarding FAT/CD36 involvement in LCFA entry into the mitochondria. In the current study we have found FAT/CD36 is localized on the outer mitochondrial membrane (OMM) and that ablating FAT/CD36 reduces palmitate, but not palmitoyl-CoA supported respiration. Therefore, we propose that FAT/CD36 is located upstream of long chain acyl-CoA synthetase (ACS) on the OMM, and has a regulatory role in LCFA oxidation. In this model FAT/CD36 accepts LCFA from FABPc at the OMM which facilitates the delivery of LCFA to ACS to promote an increase in ACS enzymatic flux rates. This would result in an increased LCFA-CoA delivery to CPT-I, the transfer of LCFA into the mitochondrial matrix, and subsequently beta oxidation. This proposed mechanism of action for mitochondrial FAT/CD36 is similar to the proposed at the plasma membrane, where FAT/CD36 is thought to facilitate the transfer of LCFA from albumin to the outer leaflet of the plasma membrane, thereby regulating LCFA transport into the cell (reviewed in [6,208]).

Specifically, we propose that FAT/CD36 accepts LCFA from FABPc at the OMM, which would facilitate the delivery of LCFA to ACS allowing this enzyme to increase enzymatic flux rates. This would result in an increased LCFA-CoA delivery to CPT-I to increase the transfer of LCFA into the mitochondria, and subsequently increase rates of mitochondrial fatty acid oxidation. In the context of this working model, CPT-I retains its essential role in LCFA transport as
classically proposed. However, unlike CPT-I, FAT/CD36 is not required for mitochondrial fatty acid oxidation, as knocking out FAT/CD36 only reduced fatty acid supported respiration rates as opposed to completely ablating them. Therefore, we propose that altering FAT/CD36 content at the mitochondrial level provides additional regulation that may be important for augmenting LCFA oxidation rates. This may be particularly important during exercise when the energetic demands from LCFA oxidation are increased substantially, a process that coincides with increases in mitochondrial FAT/CD36 [46,84,209].

3.5.5 Conclusion

Our data indicate that in skeletal muscle, FAT/CD36 is located on the OMM and influences mitochondrial fatty acid oxidation proximal to ACS. Impairments within mitochondrial fatty acid oxidation and the dysregulation of lipid metabolism have been associated with various pathologies [210-212]. Therefore, understanding the regulation of fatty acid metabolism, including mitochondrial fatty acid oxidation, may highlight potential therapeutic strategies.
CHAPTER 4:
IDENTIFICATION OF A NOVEL MALONYL-COA IC₅₀ FOR CPT-I: IMPLICATIONS FOR PREDICTING *IN VIVO* FATTY ACID OXIDATION RATES

Presented as published:

4.1 Synopsis

Published values regarding the sensitivity (IC_{50}) of carnitine palmitoyl transferase I (CPT-I) to malonyl-CoA (M-CoA) inhibition in isolated mitochondria are inconsistent with predicted in vivo rates of fatty acid oxidation. Therefore, we have re-examined M-CoA inhibition kinetics under varying palmitoyl-CoA (P-CoA) concentrations in both isolated mitochondria and permeabilized muscle fibres (PMF). PMF have an 18-fold higher IC_{50} (0.61 vs 0.034 μM) in the presence of 25 μM P-CoA and a 13-fold higher IC_{50} (6.3 vs 0.49 μM) in the presence of 150 μM P-CoA compared to isolated mitochondria. M-CoA inhibition kinetics determined in PMF predicts that CPT-I activity is inhibited by 33% in resting muscle compared to >95% in isolated mitochondria. Additionally, the ability of M-CoA to inhibit CPT-I appears to be dependent on P-CoA concentration, as the relative inhibitory capacity of M-CoA is decreased with increasing P-CoA concentrations. Altogether, the use of PMF appears to provide a M-CoA IC_{50} that better reflects the predicted in vivo rates of fatty acid oxidation. These findings also demonstrate the ratio of [P-CoA]/[M-CoA] is critical for regulating CPT-I activity and may partially rectify the in vivo disconnect between M-CoA content and CPT-I flux within the context of exercise and type II diabetes.
4.2 Introduction

The regulation of long chain fatty acid (LCFA) oxidation is a central field of study in the context of exercise and metabolic disease. The rate limiting step in LCFA oxidation has been attributed mainly to its transport across the mitochondrial outer membrane via carnitine palmitoyltransferase I (CPT-I) [8,30]. Carnitine-dependent transport of LCFA into the mitochondria was first conceptualized in the 1960s and strong evidence has now accumulated to indicate that CPT-I has an obligatory role in LCFA oxidation [27,91,202].

All previous work investigating the regulatory mechanisms surrounding CPT-I have been performed in isolated mitochondria. Although isolated mitochondria are a standard tool for certain measurements (i.e. P/O ratios), several physiologically relevant mitochondrial characteristics are altered during the isolation procedure [33,74,213]. Additionally, the removal of the cytoskeleton during the isolation procedure may alter the inherent regulatory mechanisms associated with LCFA oxidation as the cytoskeleton has previously been shown to influence CPT-I activity [214,215] and other mitochondrial regulatory processes [216,217]. In contrast to isolated mitochondria, permeabilized skeletal muscle fibres (PMF) maintain the inherent cytoskeletal architecture and mitochondrial morphology while the sarcolemma is selectively permeabilzed [218,219]. This approach enables in situ investigations into mitochondrial physiology as substrates/chemicals added to the media have direct access to the mitochondria within their native state. In addition, the provision of a myosin ATPase inhibitor (blebbistatin) to the PMF preparation enables the analysis of mitochondrial parameters under more physiological conditions (i.e. 37°C), and better represents the in vivo situation accordingly [220,221].

In skeletal muscle, malonyl-coenzyme A (M-CoA) inhibits CPT-I activity, and therefore the content of M-CoA is considered an important regulator of skeletal muscle LCFA oxidation.
However, a number of discrepancies currently exist within the literature surrounding M-CoA inhibition kinetics of CPT-I. Firstly, the reported concentration of M-CoA required to inhibit CPT-I activity 50% (IC$_{50}$) in isolated mitochondria (~0.025-0.49 µM) [30,45,46] is lower than resting M-CoA content [30,47,48] suggesting that CPT-I activity and rates of LCFA oxidation should be negligible at rest. This is inconsistent with the well characterized reliance on LCFA oxidation at rest in vivo [51,52]. Secondly, during exercise, a decrease in M-CoA content is thought to “release the brake” on CPT-I and increase LCFA transport into the mitochondria for subsequent oxidation [37,38]. However, previous studies in humans have reported unchanged [40,41] or negligible decreases [43] in skeletal muscle M-CoA concentrations during exercise despite pronounced increases in LCFA oxidation. In addition, the role of M-CoA in regulating mitochondrial LCFA entry in type II diabetes has shown disparate findings as M-CoA levels are elevated in the skeletal muscle of type II diabetic humans [53] and rats (ZDF rats) [54,55] yet LCFA entry into the mitochondria is increased in both species [56,57]. Of potential importance, during exercise and in type II diabetes, LCFA-CoA levels within skeletal muscle are increased [92,184,185] and LCFA-CoA levels have been previously shown to decrease the effectiveness of M-CoA inhibition on CPT-I [72,73]. Therefore, any change in LCFA-CoA content can influence CPT-I activity independent of changes in M-CoA content.

Therefore, to address the controversies surrounding M-CoA inhibition kinetics of CPT-I, we aimed to determine the sensitivity of CPT-I to M-CoA in isolated mitochondria and in PMF under varying concentrations of palmitoyl-CoA (P-CoA, a LCFA-CoA moiety). We report that PMF have a 13 to18-fold higher IC$_{50}$ than isolated mitochondria and that the ability of M-CoA to inhibit CPT-I is dependent on the concentration of P-CoA in both preparations.
4.3 Experimental

4.3.1 Animals

Ten-week-old female Sprague-Dawley rats (274±8 g) were bred on site at the University of Guelph, and housed in a climate control facility on a 12 h light/dark cycle and provided rat chow and water ad libitum. Malonyl-CoA decarboxylase knockout (mcd<sup>−/−</sup>) mice [223] were bred onsite at Duke University. All facets of this study were approved by the University of Guelph Animal Care Committee and the Duke University Institutional Animal Care and conform to the guide for the care and use of laboratory animals published by the US National Institutes of Health. The red gastrocnemius muscle was used for all experiments.

4.3.2 Preparation of permeabilized fibres

The preparation of PMF was adopted from prior publications [147,220], as we have previously reported [224]. Following dissection of red gastrocnemius (n=6), fibre bundles (~2 mg) were separated with fine forceps under a binocular dissecting microscope in BIOPS buffer containing, CaK2EGTA (2.77 mM), K2EGTA (7.23 mM), Na2ATP (5.77 mM), MgCl2*6H2O (6.56 mM), Na2Phosphocreatine (15 mM), Imidazole (20 mM), Dithiothreitol (0.5 mM) and MES (50 mM). Following separation, fibre bundles were placed in BIOPS containing 50µg/ml saponin, agitated for 30min and then washed in respiration buffer (MIRO5) containing EGTA (0.5 mM), MgCl2*6H2O (3 mM), K-lactobionate (60 mM), Taurine (20 mM), KH2PO4 (10 mM), HEPES (20 mM), Sucrose (110 mM) and fatty acid free BSA (1 g/L). Fibres were left in cold MIRO5 until respiration analysis.
4.3.3 Permeabilized muscle fibre respiration

Mitochondrial respiration was measured in PMF by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria) at 37°C and room air saturated oxygen tension in the presence of 25 μM blebbistatin to ensure PMF relaxation. Separate fibres from the same animal were used to determine (in duplicate) the kinetic properties of P-CoA supported respiration and the sensitivity of P-CoA respiration to M-CoA inhibition. To measure P-CoA supported respiration, MIRO5, 5 mM ADP, 2 mM malate, and 2 mM L-carnitine were used as the respiration medium. Once a baseline respiration was determined, various concentrations of P-CoA (25, 50, 75, 100, 150 and 200 μM) were titrated into the chambers. To measure M-CoA sensitivity, respiration was determined in the presence of 150 μM and 25 μM P-CoA, and various concentrations of M-CoA (0.25, 0.5, 1, 2, 10, 25 and 50 μM) were subsequently titrated into the respiration chambers. Additionally, the impact of a fixed M-CoA concentration (7 μM) on respiration supported by varying P-CoA concentrations (25, 50, 75, and 100 μM) was performed. Separate fibres were used to determine respiratory control ratios in the presence of malate (2 mM) + glutamate (10 mM) +/- 5 mM ADP in the presence and absence of exogenous cytochrome c (10 μM) and oligomycin (2 μg/ml). P-CoA and palmitoylcarnitine were made up in water whereas the palmitate was made up in MIRO5 and the palmitate stock in MIRO5 was supplemented with 10% BSA. Optimization experiments of the various substrates have previously been done to ensure high quality, consistent experiments.

4.3.4 Carnitine palmitoyl transferase-I activity

CPT-I activity was determined as described by McGarry et al [30] with minor modifications as we have previously reported [46,82]. CPT-I activity is defined as the rate at which tritiated palmitoylcarnitine is formed from tritiated L-carnitine following the addition of
isolated mitochondria to the reaction medium. The assay buffer consisted of 117 mM Tris-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 2.22 mM KCN, 0.1 mM rotenone, 0.5% BSA, 5 mM L-carnitine and 1 μCi tritiated L-carnitine (L-[\(^3\)H]carnitine Amersham Bioscience, Buckinghamshire, England). Briefly, the assay was conducted at 37ºC and initiated by the addition of 10 μl of mitochondrial suspension (final protein content of 5 μg/reaction) to 10 μl of varying P-CoA concentrations (18.75, 37.5, 75, 150, and 200 μM) and 80 μl of a standard reaction medium. The reaction containing 150 μM P-CoA was also carried out in the presence of various M-CoA concentrations (0.5, 1, 2, 10, 25 and 50 μM). The reaction was stopped after 6 minutes with the addition of ice-cold HCl. Palmitoyl-[\(^3\)H] carnitine was extracted in water-saturated butanol in a process involving three washes with distilled water and subsequent re-centrifugation steps to separate the butanol phase, in which the radioactivity was counted.

4.3.5 Isolated mitochondrial respiration

Differential centrifugation was used to isolate mitochondria from the red gastrocnemius muscle as we have previously published [224,225]. To measure M-CoA sensitivity, MIRO5, 800 μM ADP, 2 mM malate, and 750 μM L-carnitine was used as the respiration medium and respiration was stimulated with 25 μM P-CoA. Once a baseline respiration was determined, various concentrations of M-CoA (0.03, 0.05, 0.1, 0.25 μM) were titrated into the chambers. Quality control experiments were also performed with isolated mitochondria to determine respiratory control ratios and mitochondrial coupling (ADP/O). In brief, state IV respiration was determined in the presence of 10 mM pyruvate+5 mM malate and state III respiration was initiated with the addition of 100 μM ADP. Maximal state III respiration was determined in the presence of 2 mM ADP.
4.3.6 One phase exponential decay predictive equation

One phase exponential decay is defined as: \( Y = Y_0 - \text{Plateau} \cdot e^{(K \cdot X)} + \text{Plateau} \) where:

- \( Y \) = respiration rate
- \( Y_0 \) = respiration rate without exogenous M-CoA
- Plateau = where any subsequent addition of M-CoA has no effect on respiration
- \( K \) = rate constant
- \( X \) = M-CoA concentration
- Plateau was constrained to zero (Graphpad Prism 5).

4.3.7 Statistics

Michaelis-Menten kinetics and IC\(_{50}\) values for M-CoA were determined by plotting data points in GraphPad Prism 5 software following the subtraction of baseline values. The IC\(_{50}\) is defined as the concentration of M-CoA where CPT-I activity and/or P-CoA supported respiration were reduced by half. Unpaired 2-tailed t-tests were used to compare isolated mitochondria and permeabilized fibres P-CoA Km and M-CoA IC\(_{50}\). Statistical significance was accepted with a p value ≤ 0.05.

4.4 Results and Discussion

The current manuscript evaluated M-CoA inhibition kinetics (IC\(_{50}\)) of CPT-I in isolated mitochondria and PMF. The integrity of the isolated mitochondria was ensured by determining pyruvate/malate respiratory control ratio measurements (5.4±0.4 nmol O\(_2\)/mg/min) and appropriate P/O ratios (2.9±0.1). In the PMF preparation, the malate (2 mM) + glutamate (10 mM) +/− 5 mM ADP respiratory control ratio was 9.5±0.2. In addition, the exogenous provision of cytochrome c during ADP-supported respiration did not increase respiration >10%, and the addition of oligomycin fully prevented P-CoA respiration (indicating coupled respiration; data
not shown). Combined, these quality control experiments indicate that the isolated mitochondria and the mitochondria within the PMF preparation were fully coupled and intact and therefore appropriate for the current study.

4.4.1 CPT-I remains rate-limiting for fatty-acid supported respiration in permeabilzed muscle fibres

We first determined that CPT-I represented a rate-limiting step in state 3 mitochondrial fatty acid supported respiration within the PMF preparation. This was accomplished by examining the kinetics of various lipids species proximal and distal to CPT-I. The sensitivity of the PMF preparation to palmitate (Km = 90 µM) and P-CoA (Km = 80 µM), substrates that require CPT-I, were very similar (Figure 4.1). In contrast, palmitoyl carnitine supported respiration, which does not require CPT-I, displayed a marked increase in sensitivity (Km = 23 µM). Altogether, these data indicate that similar to isolated mitochondria, CPT-I is rate limiting for lipid-supported respiration in PMF.

![Figure 4.1: Palmitate, palmitoyl-CoA (P-CoA) and palmitoyl carnitine (PC) respiration kinetics in permeabilized muscle fibres.](image)

*Figure 4.1: Palmitate, palmitoyl-CoA (P-CoA) and palmitoyl carnitine (PC) respiration kinetics in permeabilized muscle fibres.* Palmitate was titrated in the presence of malate (2mM), ADP (5mM), Co-enzyme A (1mM) and L-carnitine (2mM). P-CoA was titrated in the presence of malate (2mM), ADP (5mM) and L-carnitine (2mM). PC was titrated in the presence of malate (2mM) and ADP (5mM). All respiration chambers contained 25 µM blebbistatin. All R² values are above 0.98. P-CoA was inhibitory on respiration at ~220 µM and PC was inhibitory on respiration at 100 µM. N=6 and each independent experiment was performed in duplicate, and data is expressed as mean ± SEM.
4.4.2 Use of permeabilized muscle fibres yields a higher IC₅₀ for malonyl-CoA

Prior to performing experiments to elucidate the M-CoA inhibitory kinetics on CPT-I in PMF, we determined the kinetic response of isolated mitochondria and PMF to titrations of P-CoA, a substrate for CPT-I. High P-CoA concentrations (>40 μM) prevent respiration in isolated mitochondria and therefore we determined the sensitivity of isolated mitochondria to P-CoA utilizing a CPT-I activity assay as classically performed [30]. The sensitivities of PMF and isolated mitochondria to the substrate P-CoA were similar as the Km values were 80 μM and 75 μM, respectively (Figure 4.2A and B).

Figure 4.2: Carnitine palmitoyltransferase I (CPT-I) activity in isolated mitochondria (A) and oxygen consumption in permeabilized muscle fibres (B) in the presence of various palmitoyl-CoA (P-CoA) concentrations. The Km values shown on the figures represent those determined for the mean regression lines and they are not different between methodologies. These values were used to generate the Lineweaver-Burk plots for enzyme kinetics, which are located as an inset in each graph. The slopes for these graphs were 0.41 and 0.39 for isolated mitochondria and permeabilized fibres, respectively. N=4 in isolated mitochondria, and N=6 in permeabilized fibres. Each independent experiment was performed in duplicate, and data is expressed as mean ± SEM.

In addition, the Lineweaver-Burk plots located as insets in Figures 4.2A and B, also displayed similar slopes in isolated mitochondria and permeabilized fibres. These data suggest that a diffusion limitation does not exist within PMF for saturating fatty acid concentrations, and
validate the use of identical P-CoA concentrations between methodologies in our subsequent experiments. Additionally, we performed experiments without saponin in PMF. In these experiments, although the respiration rates were ~30-50% lower, M-CoA sensitivity was again not different with or without saponification indicating that the saponin permeabilization step does not affect the IC$_{50}$ value observed in PMF. Altogether, these data support the use of PMF to assess M-CoA sensitivity.

Therefore, we next investigated the kinetic properties of M-CoA inhibition of oxygen consumption in isolated mitochondria and PMF in the presence of resting concentrations of P-CoA (25 µM) [92]. Maximal inhibition by M-CoA was similar between methodologies (~50%; Figure 4.3A and B), however this occurred at vastly different M-CoA concentrations (~10-fold higher in PMF). In these highly controlled conditions, and in the presence of a physiologically relevant P-CoA concentration, the IC$_{50}$ for M-CoA was 0.034 µM and 0.61 µM in isolated mitochondria and PMF, respectively (Figure 4.3A and B), suggesting M-CoA inhibition of CPT-I is considerably attenuated in an in situ model.

![Figure 4.3: Malonyl-CoA inhibition kinetics in isolated mitochondria (A) and in permeabilized muscle fibres (B) in the presence of 25 µM palmitoyl-CoA. The IC$_{50}$ values shown represent those determined for the mean regression line. Permeabilized fibres displayed an ~18-fold higher IC$_{50}$ compared to isolated mitochondria. N=4 in isolated mitochondria and N=4 in permeabilized fibres. Each independent experiment was performed in duplicate and data is expressed as mean ± SEM. *Significantly different from isolated mitochondria.](image_url)
In addition, the kinetics of M-CoA inhibition on P-CoA supported respiration in isolated mitochondria in the current study are virtually identical to previous reports examining M-CoA inhibition of CPT-I activity [30,30,70,72] further suggesting that CPT-I is rate-limiting for fatty acid oxidation under these experimental conditions.

We next determined the kinetic properties of M-CoA inhibition in the presence of exercise concentrations of P-CoA (150 µM) [92]. We found that the sensitivity to M-CoA remained substantially lower in PMF, as the IC$_{50}$ were 0.49 µM and 6.30 µM in isolated mitochondria and PMF, respectively (Figure 4.4A and B). However, compared to the IC$_{50}$ values found using resting concentrations of P-CoA, these exercise values represent 14-fold and 10-fold increases in isolated mitochondria and PMF, respectively.

![Figure 4.4: Malonyl-CoA inhibition kinetics in isolated mitochondria (A) and in permeabilized muscle fibres (B) in the presence of 150 µM palmitoyl-CoA. The IC$_{50}$ values shown represent those determined for the mean regression line. Permeabilized muscle fibres display a ~13-fold higher IC$_{50}$ compared to isolated mitochondria. N=4 in isolated mitochondria and N=6 in permeabilized fibres. Each independent experiment was performed in duplicate, and data is expressed as mean ± SEM. *Significantly different from isolated mitochondria.](image)

Altogether, these data indicate that the sensitivity for M-CoA inhibition is vastly lower in PMF compared to isolated mitochondria, as the IC$_{50}$ is higher in PMF in the presence of both resting (18-fold) and exercise (13-fold) P-CoA concentrations.
4.4.3 Role of palmitoyl-CoA in regulating malonyl-CoA inhibition kinetics

The pronounced difference in M-CoA IC\textsubscript{50} values in the presence of higher P-CoA concentrations in both isolated mitochondria and PMF (Figures 4.3 and 4.4) led us to further examine the notion that P-CoA concentrations can alter M-CoA sensitivity in PMF. Previous work in isolated mitochondria has highlighted the interaction between M-CoA and P-CoA [30,72,73] but considering the marked differences observed between PMF and isolated mitochondria, we decided to re-examine this concept in PMF. This was done by determining the ability of 7 µM M-CoA to inhibit P-CoA respiration at varying (25-100 µM) concentrations of P-CoA in PMF [92]. With this approach, M-CoA inhibited respiration -63% in the presence of 25µM P-CoA, but only -26% in the presence of 100 µM P-CoA (Figure 4.5A and B). These data clearly show that increasing P-CoA concentration attenuates M-CoA inhibition of CPT-I suggesting that the ratio of M-CoA:P-CoA is important in determining the overall catalytic activity of CPT-I. This implies when LCFA-CoA levels are elevated, such as during exercise or in type II diabetes, a reduction in M-CoA is not required to alter CPT-I flux. This could explain the pronounced increase in LCFA oxidation that occurs during exercise in humans in the face of little [43] or no change [40] in M-CoA levels. Additionally, a hallmark of chronic exercise training is the ability to increase fatty acid oxidation quickly at the onset of exercise, yet, following exercise training in rats, the normal exercise decline in M-CoA content is attenuated [226], suggesting additional mechanisms regulate CPT-I. In the context of the present data and others [30,72,73], the increase in the rate of fatty acid supply in the trained state would negate the need for M-CoA to substantially decrease in order to increase CPT-I flux and therefore fatty acid oxidation.
Figure 4.5: Palmitoyl-CoA (P-CoA) respiration in permeabilzed muscle fibres in the presence of 7 μM malonyl-CoA (M-CoA). Respiration of varying concentrations of P-CoA was inhibited by the addition of 7 μM. At each increased concentration of P-CoA, inhibition by M-CoA was decreased. N=4 and each independent experiment was performed in duplicate. Data is expressed as mean ± SEM.

The interaction between P-CoA and M-CoA may also explain how in type II diabetes, mitochondrial matrix fatty acid oversupply exists in the presence of elevated M-CoA concentrations [53-57,227]. In the context of the present data and others [30,72,73], this
disconnect may be explained by the increase in LCFA-CoA levels observed in type II diabetes which would render M-CoA inhibition less effective and potentially account for the increase in CPT-I flux and resultant mitochondrial matrix fatty acid oversupply present in this disease [184,185]. Considering fatty acid oversupply to the mitochondria has been associated with impaired insulin sensitivity [57,147], LCFA-CoA levels within the context of CPT-I regulation may be an important factor to consider in type II diabetes.

4.5 Perspectives and significance

4.5.1 Predicted physiological M-CoA inhibition of fatty acid oxidation

As previously documented [30,45,46,70,72], the IC₅₀ determined in isolated mitochondria would suggest that LCFA oxidation is substantially inhibited at rest. Considering the respiratory quotient across a leg muscle at rest is ~0.77 – 0.83 [51,52], these current values do not appear to represent the predicted in vivo situation. To put our results into physiological context, we applied the data from our “at rest” M-CoA inhibition curves derived from isolated mitochondria and PMF (Figure 3A and B) to a one phase exponential decay equation to predict the % inhibition of LCFA oxidation in the presence of resting concentrations of M-CoA (Table 4.1).

Table 4.1: Predicted in vivo fatty acid oxidation inhibition.

<table>
<thead>
<tr>
<th>Method</th>
<th>P-CoA concentration</th>
<th>Rate constant (K)</th>
<th>M-CoA concentration</th>
<th>Predicated rate of fat oxidation</th>
<th>Predicted % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated mitochondria</td>
<td>25 μM</td>
<td>4.076</td>
<td>0.7 μM</td>
<td>2.20 nmol/min/mg mito pr</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>PMF</td>
<td>25 μM</td>
<td>0.03707</td>
<td>0.7 μM</td>
<td>14.1 pmol/s/mg dw</td>
<td>33%</td>
</tr>
<tr>
<td>Isolated mitochondria</td>
<td>150 μM</td>
<td>1.417</td>
<td>0.7 μM</td>
<td>8.98 nmol/min/mg mito pr</td>
<td>44%</td>
</tr>
<tr>
<td>PMF</td>
<td>150 μM</td>
<td>0.03026</td>
<td>0.7 μM</td>
<td>150 pmol/s/mg dw</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

The rate constant (K) used in the one phase exponential decay equation was derived from Graphpad Prism 5. The malonyl-CoA concentration was adopted from two previous publications [49,50].
We assumed resting M-CoA concentrations to be 0.7 µM as previously documented in two independent studies using HPLC/MS [50] and HPLC/MS/MS [49]. As seen in Table 4.1, inhibition of LCFA oxidation is predicted to be >95% in isolated mitochondria at rest. However, in PMF, the predicted inhibition of LCFA oxidation is ~33% which appears to better reflect the \textit{in vivo} state as reported respiratory quotients across a leg muscle [51,52] indicates that fatty acid oxidation would contribute ~56-77% of the necessary energy at rest. In addition, independent of potential minor reductions in M-CoA content, the increase in P-CoA content (150 µM) within muscle during exercise attenuates M-CoA inhibition of LCFA oxidation to a predicted 2.1% in PMF compared to 44% in isolated mitochondria (Table 4.1). Altogether, these data suggest that in an \textit{in situ} model the predicted % inhibition of fatty acid oxidation appears to better reflect the predicted \textit{in vivo} state compared to isolated mitochondria. However, it should be acknowledged that we have not been able to account for subcellular or regional differences in M-CoA concentrations. It has been previously suggested that the $\beta$-isoform of acetyl-CoA carboxylase located on the mitochondrial outer membrane “channels” M-CoA to CPT-I [228-230] and additionally, a mitochondrial enzyme in mammals capable of synthesizing M-CoA within the mitochondria has been recently characterized [231]. Therefore, it is possible that the use of total cellular content of M-CoA (0.7 µM) as we have done is not reflective of the \textit{in vivo} exposure of CPT-I to M-CoA. While we acknowledge limitations pertaining to selecting the biologically relevant M-CoA concentration, these do not affect our interpretations, as these same limitations exist for both isolated mitochondrial and PMF preparations. We therefore hypothesize that the presence of the inherent cellular architecture within PMF may help explain the differences between methodologies. We also conclude that physiological increases in P-CoA may represent a key regulator of CPT-I activity. The mechanism by which P-CoA can alter M-CoA inhibition
appears to be by increasing the dissociation constant (Kd) of M-CoA for CPT-I (~10-fold) [73]. Therefore, P-CoA binding to CPT-I potentially alters the conformation of CPT-I such that M-CoA binding is less favorable [73]. These experiments [73] were done in the presence of maximal P-CoA and therefore the 10-fold increase in the Kd appears to match our data with “maximal” (exercise) concentrations of P-CoA as we report a 13-fold increase in the IC$_{50}$ in the presence of exercise P-CoA concentrations [73].

4.5.2 Literature comparison of IC$_{50}$ values in skeletal muscle

Previous literature displays ~70-fold range in M-CoA IC$_{50}$ values creating difficulty in interpreting the importance of M-CoA even within an isolated mitochondrial preparation. Table 2 compares our present results in the context of previous literature and highlights the vast range of reported IC$_{50}$ values. The apparent disparities within the previous literature appear to be explained by the concentration of P-CoA, as the highest values were all generated with ≥150 μM P-CoA (Table 4.2). Additionally, the IC$_{50}$ values between rat and human skeletal muscle are similar, suggesting the M-CoA kinetic properties are evolutionary conserved.

<table>
<thead>
<tr>
<th>Method</th>
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4.5.3 Potential explanations for differences between methodologies

It is currently unknown why PMF have a higher M-CoA IC$_{50}$ value compared to isolated mitochondria. However, isolating mitochondria from skeletal muscle requires homogenization followed by a series of differential centrifugation steps, a process that likely exerts substantial shear stress on the mitochondrial membranes, and therefore CPT-I [33]. This process has been shown to alter several characteristics of mitochondria, including respiration of specific substrates and susceptibility of calcium induced opening of the permeability transition pore [74]. Current models of CPT-I propose a hairpin structure, with both N and C termini located in the cytosol [233] and the interaction of the N and C termini is essential for modulating and preserving M-CoA sensitivity [33,34]. Therefore, isolating mitochondria may alter the interaction of these two cytosolic loops. However, this proposition is unlikely as current literature suggests that altering this interaction prevents M-CoA binding and therefore a challenge to this interaction would increase the IC$_{50}$ [33,34]. Currently, no published literature exists to explain structure/function alterations in CPT-I that increase M-CoA binding/sensitivity.

Alternatively, M-CoA has been previously hypothesized to be sequestered into specific compartments within the skeletal muscle and it therefore remains possible that whatever mechanism promoting the sequestering and compartmentalization of M-CoA in skeletal muscle could still be present in PMF and not in isolated mitochondria [29,47,234].

The presence of M-CoA binding proteins in PMF may also explain the discrepancy in IC$_{50}$ values between methodologies. In support of this proposal, the presence of liver M-CoA binding proteins has been previously documented [235]. Additionally, the presence of M-CoA binding proteins would be expected to decrease the effect of M-CoA in muscle at lower concentrations, while at supraphysiologica concentrations of M-CoA these binding proteins
would become saturated, and therefore CPT-I would be inhibited. This description recapitulates the observed trends in PMF as M-CoA inhibition was attenuated at all concentrations ≤10 μM in comparison to isolated mitochondria but inhibition was similar to isolated mitochondria at higher M-CoA concentrations (25 and 50 μM). Therefore, the current data supports the idea that M-CoA binding proteins exist in muscle although the identification of these proteins and the potential interaction with the cytoskeleton remains to be investigated.

4.5.4 Conclusion

In conclusion, we provide evidence that the IC_{50} value for M-CoA in PMF is much higher than in isolated mitochondria and appears to better reflect *in vivo* fatty acid oxidation rates. Therefore, we hypothesize that these functional differences between PMF and isolated mitochondria underscore the important influence of mitochondrial morphology and/or the extra-mitochondrial environment in regulating LCFA oxidation via M-CoA inhibition of CPT-I. Additionally, P-CoA levels can alter M-CoA inhibition kinetics of CPT-I which may explain some of the previous literature discrepancies. Lastly, within the context of exercise and type II diabetes, the impact of altering P-CoA concentrations may address the disconnect between M-CoA levels and CPT-I flux previously observed.
CHAPTER 5:

SUBMAXIMAL ADP-STIMULATED RESPIRATION IS IMPAIRED IN ZDF RATS AND RECOVERED BY RESVERATROL

Presented as submitted:

Smith BK, Perry CG, Herbst EAF, Ritchie IR, Beaudoin MS, Smith JC, Neuber PD, Wright DC and Holloway GP. Submaximal ADP-stimulated respiration is impaired in ZDF rats and recovered by resveratrol. Submitted to Diabetologia. Manuscript number: Diab-13-0473
5.1 Abstract

AIMS/HYPOTHESIS: Although controversial, skeletal muscle mitochondrial dysfunction and reactive oxygen species (ROS) have been implicated in the etiology of type 2 diabetes (T2DM). Notably, mitochondrial function has been traditionally assessed in the presence of saturating ADP, however ADP is thought to limit respiration *in vivo*. Therefore, we investigated the potential link between submaximal ADP-stimulated respiration rates, ROS generation and insulin sensitivity in a model of T2DM, the ZDF rat.

METHODS: Skeletal muscles of lean control, ZDF rats and ZDF rats fed a diet supplemented with resveratrol were collected to assess insulin-stimulated glucose transport. We utilized permeabilized muscle fibres to measure mitochondrial respiration and ROS production in the presence of submaximal ADP concentrations.

RESULTS: We observed that submaximal ADP-stimulated respiration rates (250-2000 μM ADP) were lower in ZDF rats compared to lean controls, which coincided with lower adenine nucleotide translocase 2 (ANT2) protein content. In contrast, electron transport chain function is not different between groups. Treating ZDF rats with resveratrol improved skeletal muscle insulin sensitivity and this was associated with increased submaximal ADP-stimulated respiration rates as well as an increase in ANT2 protein content. These results coincided with a greater ability of ADP to attenuate mitochondrial ROS emission and an improvement in cellular redox balance.

CONCLUSIONS/INTERPRETATION: Together, these data suggest that mitochondrial dysfunction is present in skeletal muscle insulin resistance when assessed at submaximal ADP concentrations and that ADP dynamics may influence skeletal muscle insulin sensitivity through alterations in the propensity for mitochondrial ROS emission.
5.2 Introduction

Skeletal muscle, by virtue of its mass and overall rate of insulin-stimulated glucose disposal, is a highly important tissue in the etiology of insulin resistance and Type II Diabetes Mellitus (T2DM). The mechanisms that result in skeletal muscle insulin resistance remain poorly elucidated and controversial however a number of hypotheses have been proposed. For instance, a causal role for mitochondrial dysfunction in the progression of insulin resistance has been speculated, however there is significant discrepancy within the literature surrounding this concept [127,129,130,132-135,236-239]. Of potential importance, mitochondrial function has been traditionally assessed as a capacity measurement as maximal ADP concentrations have been utilized to promote mitochondrial respiration [129,130,132]. However, these previous examinations of mitochondrial function may not reflect the in vivo situation as under most physiological conditions ADP is thought to limit respiration. Therefore, from a physiological standpoint, it may be more appropriate to examine mitochondrial function in the presence of submaximal concentrations of ADP, however this has yet to be considered within the context of skeletal muscle insulin resistance.

In addition to mitochondrial dysfunction, a causal role of mitochondrial reactive oxygen species (ROS) production has been proposed to explain the progression of insulin resistance and T2DM [147,153,240]. In support of this concept, mitochondrial ROS generation is elevated in the over-fed and insulin resistant states and attenuating mitochondrial ROS emission using mitochondrial targeted antioxidant approaches prevents diet-induced insulin resistance [147,155]. Therefore, numerous studies [147,150,152-155] have suggested that by decreasing mitochondrial derived ROS, insulin sensitivity can be maintained or improved. However, a
relationship between mitochondrial ROS and insulin resistance is not universally found indicating there is still some disparity [156].

Importantly, the supply of ADP to mitochondria decreases the propensity for ROS emission [94]. Therefore, the ability of the mitochondria to transport ADP into the mitochondrial matrix to subsequently decrease ROS emission may have a role in the protection of insulin sensitivity. In this regard, adenine nucleotide translocase (ANT) has an obligatory role in counter-transporting ADP and ATP across the inner mitochondrial membrane [162]. Skeletal muscle expresses two primary ANT isoforms, ANT1 and ANT2, however ANT2 possesses a higher transport efficiency and has a higher $V_{\text{max}}$ [175]. In addition to the anti-port function, ANT is involved in the dissipation of basal membrane potential by allowing proton leak from the intermembrane space into the matrix [241]. Therefore, ANT can potentially reduce mitochondrial ROS emission by transporting ADP into the matrix as well as through the induction of basal proton leak. The transport of ADP is also the governing factor for oxidative phosphorylation [165] and previous studies have shown that alterations in ANT content or a shift in ANT isoform expression directly impacts mitochondrial function [175]. Therefore, ANT may sit as a nexus between mitochondrial function/dysfunction and mitochondrial ROS production. Intriguingly, the polyphenolic compound resveratrol, has been previously shown to improve the oxidative status of the muscle [242] concomitantly with improving insulin sensitivity [243] and may increase ANT expression [244,245].

Therefore, we investigated the potential link between submaximal ADP-stimulated respiration rates, ROS generation and insulin sensitivity in a widely used model of T2DM, the ZDF rat. We hypothesized that submaximal ADP-stimulated respiration would be repressed in
the ZDF rat and that resveratrol would prevent this decrease and this would be associated with an improved cellular redox state.

5.3 Methods

5.3.1 Animals

Male ZDF rats (Charles River) were housed in individual cages, with a reverse 12:12 h light-dark cycle, and were provided with food and water *ad libitum*. Rats were acclimatized to the animal housing unit for 10 days prior to the start of the feeding protocol. Lean control (LC) rats (n=13) were fed a stock diet. Twenty six ZDF rats (n=13 each group) were randomly assigned to either a stock diet (ZDF) or a stock diet supplemented with resveratrol (Cayman Chemicals, 200mg/kg body weight/day (ZDF+RESV)) for 6 weeks. Previous work has shown that ZDF rats are insulin resistant at 6 weeks and become T2DM by 12 weeks, therefore we attempted to delay the onset of T2DM by treating with resveratrol from 5 weeks until 11 weeks of age [246]. Thereafter, animals were anaesthetized with isoflurane and the soleus (for glucose uptake) and red gastrocnemius (all other experimental procedures) muscles were removed. Originally, this study was planned with n=8 for all experiments. However, upon completing the first set of experiments additional animals (n=5) were acquired to determine a link between decreased ADP transport and mitochondrial ROS emission (i.e. ROS in presence of ADP (Figure 6B). Additionally, ADP titrations (Figure 3), and maximal ROS (Figure 6A) measurements were repeated as these are key findings and we aimed to ensure repeatability (n=13). This study was approved by the University of Guelph Animal Care Committee, and conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health.
5.3.2 *Basal and insulin-stimulated glucose transport*

Glucose transport was measured as previously described [247]. Briefly, pregassed (95% O₂-5% CO₂) Medium 199 containing 0.1% BSA was warmed to 30°C and used as a base for all glucose uptake buffers. Insulin (10 mU/mL) (Humulin R; Eli Lilly, Toronto, Ontario, Canada) was added to all buffers for the insulin-stimulated condition. Excised soleus strips were placed in glass vials containing preincubation buffer for 30 minutes in the presence or absence of insulin (10 mU/mL; maintained in all subsequent buffers). The preincubation buffer consisted of base buffer with 8 mM glucose and 32 mM mannitol. The soleus strips were then washed in two glucose-free buffers, which contained 4 mM pyruvate and 36 mM mannitol, for 10 minutes each. The soleus strips were then incubated in base buffer with 4 mM pyruvate, 8 mM 3-O-[³H]methyl-d-glucose (0.5 μCi/mL; ARC, St. Louis, MO), and 28 mM [¹⁴C] mannitol (0.2 μCi/mmol; GE Healthcare, Baie d'Urfe, Quebec, Canada) for 20 minutes (insulin stimulated) or 40 minutes (basal) conditions. Soleus strips were then weighed and digested for 10 minutes in 1 mL of 1 M NaOH at 95°C. Muscle from each sample was sampled in duplicate and analyzed by liquid scintillation counting, from which glucose transport was calculated.

5.3.3 *Preparation of permeabilized fibres*

The preparation of permeabilized muscle fibre bundles (PmFB) was adopted from prior publications [220], as we have previously reported [248]. Following dissection of red gastrocnemius, fibre bundles (~2 mg) were separated with fine forceps under a binocular dissecting microscope in BIOPS buffer containing, CaK₂EGTA (2.77 mM), K₂EGTA (7.23 mM), Na₂ATP (5.77 mM), MgCl₂·6H₂O (6.56 mM), Na₂Phosphocreatine (15 mM), Imidazole (20 mM), Dithiothreitol (0.5 mM) and MES (50 mM). Following separation, fibre bundles were placed in BIOPS containing 40 μg/mL saponin, agitated for 30min and then fibres prepared for
respiration were washed in respiration buffer (MIRO5) containing EGTA (0.5 mM), MgCl₂·6H₂O (3 mM), K-lactobionate (60 mM), KH₂PO₄ (10 mM), HEPES (20 mM), Sucrose (110 mM) and fatty acid free BSA (1 g/L). Fibres prepared for H₂O₂ emission were washed in Buffer Z containing K-MES (110 mM), KCl (30 mM), EGTA (1 mM), K₂HPO₄ (10 mM), MgCl₂·6H₂O (10 mM). PmFB were left in cold MIRO5 or Buffer Z until analysis.

5.3.4 Permeabilized muscle fibre respiration

Mitochondrial respiration was measured in PmFB by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria) at 37°C and room air saturated oxygen tension in the presence of 25 µM blebbistatin to ensure PmFB relaxation [220]. In the presence of 5 mM ADP and 2 mM malate, separate PmFB from the same animal were used determine complex I (pyruvate) and complex I+II (pyruvate+succinate) respiration rates. Separate PmFB from the same animal in the presence of 5 mM ADP and 2 mM malate were then used determine the kinetic properties of glutamate (0, 100, 175, 250, 500, 2000, 4000 µM) and pyruvate (0, 15, 30, 50, 75, 150, 500, 1000 µM) stimulated respiration. ADP (0, 100, 175, 250, 500, 1000, 2000, 4000, 6000 µM) stimulated respiration was determined in the presence of 10 mM pyruvate and 2 mM malate. To measure palmitoyl-CoA (P-CoA) supported respiration, MIRO5 + 5 mM ADP, 2 mM malate, 2 mM L-carnitine was used as the respiration medium. P-CoA (150 µM) was added to initiate respiration. To measure palmitate supported respiration, MIRO5 + 1 mM ATP + 5 mM ADP, 2 mM malate, 2 mM L-carnitine + 1 mM CoA was used as the respiration medium. Palmitate (250 µM) was added to initiate fatty acid-supported respiration. Exogenous cytochrome c (10 µM) was added at the end of all respiration experiments to ensure outer mitochondrial membrane integrity. A cytochrome c response was not observed for any respiration experiment.
5.3.5 Permeabilized muscle fibre mitochondrial $H_2O_2$ emission

Measurement of mitochondrial $H_2O_2$ emission was similar to previously described [147]. Briefly, mitochondrial $H_2O_2$ emission was determined fluorometrically (Lumina, Thermo Scientific) in a constantly stirring cuvette at 37°C (peltier controlled). PmFB were placed in a cuvette containing Buffer Z supplemented with 25 μM blebbistatin, 40 U/mL of CuZnSOD, 10 μM Amplex Red (Invitrogen), 0.5 U/mL horseradish peroxidase with or without 100 μM ADP. Mitochondrial $H_2O_2$ emission was initiated by the addition of 10 mM succinate. The rate of $H_2O_2$ emission was calculated from the slope (fluorescence/min), after subtracting the background, from a standard curve established with the same reaction conditions and normalized to dry muscle weight.

5.3.6 Glutathione measurements

GSH and GSSG measurements were determined as previously described [147]. Briefly, 2 muscle chips (in triplicate wells) were used to determine GSH and 2 separate muscle chips (in triplicate wells) were used to determine GSSG in the presence of the scavenger methyl-2-vinylpyridinium triflate (M2VP). Total GSH and GSSG were measured as per manufacturer’s instructions (Oxis International, Inc).

5.3.7 Western Blotting

All samples were analyzed for total protein (BCA protein assay), and samples were separated by electrophoresis on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes as we have previously reported [224]. Commercially available antibodies were used to detect complex I subunit NDUFB8 (Mitosciences), complex II subunit 30kDa (Mitosciences), complex III subunit core 2 (Mitosciences), complex IV subunit I (Mitosciences), ATP synthase subunit alpha (Mitosciences), ANT1 (Mitosciences), ANT2 (abcam) and
cytochrome c oxidase complex IV (Invitrogen). All samples for a given protein were transferred and developed on the same membrane to limit variation. Blots were quantified using chemiluminescence and the FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA, USA).

5.3.8 Statistics

A one-way ANOVA with Newman-Keuls Multiple Comparison Test post-hoc analysis was utilized for all comparisons except for Table 1 where a Student’s t-test was used to compare ZDF and ZDF+RESV groups and Figure 1A where a two-way ANOVA was used. Statistical significance was accepted with a p value ≤ 0.05.

5.4 Results

5.4.1 Resveratrol recovers skeletal muscle insulin sensitivity in ZDF rats

We first utilized an incubated soleus muscle preparation to examine basal and insulin-stimulated glucose transport to validate our experimental animals. Basal glucose uptake was not different in any condition (Figure 5.1A). Insulin increased glucose uptake in lean control animals (~40%), while insulin did not stimulate glucose uptake in untreated ZDF rats (p>0.05). In contrast, administration of resveratrol in ZDF rats increased insulin-stimulated glucose uptake ~40%. As a result, insulin-stimulated glucose uptake was only repressed in the soleus muscle of untreated ZDF animals (Figure 5.1A).
Figure 5.1: Depressed insulin-stimulated glucose uptake is not associated with decreased mitochondrial respiratory capacity. A) Soleus strips from control ZDF rats (ZDF) were unable to significantly promote glucose (3-O-methyl-glucose) uptake in response 10 mU/mL of insulin and resveratrol treatment (ZDF+RESV) recovered this decrement. B) Mitochondrial complex I and complex I+II supported respiratory capacity is not different between lean control (LC), ZDF, and ZDF+RESV. Respiratory control ratios were also unchanged: LC=7.59±0.63, ZDF=7.65±0.63 and ZDF+RESV=8.08±0.27. Values represent means±SEM. N=8. *Significantly different (p<0.05) from basal value. φSignificantly different from corresponding insulin-stimulated values.
PM; pyruvate+malate,
PMD; pyruvate+malate+ADP,
PMDS; pyruvate+malate+ADP+succinate.
PMDSc; pyruvate+malate+ADP+cytochrome c
5.4.2 Submaximal ADP-stimulated respiration is impaired in ZDF rats

Mitochondrial dysfunction has been suggested to be a cause of skeletal muscle insulin resistance, and is classically defined as a reduction in mitochondrial content and/or an intrinsic impairment within mitochondria [127,239]. To examine this hypothesis we utilized PmFB, an *in situ* mitochondrial preparation that retains the inherent cellular architecture, to assess mitochondrial function [218]. Insulin sensitivity was not associated with alterations in the maximal capacity of the electron transport chain (complex I and complex I+II) as LC, ZDF and ZDF+RESV displayed similar maximal rates (Figure 5.1B). To delineate mitochondrial respiratory “capacity” from mitochondrial respiratory “sensitivity” we ran titration curves for glutamate and pyruvate which supply reducing equivalents to complex I. Neither substrate showed a difference in $V_{\text{max}}$ or a difference in sensitivity indicating that electron transport chain function was similar between groups (Figure 5.2A and B).

![Figure 5.2: Glutamate (A) and pyruvate (B) titrations reveal similar respiration rates across all concentrations examined.](image)

In contrast, submaximal ADP-stimulated respiration rates (250, 500, 2000 µM ADP (Figure 5.3)) in ZDF rats were significantly lower compared to LC and treatment with resveratrol recovered submaximal ADP-stimulated respiration back to LC values. These data are the first to analyze submaximal ADP-stimulated respiration in a model of T2DM and suggest that
mitochondrial dysfunction is present when assessed in this manner (Figure 5.3), while electron transport capacity is not altered.

**Figure 5.3: Submaximal ADP-stimulated respiration is significantly lower in ZDF animals and resveratrol recovers this decrease.** At ADP concentrations of 250, 500 and 2000 μM, ZDF respiration rates are significantly lower than both lean control (LC) and resveratrol supplemented (ZDF+RESV) groups. Black circles with solid line represent LC, black squares with dotted line represent ZDF and black triangles with a solid line represent ZDF+RESV. Values represent means±SEM. N=13. *Significantly (p<0.05) different from LC and ZDF+RESV.

5.4.3 Palmitate supported respiration is elevated with resveratrol treatment

The transport of long chain fatty acids into the mitochondria represents a rate limiting step for long chain fatty acid oxidation and involves a series of reactions; i) the activation of a long chain fatty acids to an acyl-CoA moiety by acyl-CoA synthetase (ACS) ii) the conversion of acyl-CoA to fatty acylcarnitine via carnitine palmitoyltransferase I (CPT-I), iii) transport of acylcarnitine across the outer and inner mitochondrial membrane via carnitine:acylcarnitine translocase and iv) the re-conversion of acyl-CoA from acylcarnitine within the mitochondrial matrix via CPT-II [8,27]. This transport process is traditionally viewed as being governed by CPT-I activity and both palmitate and P-CoA supported respiration rates are regulated by this enzyme [28], however palmitate is also regulated by ACS. Therefore, we assessed both lipids
with respect to maximal respiration. Palmitate and P-CoA supported state III (5 mM ADP) respiration rates were not different between LC and ZDF (Figure 5.4A and B, respectively). In contrast, state III palmitate (p<0.05) and P-CoA (p=0.08) supported respiration rates were higher in resveratrol treated animals in the face of unchanged respiratory capacity (Figure 5.4).

Figure 5.4: State III fatty acid supported respiration rates are elevated with resveratrol treatment. A) Resveratrol supplementation increased state III (5 mM ADP) palmitate (250 μM) and B) palmitoyl-CoA (P-CoA, 150 μM) supported respiration rates. Values represent means±SEM. N=5 for (A) and n=8 for (B). *Significantly (p<0.05) different from LC.

5.4.4 Mitochondrial and adenine nucleotide translocase content

Previous reports have shown that resveratrol treatment can induce mitochondrial biogenesis [243]. Therefore, to examine whether our respiration data could be explained by changes in mitochondrial content we examined the content of six mitochondrial proteins (representative blots shown in Figure 5.5A). In ZDF rats treated with resveratrol there was no induction of mitochondrial biogenesis (supported by unchanged maximal respiration in Figure 1B) to account for the increased submaximal ADP-stimulated respiration observed in the current study. Considering submaximal ADP-stimulated respiration appears to be depressed in the ZDF rat and recovered with resveratrol supplementation, we next measured the protein expression of ANT1 and 2 to investigate if these differences in ADP-stimulated respiration were affiliated with the content of ADP transport proteins. ANT1 protein abundance was not different across the
three groups (Figure 5.5B), however, ANT2 protein content was significantly reduced in the ZDF group compared to LC (-34%; Figure 5.5C) and following resveratrol supplementation, ANT2 protein content was not different from LC values. The increase in ANT2 protein content may help account for the improved submaximal ADP-stimulated respiration observed with resveratrol supplementation.

Figure 5.5: Mitochondrial content and adenine nucleotide translocase isoform content. A) Muscle homogenates from lean control (LC), ZDF control rats (ZDF) and ZDF rats supplemented with resveratrol (ZDF+RESV) were analyzed for protein content of mitochondrial markers (30 μg loaded); complex I subunit NDUFB8 (Mitosciences), complex II subunit 30kDa (Mitosciences), complex III subunit core 2 (Mitosciences), complex IV subunit I (Mitosciences), ATP synthase subunit alpha (Mitosciences). No significant differences were found. Two representative blots shown. B) There were no differences in ANT1 content between groups (20 μg protein loaded). C) ZDF rats have a decrease in ANT2 expression and supplementation with resveratrol increases ANT2 expression up to LC values (40 μg protein loaded). Values represent means±SEM. N=8. *Significantly (p<0.05) different from LC and ZDF+RESV.
5.4.5 Mitochondrial $H_2O_2$ emission and cellular redox state

We next speculated a potential mechanism of action to account for how submaximal ADP-stimulated respiration could be related to insulin sensitivity. Mitochondrial derived ROS have been linked to insulin resistance and the provision of ADP to mitochondria can decrease ROS production \([94,147]\). Therefore we examined mitochondrial $H_2O_2$ emission rates (a marker of mitochondrial ROS production) in the presence and absence of a submaximal ADP concentration (100 µM). Between groups, the maximal capacity of mitochondria to emit $H_2O_2$ was not different (Figure 5.6A). In contrast, in the presence of a submaximal ADP concentration, $H_2O_2$ emission rates were ~34% higher in the ZDF group compared to LC (p=0.06) (Figure 5.6B). Following treatment with resveratrol, $H_2O_2$ emission rates were ~40% lower (p<0.05) in the ZDF+RESV group compared to ZDF (Figure 5.6B).

![Figure 5.6](image)

**Figure 5.6:** In ZDF rats, supplementation with resveratrol decreases the propensity for $H_2O_2$ emission in the presence of 100 µM ADP. A) The maximal capacity of the mitochondria to emit $H_2O_2$ was not different between lean controls (LC), ZDF control (ZDF) and ZDF supplemented with resveratrol (ZDF+RESV). B) In the presence of 100 µM ADP, the capacity of the mitochondria to emit $H_2O_2$ was significantly lower in the ZDF+RESV group compared to ZDF. N=13 for (A) and n=5 for (B). All $H_2O_2$ emission measurements were done in duplicate. *Significantly different (p<0.05) different from ZDF.
These data suggest that resveratrol treatment may improve insulin sensitivity by enhancing ADP transport which results in a decrease in mitochondrial ROS production. To determine if these changes in mitochondrial H$_2$O$_2$ emission rates correlated with the cellular redox state we measured the ratio of reduced glutathione to oxidized glutathione (GSH:GSSG ratio). The LC animals surprisingly had the lowest GSH/GSSG ratios, suggesting they had the greatest oxidative stress despite not being overfed (Table 5.1). It is therefore likely that the ZDF animals have evolved compensatory adaptations to accommodate the chronic fuel oversupply. Therefore, we have performed statistical analysis to determine the effect of resveratrol supplementation in ZDF animals only (Table 5.1).

<table>
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<td>40.6 ± 2.51</td>
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<tr>
<td>ZDF</td>
<td>6.46 ± 0.53</td>
<td>0.11 ± 0.02</td>
<td>64.1 ± 7.95</td>
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<tr>
<td>ZDF+RESV</td>
<td>6.72 ± 0.62</td>
<td>0.08 ± 0.01</td>
<td>86.1 ± 13.3*</td>
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ZDF rats supplemented with resveratrol (ZDF+RESV) have a higher reduced glutathione (GSH):oxidized glutathione (GSSG) ratio compared to ZDF. Values represent means±SEM. N=8. *Significantly (p<0.05) different from ZDF.

Importantly, resveratrol treatment increased the GSH:GSSG ratio, independent of altering total GSH content within the ZDF animal (Figure 5C), suggesting that the skeletal muscle of the ZDF+RESV group was less oxidized than the ZDF group, supporting our functional PmFB data. Altogether, these data demonstrate a potential link between mitochondrial function and mitochondrial ROS production in the etiology of skeletal muscle insulin resistance.
5.5 Discussion

In the current study we provide evidence for a potential link between mitochondrial function and ROS emission in the context of skeletal muscle insulin sensitivity. Specifically, we show that mitochondrial respiration at submaximal ADP concentrations is impaired in a model of T2DM, an observation that coincided with decreased ANT2 protein content and a decreased ability of ADP to attenuate mitochondrial ROS emission. We further show that resveratrol treatment in ZDF rats recovers insulin sensitivity, submaximal ADP-stimulated respiration and ANT2 protein content in concert with improving ADP attenuation of mitochondrial ROS and cellular redox balance (GSH/GSSG ratio). Altogether, our data suggest a novel link between mitochondrial function, ROS emission and insulin sensitivity, and places a new emphasis on ANT2 protein content as a potential regulator of insulin sensitivity.

5.5.1 Mitochondrial dysfunction in T2DM

There is considerable controversy surrounding the notion of mitochondrial dysfunction as a cause of insulin resistance [127,129,130,132-135,236-238] which may be reflective of differences in methodologies and experimental approaches. In the current study, there were no differences in maximal ADP-stimulated respiration, supporting three previous studies in ZDF rats that utilized in vivo P-MRS and ex vivo determinations of mitochondrial function [133-135]. In contrast, titrating ADP revealed attenuated respiration rates in ZDF animals at submaximal ADP concentrations. Therefore we suggest that mitochondrial dysfunction, as defined by a decrease in submaximal ADP-stimulated respiration, is associated with the progression of T2DM. While this method of ADP titration has not previously been utilized to study skeletal muscle mitochondrial function in insulin resistant muscle, another research group has utilized titrations of various substrates in human muscle from type II diabetics [249]. In their study,
Larsen et al (2011) unexpectedly found that complex I and complex II sensitivity were increased in humans with T2DM [249]. Within the context of mitochondrial ROS production, increased mitochondrial substrate sensitivity may be detrimental as proton motive force would be generated more rapidly per reducing equivalent supplied, a suggestion that would be exacerbated in the presence of attenuated submaximal ADP transport. Clearly, future work should examine submaximal ADP-stimulated respiration in humans with T2DM to address this speculation.

5.5.2 Resveratrol and mitochondrial biogenesis

Resveratrol is a polyphenol compound found primarily in the skin of grapes and the beneficial effects of resveratrol have been associated with enhanced mitochondrial function/biogenesis in skeletal muscle through a SIRT/AMPK/PGC-1α axis [250-252]. In contrast to previous reports, resveratrol supplementation in the ZDF rat did not induce mitochondrial biogenesis [242,243]. In an attempt to reconcile this disparity, previous work in the ZDF rat model has shown that AMPK activity is repressed [54] and AMPK activity has been shown to be essential for resveratrol-induced mitochondrial biogenesis [252]. Additionally, in ZDF rats, PGC-1α is re-distributed to the nucleus indicating that one of the primary molecular mechanisms to induce mitochondrial biogenesis is already present in the ZDF rat [135]. Therefore, supplementation with resveratrol to induce mitochondrial biogenesis in the ZDF rat may be ineffectual as the biogenesis stimulus is already present. We are unaware of previous work examining resveratrol-induced mitochondrial biogenesis in the ZDF rat model.

5.5.3 Resveratrol and mitochondrial fatty acid supported respiration

Following resveratrol supplementation, we observed an increase in maximal fatty acid supported respiration (both P-CoA and palmitate) in the face of unchanged respiratory capacity. These data match previous work in resveratrol supplemented humans examining mitochondrial
bioenergetics utilizing PmFB [253]. Timmers et al (2011) observed that octanoylcarnitine supported respiration and citrate synthase activity were elevated while respiratory capacity was unchanged in obese humans following 30 days of resveratrol supplementation [253]. Octanoylcarnitine bypasses CPT-I and therefore has direct access to the mitochondrial matrix where beta oxidation takes place. Considering that all fatty acid supported (palmitate, P-CoA and octanoylcarnitine) respiration rates increase with resveratrol supplementation, resveratrol appears to influences fatty acid oxidation independent of CPT-I. Although CPT-I has traditionally been viewed as the primary rate-limiting step for mitochondrial fatty acid oxidation [28] an earlier study pointed out that tissue acylcarnitine levels are increased under conditions known to involve increased rates of fatty acid oxidation [91]. The increase in acylcarnitine concentration suggests that the rate-limiting step must be downstream of CPT-I because if CPT-I was rate-limiting there would be no excess substrate to undergo the carnitine acetyltransferase (CrAT) reaction to produce the acylcarnitines. Therefore, altered regulation of fatty acid oxidation downstream of CPT-I (potentially beta oxidation) is likely with resveratrol supplementation and has been previously observed [242]. Classically, the regulation of beta oxidation has been attributed to the balance of substrates and products, however, beta oxidation can also be regulated by the cellular redox state [254] suggesting a possible mechanism by which resveratrol could promote fatty acid oxidation independent of CPT-I.

5.5.4 Proton leak and post-translational modifications of ANT

ANT has previously been suggested to account for 50-66% of basal proton leak and it has been speculated that an increase in ANT protein could lower ROS emission through the dissipation of proton motive force [241]. However, in the current study, non ADP-stimulated respiration (state IV) was unchanged following resveratrol administration despite increased
ANT2 protein content. These data suggest that ANT protein does not independently determine basal proton leak. To explain this discrepancy, we hypothesize that post-translational regulation of ANT may induce the uncoupling function of ANT similar to the mechanism surrounding the induction of UCP2 and UCP3 mediated uncoupling [102]. Indeed, considering the critical function ANT has in regulating energy homeostasis, it is not surprising that numerous studies have observed extensive post-translational regulation of ANT function. Evidence now exists that ANT1 can be phosphorylated on tyrosine 194 and phosphorylation is associated with an increase in activity [178]. Exposure to high oxygen tension or oxidative stress-induced glutathionylation result in decreased ADP transport into the mitochondrial matrix [179,180]. Additionally, long chain fatty acyl-CoA moieties can directly suppress ANT function [181]. While these data indicate that ANT is externally regulated, this does not diminish the physiological implications of increasing ANT2 protein in the current study. Instead, the improvements in cellular redox balance and rates of fatty acid supported respiration following resveratrol supplementation likely further improve ANT function as resveratrol has been linked to reducing oxidative stress and increasing fatty acid metabolism (current study and others [242,253]).

5.5.5 Adenine nucleotide translocase content, oxidative stress and insulin resistance

Taking into account our finding that submaximal ADP-stimulated respiration is depressed in T2DM, we attempted to identify a mechanism by which ADP dynamics could be related to insulin resistance. Previous work has highlighted a link between skeletal muscle oxidative stress and insulin sensitivity as mitochondrial targeted antioxidant interventions and genetic approaches that over-express antioxidant enzymes within mitochondria protect against diet-induced skeletal muscle insulin resistance [147,155]. Our work extends these findings by demonstrating that improved ADP dynamics are associated with decreased H$_2$O$_2$ emission and improved cellular
redox state, which are all associated with improved insulin sensitivity. Our hypothesis is as follows: increasing ANT2 expression generates an environment whereby mitochondrial ROS emission is mitigated at a faster rate in the presence of low ADP concentrations. Considering ADP levels in skeletal muscle are low in the absence of exercise, an increase in ANT content and the resultant augmented ADP sensitivity would protect against ROS-induced insulin resistance [147,153,240]. To further support our hypothesis that ADP dynamics are linked to ROS production and therefore insulin resistance, previous work in a liver cell line has shown that overexpression of ANT2 decreases oxidative stress and enhances mitochondrial function [255]. Additionally, mitochondria isolated from ANT1-deficient mice produce greater amounts of ROS, resulting in damaged mtDNA and cellular dysfunction [256], while in cardiac tissue, overexpression of ANT1 protects against the development of diabetes-induced cardiomyopathy [257]. Altogether, we propose a novel mechanism whereby improved ADP dynamics decrease mitochondrial ROS emission and maintain cellular redox balance resulting in enhanced insulin sensitivity. While ZDF animals share a number of similar traits with human T2DM including hyperglycemia, insulin resistance, hyperlipidemia [258] and elevated acylcarnitines [56], we recognize that the ZDF animal model is not fully representative. This is apparent as the GSH:GSSG is higher in ZDF animals compared to LC while in insulin resistant and type II diabetic humans a reduction in this ratio has been reported [147]. Previous studies showing a protection of insulin sensitivity by mitigating mitochondrial ROS production have utilized acute (6 hours [152]) or short term (4-6 weeks [147,153]) antioxidant interventions. However, a recent study has divorced the relationship between the oxidative state of the muscle and insulin sensitivity when treating with a mitochondrial targeted antioxidant chronically (16 weeks [156]). Therefore, our work, in combination with others [147,152,153,156] may suggest that ROS is
important as an acute barometer of fuel oversupply to induce insulin resistance, however, this barometer function becomes disconnected from insulin sensitivity chronically. Nonetheless, resveratrol improved the redox state within the ZDF animal concomitantly with improved skeletal muscle glucose uptake. Therefore, it will be important to discern whether our current observations regarding ADP dynamics and ANT2 content in ZDF animals extend to human type II diabetic individuals.

5.5.6 Conclusion

In summary, our data suggest that mitochondrial dysfunction is present in skeletal muscle insulin resistance when assessed at submaximal ADP concentrations and that ADP dynamics may influence skeletal muscle insulin sensitivity through alterations in the propensity for mitochondrial ROS emission. We found that submaximal ADP-stimulated respiration is impaired in ZDF animals and resveratrol supplementation improved submaximal ADP-stimulated respiration concomitantly with enhancing insulin sensitivity. The favorable effects of resveratrol may be mediated through an improved ability of ADP to attenuate mitochondrial ROS emission.
CHAPTER 6:

INTEGRATIVE DISCUSSION
6.1 Discussion

This thesis focused on two broad components within mitochondrial metabolism; the regulation of mitochondrial fatty acid transport and the regulation of ADP supply in the context of ROS emission and insulin sensitivity. A number of findings have been presented, specifically, 1) a functional role for mitochondrial FAT/CD36 in mitochondrial LCFA oxidation was confirmed and the topology of this protein along the OMM was expanded upon, 2) M-CoA inhibition kinetics of CPT-I were re-evaluated in PmFB and a regulatory role of LCFA-CoA on M-CoA inhibition kinetics was established and 3) submaximal ADP-stimulated respiration rates and ANT2 content are depressed in the ZDF rat and resveratrol supplementation prevents these decrements.

The first two projects (Chapters 3 and 4) were centered on mitochondrial LCFA transport and oxidation which, as mentioned in Chapter 1, may be related to insulin resistance and T2DM. The third study (Chapter 5) contained a T2DM component as the ZDF rat model was utilized. Therefore, to coordinate this discussion, I hope to integrate the results from Chapters 3 and 4 within the context of T2DM, in addition to expanding on the results from Chapter 5. In order to do this, an overarching view of insulin resistance has to be defined. In general, my belief is that the cause of insulin resistance is a result of excess fuel intake in the absence of a similar increase in demand. The balance between supply and demand at the cellular level is primarily mediated by the mitochondria such that any system involved in regulating mitochondrial fuel supply/demand is important. Within the studies presented, FAT/CD36, CPT-I and ANT2 may all have intricate roles in mediating supply/demand and appear to be of significant importance.
6.2 Direct influence of fatty acid oversupply: implications for T2DM

6.2.1 Role of mitochondrial FAT/CD36

Evidence is provided in Chapter 3 that FAT/CD36 is located upstream of both CPT-I and ACS on the outer mitochondrial membrane [224]. As such, FAT/CD36 has emerged as a unique protein, as it can be induced to translocate to the mitochondria to regulate mitochondrial fatty acid oxidation [5,46,84]. This effect may be particularly advantageous during exercise when the energetic demand for LCFA oxidation is increased substantially. Additionally, previous work in ZDF rats found that FAT/CD36 content on the mitochondria is elevated in the basal condition [135]. An increase in FAT/CD36 on the mitochondrial membrane in the obese state may be detrimental as this would increase the supply of lipid substrate and could exacerbate the negative consequences of mitochondrial fatty acid oversupply. It is currently unknown if this chronic redistribution of FAT/CD36 is influencing the reported mitochondrial fatty acid oversupply in the obese and type II diabetic state and future work evaluating the role of mitochondrial FAT/CD36 in T2DM is warranted [56,57].

6.2.2 Cellular elevation of LCFA-CoA as a perilous side effect of lipid oversupply

In Chapter 5 it was argued that ANT2 content is accounting for the decrease in submaximal ADP-stimulated respiration in ZDF rats, however, other factors have been shown to influence ADP transport and regulate ANT function independent of ANT content. Firstly, LCFA-CoA have been consistently shown to inhibit ANT function as these compounds are competitive for ADP binding on ANT [181-183]. Although this phenomenon was observed 39 years ago, I have recently generated data in respiring PmFB to show how fast and dynamic this inhibition can occur (Figure 6.1).
Figure 6.1: The addition of P-CoA (60 μM) rapidly inhibits ADP transport in PmFB.

In mouse skeletal muscle, during state III respiration (1), the addition of P-CoA (2) decreases respiration rapidly by inhibiting ANT. Addition of an uncoupler (3) promotes respiration independent of ADP transport and rapidly recovers respiration proving that the decrease was due to ADP transport.

Blue line represents oxygen concentration
Red line represents rate of oxygen consumption
GM = glutamate+malate
GMD = glutamate+malate+ADP
P-CoA = addition of 60 μM palmitoyl-CoA (P-CoA)
DNP = addition of uncoupler 2,4-Dinitrophenol (DNP)

The inhibitory effect of LCFA-CoA has been observed when examining both the mitochondrial and cytosolic side of ANT [182,259]. This is an important point, as it was observed in Chapter 4 that elevations in cytosolic LCFA-CoA can promote an increase in mitochondrial LCFA entry. Therefore, any increase in cytosolic LCFA-CoA could inhibit ANT and these elevated cytosolic levels would promote increases in mitochondrial LCFA-CoA which could also inhibit ANT. Thus, by inhibiting ANT from both the cytosolic and mitochondrial sides, LCFA-CoA could promote mitochondrial ROS and act as an amplifier for the proposed barometer function of mitochondrial ROS [95].

It should also be noted that membrane potential is a primary regulator of the intrinsic function of ANT as the energization state of the mitochondria strongly predicts the rate of ADP/ATP antiport [260]. In the uncoupled state (de-energized), ADP and ATP are transported in both directions through ANT at equal rates [261]. However, as membrane potential rises, ADP influx (compared to efflux) is more and more preferred to the point where ANT is 20 fold more likely to influx ADP vs. ATP at a physiological membrane potential (+180 mV).
characteristic effectively coordinates oxidative phosphorylation with ETC activity [260]. In the context of the overfed state, membrane potential is elevated which would actually promote ADP uptake. Therefore, a chronically elevated membrane potential may be compensatory in an attempt to maintain ADP transport in the face of depressed ANT2 protein content. However, in contrast to the overfed state, in diabetic mitochondrial heart tissue, membrane potential is reduced [262] which would exacerbate the potential repressed ADP transport in T2DM. Investigations into mitochondrial membrane potential in diabetic skeletal muscle are currently lacking.

In addition to inhibiting ANT [183] and M-CoA binding [30,73], LCFA-CoA have also been shown to influence the activity of glycogen synthase [263], glucose-6-phosphatase [264], ACC [265], hormone sensitivity lipase [266] and promote calcium release from the terminal cisternae [267]. With respect to insulin sensitivity, LCFA-CoA can also influence glucose uptake into skeletal muscle by decreasing hexokinase activity [268] and promote DAG and ceramide synthesis [269,270]. Therefore, LCFA-CoA are highly reactive by nature and methods to reduce the levels of these lipid intermediates is of importance. Triacin C is a potent inhibitor of ACS [271] and investigations into triacin C treatment could be utilized to examine if decreasing the conversion of LCFA to LCFA-CoA is protective against diet-induced insulin resistance. Interestingly, PPARλ agonists such as thiazolidinediones (TZD) have also been shown to significantly inhibit ACS activity [272]. Therefore, inhibition of ACS by TZD may help explain the PPARλ independent effects of TZD treatment [273,274] as LCFA-CoA levels decrease following TZD treatment [53].

To link the previous two sections regarding FAT/CD36 and LCFA-CoA (6.2.1 and 6.2.2), it could be argued that the redistribution of FAT/CD36 to the mitochondrial membrane in
T2DM [135] could result in elevated mitochondrial LCFA-CoA levels. This connection suggests that if mitochondrial FAT/CD36 does have a detrimental impact in the context of T2DM, the effect may be mediated through increasing LCFA-CoA.

### 6.2.3 Potential role for palmitoylation

In addition to regulating CPT-I and ANT, LCFA-CoA is also a substrate for palmitoylation. Palmitoylation is a posttranslational modification whereby fatty acids (predominantly palmitate) are covalently bonded to cysteine residues through a thioester linkage [275].

![Figure 6.2: Mechanism of palmitoylation/acylation.](http://lipidlibrary.aocs.org/lipids/protlip/index.htm)

A wide variety of proteins that play key roles in cellular function are palmitoylated and palmitoylation is rapidly reversible and can positively or negatively influence cellular function illustrating a dynamic role for this reaction [276,277]. The mechanism by which this reversible posttranslational modification occurs has not been well characterized, however current evidence indicates that palmitoylation can occur enzymatically via a family of protein acyltransferases and
thioesterases (Figure 6.2) [278,279]. In addition, a non-enzymatic, mass action reaction known as autoacylation has been shown to occur [276]. Importantly, autoacylation of a protein occurs in the presence of elevated LCFA-CoA concentrations, suggesting that a given increase in LCFA-CoA will influence the propensity for palmitoylation [276]. Furthermore, although not directly relevant to energy metabolism, a mitochondrial enzyme involved in urea metabolism known as carbamoyl-phosphate synthetase I (CPS-I) can be palmitolyated within its active site, causing inhibition of enzymatic activity [280]. Palmitoylation of purified CPS 1 occurs at P-CoA concentrations within the physiological range found in mitochondria, indicating that autoacylation may occur in vivo [281]. Therefore, evidence does exist that an increase in mitochondrial LCFA-CoA could be detrimental via promotion of palmitoylation. In the context of Chapter 4 whereby an increase in LCFA-CoA causes an increase in LCFA-CoA entry into the mitochondria, the negative consequences of increased LCFA-CoA are apparent.

FAT/CD36 can be palmitoylated and previous work has shown that a mutation within the palmitoylation site on FAT/CD36 prevents this protein from correctly inserting into lipid rafts [282,283]. However, this investigation was done in COS-7 cells (monkey kidney tissue) and therefore FAT/CD36 palmitoylation at the level of the mitochondria in mature mammalian tissue has yet to be examined. If we assume that palmitoylation is required for optimal FAT/CD36 function, the hypothesis may be that when fatty acid supply is elevated, FAT/CD36 can be palmitoylated and further activated to promote fatty acid uptake into the mitochondria. During exercise this would be advantageous, however, in the absence of cellular demand, this effect would be deleterious. Websites have been designed to predict palmitoylation sites on various proteins [284,285] and future work with mitochondrial protein palmitoylation is warranted.
6.3 Indirect effect of fatty acid oversupply – increased mitochondrial ROS

In the absence of ATP demand and/or uncoupling, provision of excess reducing equivalents will increase membrane potential and create a “back pressure” on electron transport within the ETC [95]. This back pressure is a result of an imbalance between energy supply and demand. However, various studies have been designed to promote fatty acid oxidation without considering the nature of metabolic balance. Mouse models with elevated fatty acid oxidative potential, such as PPARα [286] and PGC-1α overexpressing [287] mice, show that despite an increase in fatty acid oxidative potential (including a 2.4 fold increase in mitochondrial content [287]), if supply outstrips demand, insulin resistance will occur regardless of the “potential” to burn the fuel. This excess energy supply will increase mitochondrial ROS and in light of the association between mitochondrial ROS and insulin sensitivity [137-139,147,150-155,240], we can view mitochondrial ROS as a mediator between fuel oversupply and insulin resistance. This mitochondrial-centric view of insulin resistance is supported by various lines of evidence which are intrinsically connected to a balance between energy supply and demand and are shown/described in Figure 6.3:

1) Increase proton motive force turnover
2) Reduce energy supply
3) Divert lipid-derived substrate away from mitochondria
4) Mitigate \( \text{O}_2^- \) and/or \( \text{H}_2\text{O}_2 \) with antioxidants
Figure 6.3: Mitochondrial-centric view of mechanisms to improve insulin sensitivity.

1) Mitochondrial uncoupling via pharmacologic agents such as 2, 4 dinitrophenol (DNP) [157,158] or overexpression of uncoupling proteins (UCP3) [159] will increase proton motive force turnover and are associated with improved insulin sensitivity. ATP consumption via exercise [288], βGPA supplementation [289,290] or metformin [291] promotes proton motive force turnover and is associated with improved insulin sensitivity. 2) Lowering energy intake is associated with improved insulin sensitivity and possibly the recovery of beta cell function [292]. 3) Diversion of fatty acids away from mitochondria via overexpression of the carnitine acetyltransferase (CrAT) [69], L-carnitine supplementation [69], etomoxir treatment [57,126] or a mouse model with elevated M-CoA (MCD KO mouse) [57] improves skeletal muscle insulin sensitivity. 4) Mitochondrial targeted antioxidant and overexpression of mitochondrial antioxidant enzymes are associated with improved insulin sensitivity [147,150-155,240]. ETF; electron transfer flavoprotein, MnSOD; manganese superoxide dismutase, GSH: glutathione transferase, Trx; thioredoxin, Prxs; peroxiredoxin, Cat; catalase, V: ATPsynthase, βGPA: Beta-guanidopropionic acid.

6.4 Promotion of glucose oxidation is an effective treatment option for T2DM

From a teleological standpoint, the induction of insulin resistance is a result of feedback signals due to fuel oversupply. However, the induction of insulin resistance and the inhibition of
glucose uptake has significant negative consequences due to the resultant elevations in circulating glucose. Therefore, strategies to improve glucose disposal into skeletal muscle are paramount in order to alleviate the stress of high circulating blood glucose.

The oxidation of carbohydrate and LCFA exists in a reciprocal relationship at rest. Therefore, any regulatory mechanism that increases LCFA oxidation will decrease carbohydrate oxidation. In light of western society’s high fat diet, it should be noted that a higher fat intake will promote fat oxidation and therefore blunt carbohydrate oxidation. Our body’s attempt to deal with excess fat results in a sustained lipid challenge to the mitochondria which may play a role in the induction of insulin resistance. The data from the literature [69,126] show that in humans, strategies that divert lipid substrate away from mitochondria (point 3 in Figure 6.3, L-carnitine and etomoxir) show a reciprocal elevation in glucose oxidation concomitantly with improved whole body insulin sensitivity. Moreover, carbohydrate oxidation is associated with lower ROS production compared to fatty acids [293] thus substantiating the potential positive effects. To further evaluate this potential mechanism, treatment with the beta oxidation inhibitor trimetazidine in combination with L-carnitine may be an effective way to divert lipid-derived substrate out of the mitochondria and promote glucose oxidation.

6.5 Unresolved questions

6.5.1 Exercise and mitochondrial ROS

The most potent strategy to improve insulin sensitivity appears to be via an increase in physical activity. Even 150 minutes of exercise over the course of a week has shown to be beneficial regarding insulin sensitivity [288]. The mechanism(s) of action stretch across many tissue beds but at the level of the skeletal muscle, the mechanism may be related to ATP turnover and creating a more appropriate metabolic balance and lower ROS production. However,
previous studies have suggested that exercise promotes mitochondrial ROS production and considering the argument that exercise improves insulin sensitivity, this seems counterintuitive. To recapitulate this finding, it is now believed that exercise-induced mitochondrial ROS is a result of the cessation of exercise, not the exercise itself. During exercise, the metabolic pathways are significantly unregulated to supply reducing equivalents for ATP synthesis. The electrons supplied are not likely to leak out of the ETC as ADP is present to pull the electrons through the ETC at a rapid rate ([186] and Chapter 5). Conversely, at the cessation of exercise, ADP is no longer present but the inertia within the metabolic pathways causes an imbalance between supply and demand and mitochondrial ROS is produced [101]. This exercise-derived mitochondrial ROS may be imperative for orchestrating the upregulation of antioxidant genes and the promotion of mitochondrial biogenesis [295,296]. Therefore, taking into account the consistently observed beneficial effects of exercise, exercise-induced ROS seems to be physiologically appropriate. The acute nature of exercise-induced ROS vs. the chronic nature of overfeeding-induced ROS may play a role in explaining this inconsistency, however this speculation has yet to be fully examined.

6.5.2 Drastic differences in M-CoA inhibition kinetics between permeabilzed fibres and isolated mitochondria

In Chapter 4, the reported M-CoA IC$_{50}$ utilizing PmFB was 13-18 fold higher vs. isolated mitochondria. This significant difference is attributable to the difference in sample preparation and therefore highlights a potential regulatory role played by the cytoskeleton. Unlike isolated mitochondria, PmFB retain the inherent cellular architecture which may include the maintenance of VDAC and β-tubulin linkages. I hypothesize that retaining the linkage between β-tubulin and VDAC may help explain the differences between these methodologies. In support of this
hypothesis, in permeabilized cardiomyocytes, the Km for ADP is significantly higher compared to isolated cardiac mitochondria somewhat paralleling our Chapter 4 observation [297]. To explain this finding in heart mitochondria, it has been shown that β-tubulin binding to VDAC restricts the availability of ADP for oxidative phosphorylation which raises the apparent Km for ADP [217]. As proof of principle, the addition of β-tubulin to an isolated mitochondrial preparation raises the Km for ADP and protease treatment of permeabilized cardiomyocytes to remove β-tubulin-VDAC interaction lowers the elevated Km [217]. In support, it has recently been found that VDAC, CPT-I and ACS exist as a heterotrimERIC complex and this “interactosome” is proposed to regulate the activity of CPT-I [76]. Thus, in the context of Chapter 4, the finding that VDAC and CPT-I cooperate as an interactosome [76], combined with previous findings demonstrating that β-tubulin-VDAC interaction explains the differences in Km for ADP [217], may suggest that the β-tubulin-VDAC interaction is the reason for the disparate IC50 values between PmFB and isolated mitochondria. To investigate this hypothesis, incubating isolated skeletal muscle mitochondria with β-tubulin and re-evaluating the kinetics of M-CoA inhibition could be performed. In addition, treatment of PmFB with trypsin to break the β-tubulin-VDAC linkage could also be performed.

6.5.3 Resveratrol: Indirect or direct regulation of ANT2

In Chapter 5, I suggest that resveratrol promotes an increase in ANT2 in the ZDF rat and this induction of ANT2 plays a functional role in the mechanism by which resveratrol improves skeletal muscle insulin resistance. Resveratrol appears to activate many signalling cascades related to mitochondrial function including SIRT1, PPARα, AMPK and PGC-1α indicating that it likely also induces ANT2 expression [243-245]. However, as opposed to directly regulating ANT2 content, resveratrol may improve insulin sensitivity (independent of ANT2) and the
improved insulin signalling is what regulates ANT2. In support of this idea, resveratrol has been shown to increase MnSOD activity 14 fold [298] and overexpression of MnSOD within skeletal muscle has been shown to be protective against diet-induced skeletal muscle insulin resistance [155,298]. Therefore, by improving the oxidative state of the muscle, insulin signalling could proceed to initiate (or maintain) ANT2 expression. In an insulin deficient state (streptozotocin-induced diabetes in rats, 2 months), ANT1 content was decreased by 50% [299] (ANT2 was not measured). Also, in diabetic cats, liver mitochondrial P:O ratios were recovered with insulin treatment [300]. These data indicate that the complete removal of insulin has deleterious effects on mitochondrial function which could be related to altered ANT2 content, although this has yet to be investigated.

In addition to ANT2 content, it is currently unknown whether resveratrol may influence ANT activity. In Chapter 5, it was observed that resveratrol supplementation promoted an increase in state III mitochondrial LCFA oxidation (palmitate and P-CoA). Hypothetically, an increase in LCFA oxidation in T2DM would reduce the elevated skeletal muscle LCFA-CoA concentration. Therefore, by lowering LCFA-CoA with resveratrol supplementation, ANT would be less inhibited and ADP transport would be promoted, matching the Chapter 5 data on submaximal ADP-stimulated respiration. Furthermore, lowering LCFA-CoA would increase the M-CoA binding affinity to CPT-I such that mitochondrial lipid oversupply (and subsequent inhibition of ANT2) would be reduced. Previous work has yet to measure LCFA-CoA in skeletal muscle following resveratrol supplementation but if LCFA-CoA levels are reduced, resveratrol may increase (directly or indirectly) both the content and activity of ANT2.
6.6 Conclusion

In summary, this thesis highlights a number of non-traditional regulatory processes within skeletal muscle mitochondrial metabolism. A number of findings have been presented, including, 1) a functional role for mitochondrial FAT/CD36 in mitochondrial LCFA oxidation was observed and this protein was found to be located upstream of ACS on the OMM, 2) M-CoA inhibition kinetics of CPT-I in PmFB present a more physiologically appropriate IC$_{50}$ and a regulatory role of LCFA-CoA on M-CoA inhibition kinetics was ascertained and 3) submaximal ADP-stimulated respiration rates and ANT2 content are depressed in the ZDF rat and recovered by resveratrol treatment.


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