

THE ROLE OF PROTEIN MEDIATED TRANSPORT IN REGULATING  
MITOCHONDRIAL LONG CHAIN FATTY ACID OXIDATION

A Thesis

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## ABSTRACT

### THE ROLE OF PROTEIN MEDIATED TRANSPORT IN REGULATING MITOCHONDRIAL LONG CHAIN FATTY ACID OXIDATION

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Professor L.L. Spriet

This thesis is an investigation of the role of fatty acid translocase (FAT/CD36), plasma membrane associated fatty acid binding protein (FABPpm) and carnitine palmitoyltransferase I (CPTI) in transporting long chain fatty acids (LCFA) across mitochondrial membranes.

Maximal CPTI activity, as well as the sensitivity of CPTI for its substrate palmitoyl-CoA (P-CoA) and its inhibitor malonyl-CoA (M-CoA), were measured in mitochondria isolated from human vastus lateralis muscles at rest and following muscle contraction. Exercise did not alter maximal CPTI activity, or the sensitivity of CPTI for P-CoA. In contrast, exercise progressively attenuated the ability of M-CoA to inhibit CPTI activity. Mitochondrial FAT/CD36 protein content was also measured at rest, during, and following 2 hours of cycling at ~60% maximal oxygen uptake. Exercise progressively increased the content of mitochondrial FAT/CD36 (+59%), which was significantly ( $P < 0.05$ ) correlated with palmitate oxidation during exercise ( $r = 0.52$ ), while palmitate oxidation was inhibited ~80% by the administration of a specific FAT/CD36 inhibitor. These data suggest that alterations in CPTI M-CoA sensitivity and increases in mitochondrial FAT/CD36 coordinate exercise induced increases in fatty acid oxidation.

FABPpm, another plasma membrane transport protein, has identical amino acid sequence to mitochondrial aspartate aminotransferase (mAspAT). Since FABPpm

contributes to plasma membrane fatty acid transport, the role of FABPpm with respect to mitochondrial LCFA transport was investigated. However, unlike FAT/CD36, muscle contraction did not induce an increase in mitochondrial FABPpm protein in rat or human skeletal muscle. In addition, electrotransfecting FABPpm cDNA into rat skeletal muscle upregulated this protein in mitochondria +80% without altering mitochondrial palmitate oxidation. In contrast, electrotransfection increased mAspAT activity +90%, and this was correlated ( $r=0.75$ ;  $P<0.01$ ) with FABPpm protein. These data suggest that FABPpm does not contribute to the regulation of mitochondrial LCFA transport.

Previously, it has been suggested that mitochondria from obese individuals contains an inherent dysfunction to oxidize LCFAs. In age matched lean ( $BMI = 23.3 \pm 0.7 \text{ kg}\cdot\text{m}^{-2}$ ) and obese ( $BMI = 37.6 \pm 2.2 \text{ kg}\cdot\text{m}^{-2}$ ) individuals, isolated mitochondrial palmitate oxidation was not altered. In addition, mitochondrial FAT/CD36 content were not different in lean and obese individuals. In contrast, citrate synthase and beta-hydroxyacyl-CoA dehydrogenase, common markers of total mitochondrial content, were decreased with obesity. Therefore, the decrease in mitochondrial content appeared to account for the observed reductions in whole muscle LCFA oxidation.

## PUBLICATIONS

**This thesis is based on the following publications:**

**Holloway GP**, Bezaire V, Heigenhauser GJF, Tandon NN, Glatz JFC, Luiken JJFP, Bonen A, Spriet LL (2006). Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. J Physiol. 15; 571(Pt 1): 201-210.

**Holloway GP**, Lally J, Nickerson JG, Alkhateeb H, Snook LA, Heigenhauser GJF, Calles-Escandon J, Glatz JFC, Luiken JJFP, Spriet LL, Bonen A (2007). Fatty acid binding protein facilitates sarcolemmal fatty acid transport but not mitochondrial oxidation in rat and human skeletal muscle. J Physiol. In press.

**Holloway GP**, Thrush AB, Heigenhauser GJF, Tandon NN, Calles-Escandon J, Dyck DJ, Bonen A, Spriet LL (2007). Skeletal muscle mitochondrial FAT/CD36 and palmitate oxidation are not decreased in obese women. Am J Physiol Endocrinol Metab. 292: E1782-1789.

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**Jamie Lally:** Performed all of the electrotransfection surgeries

**Dr. James G. Nickerson:** Completed Western blot analysis on whole muscle and plasma membranes, in addition to palmitate transport into vesicles following electrotransfection.

**Dr. Hakam Alkhateeb:** Completed the whole muscle palmitate oxidation analysis following electrotransfection.

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## LIST OF ACRONYMS

<b>Acetyl-CoA carboxylase</b>	<b>ACC</b>
<b>Acyl-CoA synthetase</b>	<b>ACS</b>
<b>5-aminoimidazole-4-imidazole-carboxamide-1-<math>\beta</math>-D-ribose</b>	<b>AICAR</b>
<b>AMP-activated protein kinase</b>	<b>AMPK</b>
<b><math>\beta</math>-hydroxyacyl-CoA dehydrogenase</b>	<b><math>\beta</math>-HAD</b>
<b>Calcium calmodulin dependent protein kinase II</b>	<b>CaMKII</b>
<b>Carnitine acyl-carnitine translocase</b>	<b>CACT</b>
<b>Cyclic AMP</b>	<b>cAMP</b>
<b>Carnitine acetylcarnitine transferase</b>	<b>CAT</b>
<b>Caveolin-3</b>	<b>Cav-3</b>
<b>Carbohydrates</b>	<b>CHO</b>
<b>Cytochrome c oxidase IV</b>	<b>COX-IV</b>
<b>Carnitine palmitoyltransferase-I</b>	<b>CPTI</b>
<b>Carnitine palmitoyltransferase-II</b>	<b>CPTII</b>
<b>Citrate synthase</b>	<b>CS</b>
<b>Diacylglycerol</b>	<b>DAG</b>
<b>Diglyceride lipase</b>	<b>DG Lipase</b>
<b>Dimethylsulfoxide</b>	<b>DMSO</b>
<b>Electron transport chain</b>	<b>ETC</b>
<b>Extra cellular signal regulated kinase</b>	<b>ERK</b>
<b>Cytosolic fatty acid binding protein</b>	<b>FABPc</b>
<b>Plasma membrane associated fatty acid binding protein</b>	<b>FABPpm</b>

<b>Fatty acid translocase</b>	<b>FAT/CD36</b>
<b>Fatty acid transport proteins 1-6</b>	<b>FATP1-6</b>
<b>Free fatty acid</b>	<b>FFA</b>
<b>Hormone sensitive lipase</b>	<b>HSL</b>
<b>Intermyofibrillar</b>	<b>IMF</b>
<b>Intramuscular triacylglycerol</b>	<b>IMTG</b>
<b>Michaelis constant</b>	<b>K<sub>M</sub></b>
<b>Long chain fatty acid</b>	<b>LCFA</b>
<b>Mitochondrial aspartate aminotransferase</b>	<b>mAspAT</b>
<b>Malonyl-CoA</b>	<b>M-CoA</b>
<b>Malonyl-CoA decarboxylase</b>	<b>MCD</b>
<b>Monoglyceride lipase</b>	<b>MG Lipase</b>
<b>Sodium potassium ATPase</b>	<b>Na<sup>+</sup>K<sup>+</sup> ATPase</b>
<b>Non-esterified fatty acid</b>	<b>NEFA</b>
<b>Perchloric acid</b>	<b>PCA</b>
<b>phosphoinositol triphosphate kinase</b>	<b>PI3-K</b>
<b>Protein kinase A</b>	<b>PKA</b>
<b>Protein kinase C</b>	<b>PKC</b>
<b>phorbol 12-myristate 13-acetate</b>	<b>PMA</b>
<b>Respiratory control ratios</b>	<b>RCR</b>
<b>Respiratory exchange ratio</b>	<b>RER</b>
<b>Sarcoplasmic reticulum calcium ATPase</b>	<b>SERCA</b>
<b>Subsarcelommal</b>	<b>SS</b>
<b>Sulfo-N-succinimidyl oleate</b>	<b>SSO</b>

**Triacylglycerol**

**TAG**

**Tricarboxylic acid cycle**

**TCA**

**Peak oxygen uptake**

**VO<sub>2peak</sub>**

**CHAPTER ONE**  
**INTRODUCTION**

## 1.1 Introduction

Fatty acids participate in many cellular processes including membrane synthesis, intracellular signaling, transcriptional regulation and energy provision. These lipids can be derived from several sources; including exogenous dietary sources, and absorption from the intestine, or release from triacylglycerol (TAG) stores located in either adipose or skeletal muscle tissue. It has been known for almost 70 years that fatty acids contribute a large portion of whole body energy at rest, and to varying degrees during exercise (4). Although fatty acids cannot provide energy as rapidly as carbohydrates (CHO), and are less efficient per unit of oxygen, they provide more energy per gram wet weight due to their hydrophobic nature and lack of interaction with water. This makes them an invaluable source of energy for exercise at low to moderate intensities [for review see (180)]. The absolute and relative amount that fatty acids contribute to the overall energy requirement varies with the intensity of exercise (144, 180). Generally, plasma non-esterified fatty acids (NEFAs) contribute the majority of the required energy at rest and during low-intensity exercise [ $<30\%$  peak oxygen uptake ( $VO_{2peak}$ )]. Although at rest energy from fatty acids can be derived from circulating TAG, plasma NEFAs and intramuscular triacylglycerols (IMTGs), during exercise the proportion that circulating TAGs contribute to the overall energy requirements is negligible (76, 120). Current estimates suggest that during moderate-intensity exercise  $\sim 70\%$  of the fatty acids oxidized are derived from plasma NEFA (180). As a result, it is easy to imagine why regulating the entry of fatty acids into muscle cells would be advantageous (Figure 1.1).

QuickTime™ and a  
TIF (LZW) decompressor  
are needed to see this picture.

**Figure 1.1. Energy expenditure and fuel selection as a function of exercise intensity.**

Free fatty acid (FFA); From (180).

The literature now suggests that there are multiple sites regulating fatty acid uptake by various tissues. Specifically, skeletal muscle fatty acid oxidation appears to depend on: i) lipolysis and fatty acid release from the adipose tissue; ii) delivery of fatty acids to the skeletal muscle, and dissociation from albumin; iii) transport across the plasma membrane; iv) lipolysis of IMTG; v) activation of fatty acids with the addition of CoA thioester, and transport through the cytosol to the mitochondria; and vi) transport into mitochondria, and ultimately oxidation (reviewed in (13, 70). This review will focus on the recent literature that has expanded our understanding of the regulation of fatty acid metabolism, with particular emphasis on skeletal muscle.

## 1.2 Delivery of FFA to Skeletal Muscle

Lipids that are absorbed from the gut, circulate through the lymphatic system as chylomicrons, and are eventually taken up by liver and repackaged into lipoproteins (very low density and low density lipoproteins). These lipoproteins are released back into the circulation, travel to skeletal muscle and adipose tissue, where an endothelial located enzyme (lipoprotein lipase) degrades TAGs to NEFA and glycerol. The NEFA are then taken up and re-esterified to TAGs. The TAGs located in adipose tissue represent a large storage of energy (estimated to represent around 100,000 kcal) that can be released into the circulation, while IMTG storage represents a significantly smaller storage (~4700-23000 kcal/kg wet wt) (180). The NEFA concentration in blood represents a balance between release and clearance. At rest, the blood NEFA concentration is in excess of the metabolic demand, and as such a large majority of the TAG that undergo lipolysis are re-esterified. In contrast, during exercise there is an increase in energy demand and as a result more fatty acids are oxidized, and less are recycled back to TAGs in the adipose tissue.

Due to the hydrophobic hydrocarbon chain of fatty acids, they are insoluble in an aqueous solution such as blood plasma. As a result, fatty acids bind noncovalently with proteins located within the blood. Although fatty acids can bind with several proteins to render them water soluble, due to the high affinity of albumin for fatty acids as a ligand, under normal physiological conditions, albumin is the only protein of consequence. Albumin has several binding sites for fatty acids, each with a unique ligand binding affinity. Despite the high capacity of albumin to bind fatty acids, a small percentage of the circulating fatty acids are found unassociated, and are deemed to be free fatty acids (FFA). It is the FFA that represents the physiologically relevant component that can be

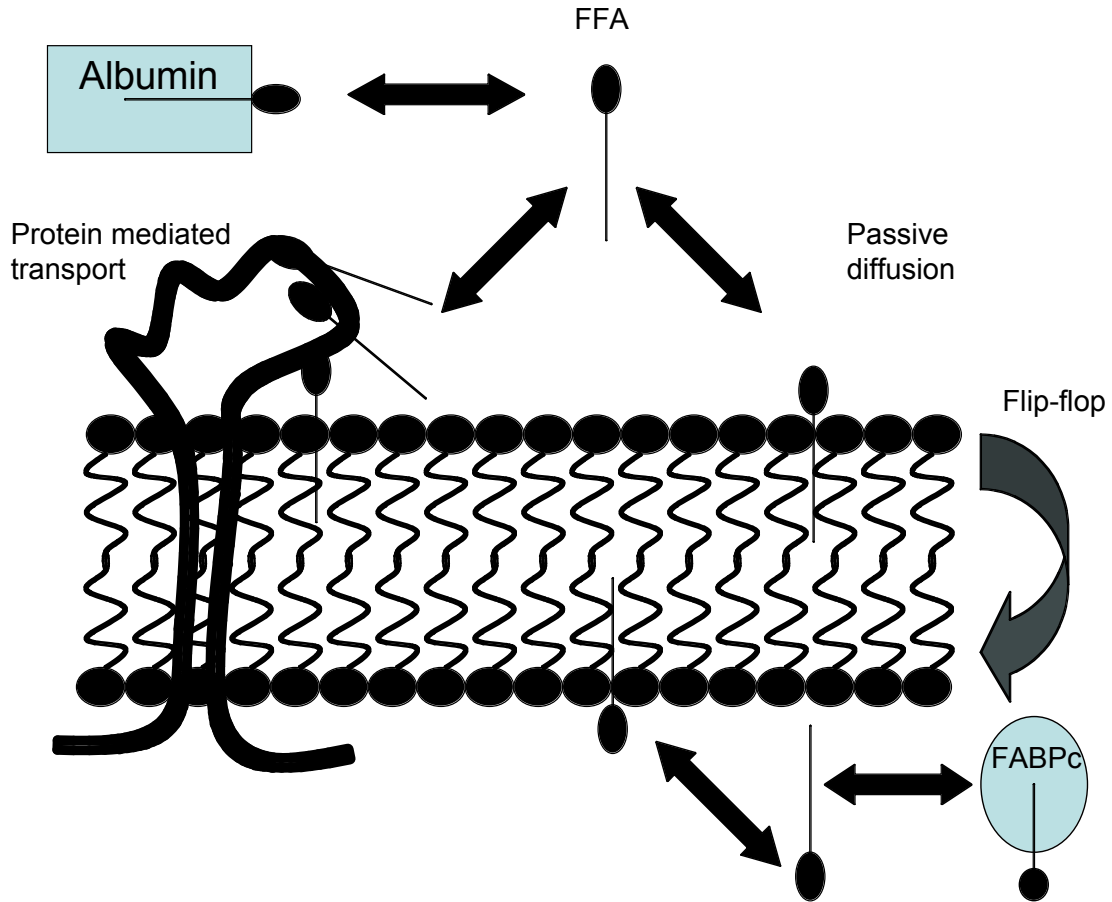
taken up and utilized by various tissues. While the concentration of FFA is therefore of interest to physiologists, it has remained difficult to directly measure since it represents an equilibrium between ligand (fatty acid) and carrier (albumin) that is constantly changing. Nevertheless, Richieri and colleagues have used a fluorescent probe to measure FFA concentration in plasma to develop a stepwise equilibrium model, enabling FFA levels to be estimated from total plasma NEFA levels (141). This model suggests that the FFA concentration is a function of the ratio between albumin and fatty acids. They have shown that FFA concentration increases linearly with small fatty acid:albumin ratios (<4:1), and exponentially with large fatty acid:albumin ratios (>4:1), enabling FFA concentrations to be calculated under various physiological conditions (fed vs. fasted and resting vs. during muscle contraction). Under normal physiological conditions the molar ratio between fatty acids and albumin rarely exceeds 4:1, and typically is between 1:2 and 2:1, and therefore FFA concentrations increase linearly with plasma NEFA concentrations. In addition, by using the stepwise equilibrium model, FFA concentrations can be calculated if the NEFA and albumin plasma contents are known. Normal plasma albumin concentrations are ~600  $\mu\text{M}$ , and average NEFA concentrations range between ~200 and 2000  $\mu\text{M}$ , at rest and following prolonged exercise, respectively. Therefore, FFA concentration ranges between 5-50 nM depending on diet and exercise, and represent <0.01% of the total circulating NEFA (141). Although only FFA can be transported into various tissues, given that FFA are dependent on the amount of NEFA, and represent such a small proportion of circulating fatty acid, NEFA contents are still of interest to physiologists.

Prior to fatty acid delivery to skeletal muscle, the unbound FFA must exit the lumen of the blood vessel, by crossing a layer of simple squamous cells lining the capillaries known as the endothelium. While the exact mechanism of how fatty acids cross these cells remains unknown, there is evidence to suggest that both passive diffusion and protein mediated transport participates. With respect to protein-mediated transport, there are indications in the literature that fatty acid translocase (FAT)/CD36, plasma membrane associated fatty acid binding protein (FABPpm) and cytosolic fatty acid binding protein (FABPc) participate in mediating fatty acid transport in a similar fashion to that in the plasma membrane (see below). Regardless of the transport mechanism out of the circulation, once fatty acids are located in the interstitial space they are rebound to albumin. Once again, the metabolically active concentration of fatty acids is the unbound FFA component, which can be calculated using the stepwise model. While more difficult to measure, estimations of interstitial albumin are  $\sim 200 \mu\text{M}$  in skeletal muscle, and assuming a similar fatty acid:albumin ratio as found in plasma, the resulting interstitial FFA concentration is  $\sim 7.5 \text{ nM}$ . [reviewed in (70)].

### **1.3 FFA Transport Across Plasma Membranes**

Transport of fatty acids across the plasma membrane appears to involve 6 steps, including: 1) dissociation of FFA from albumin; 2) movement to the outer phospholipid bilayer; 3) insertion into the outer phospholipid bilayer; 4) 'flip-flop' across the membrane; 5) dissociation from the inner phospholipid bilayer, rendering the FFA inside the cell; and 6) binding to intracellular proteins. This transport process has traditionally been considered to be limited by the concentration or driving gradient of FFA, and passive

diffusion across plasma membranes, because the speed of ‘flip-flop’ across membranes appear too rapid to involve regulatory proteins. This ‘physical-chemical model’ of membrane transport essentially argued that regulation was not required because it was not needed (71, 132). The model proposed that fatty acids transfer easily from albumin to the outer leaflet of plasma membrane. Once imbedded in the membrane, protonation is increased as a result of the shift in the fatty acid pKa from 5 to ~7. Protonated fatty acids can rapidly flip-flop to the inner leaflet, and dissociate. However, it now appears that both passive diffusion, and protein-mediated transport contribute to fatty acid transport (2, 13, 85, 100). Despite the overwhelming evidence to suggest proteins facilitate fatty acid transport, current insight into membrane topology does not suggest these proteins act like traditional transporters that create a pore or channel for fatty acids [reviewed in (70)]. Instead, transport proteins may facilitate: 1) dissociation of NEFA from albumin; 2) movement to the outer phospholipid bilayer; 3) insertion into the outer phospholipids bilayer; and 4) desorption from the inner phospholipids bilayer (Figure 1.2).

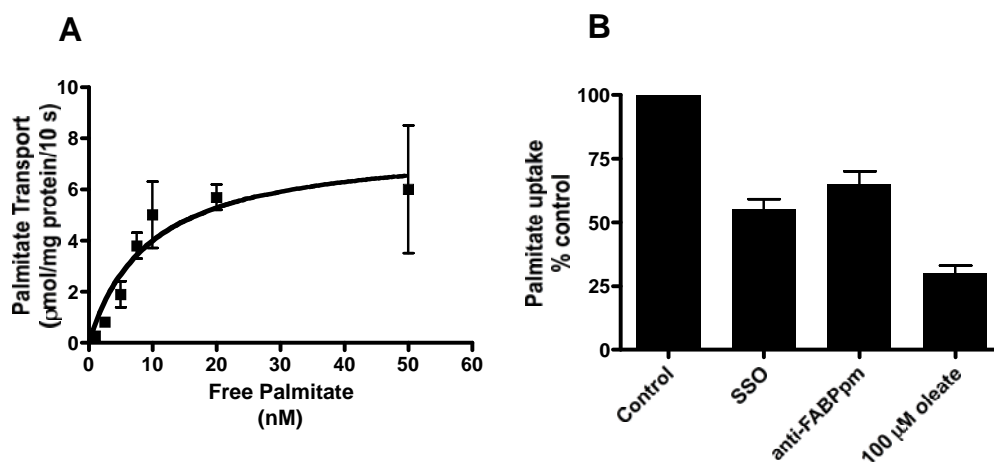


**Figure 1. 2. Schematic representation of theoretical steps in passive diffusion in protein mediated fatty acid transport.** Cytosolic fatty acid binding protein (FABPc); Free fatty acid (FFA).

Recent evidence in support of a protein mediated role in fatty acid transport includes: i) the measured rate of ‘flip-flop’ has been overestimated, and the actual rate is much slower and could be limiting (98, 99). Current estimations for the rate of palmitate and oleate passive diffusion are ~2-50 times slower than the maximal demands of myocytes (98); ii) the transport of FFA appears to be a saturable process (25, 118, 172) (if passive diffusion was the primary mechanism high concentrations of FFA would elicit higher transport rates, but saturation plateaus indicate a finite number of transport proteins); iii) transport

appears to exhibit competitive inhibition (25); and iv) specific inhibitors of various putative transport proteins drastically decrease the rate of fatty acid transport, indicating that proteins mediate ~80% of transport (25, 39, 46, 118) (Figure 1.3).

Three types of transport proteins have been identified: a ~40 kDa FABPpm located on the outer leaflet of the plasma membrane, a family of at least six ~70 kDa fatty acid transport proteins (FATP1-6) that have at least six transmembrane domains, and a highly glycosylated ~88 kDa FAT/CD36 (CD36 is the human homolog) that has at least two transmembrane domains.



**Figure 1.3. Evidence for a protein mediated transport component in plasma membrane fatty acid transport.** Sulfo-*N*-succinimidyl oleate (SSO); Plasma membrane associated fatty acid binding protein (FABPpm); Redrawn from (25).

### 1.3.1 Fatty acid translocase/CD36

FAT/CD36 is an integral membrane protein that is found on the surface of a variety of cells, including monocytes, epithelial cells, small intestine, adipocytes, cardiac

and myocytes. Originally, the primary function of FAT/CD36 was attributed to cell adhesion and the trafficking of monocytes to damaged tissues by working as a class B scavenger receptor [reviewed in (70)]. However, Harmon and Abumrad utilized labeled reactive succinimidylesters, that irreversibly bind to FAT/CD36, to show a role for FAT/CD36 in fatty acid transport, as inhibition greatly reduced fatty acid transport into adipocytes (75). In addition, FAT/CD36 null mice had a pronounced reduction in fatty acid transport into adipose tissue (54). Although these data suggested a role for proteins in mediating plasma membrane transport, it was important to also ascertain the role of FAT/CD36 in muscle cells, given that adipose tissue and skeletal muscle metabolism are very different, and muscle cells contain lower intracellular concentrations of lipids, and therefore could have a greater capacity for passive diffusion. In support of FAT/CD36 mediating fatty acid transport, Coburn and colleagues demonstrated that null mice have an ~60% reduction in fatty acid uptake into adipose tissue, heart and red skeletal muscle (44). In addition, FAT/CD36 null mice oxidize less fat, and have a decreased ability to perform exercise. In contrast, mice overexpressing FAT/CD36 have an enhanced ability to perform exercise (1).

To ascertain a true measure of fatty acid transport into muscle, cytosolic metabolism must be removed as any oxidation or FFA release can alter the apparent transport rate. Importantly, such a technique has been created, and the giant sarcolemmal vesicles offers the advantages of: 1) the membranes being 'right' side out; 2) the removal of all cytosolic organelles (and therefore metabolic pathways); and 3) containing a large amount of FABPc, ensuring that back inhibition does not limit transport (25). This technique has revealed several significant findings, most importantly that transport

correlates with the amount of transport proteins on the plasma membrane in rat (100) and human (26) skeletal muscle [for review see (20)].

Many insights into the role of FAT/CD36 in fatty acid transport and metabolism have been obtained. Using giant sarcolemmal vesicles it has been shown that the  $K_m$  for palmitate is ~6 nM in both red and white muscle (25). However, under basal conditions the maximal transport into vesicles generated from red muscle is ~2 fold higher (25), indicating a greater capacity to transport fatty acids into red skeletal muscle. In addition, a hierarchial protein expression pattern has emerged for FAT/CD36, with heart>>red>>white muscle, and the abundance of FAT/CD36 has been correlated with fatty acid transport (118). The addition of sulfo-*N*-succinimidyl oleate (SSO), a specific inhibitor of FAT/CD36, has also been shown to reduce fatty acid transport by ~50% into vesicles generated from heart and skeletal muscle (25, 117, 118), similar to that originally reported by Harmon and Abumrad in adipocytes (75). Together, these data strongly suggest a role for FAT/CD36 in plasma membrane fatty acid transport.

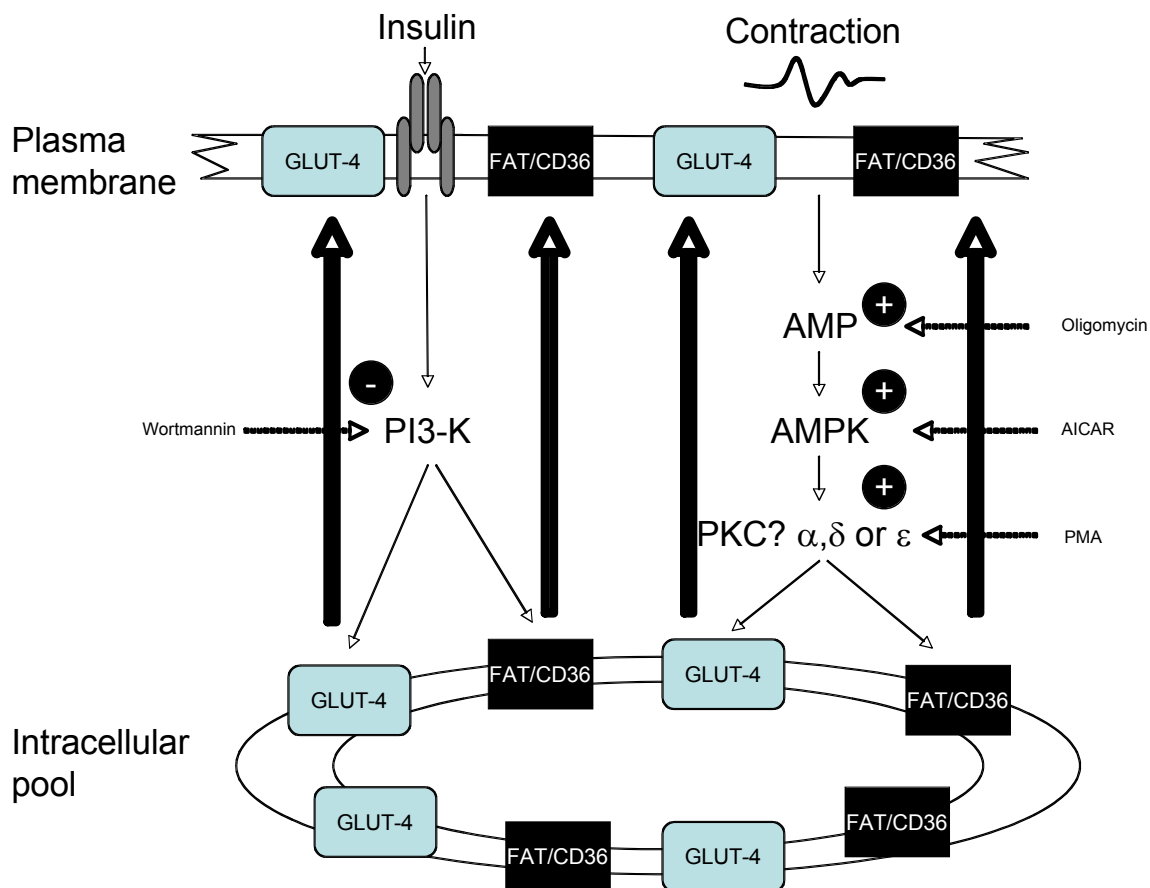
Situations that alter the content of plasma membrane fatty acid transport proteins can have dramatic effects on cell metabolism. In rats, the fatty acid transport capacity of skeletal muscle can be increased by chronic training, and decreased by denervation (100). Importantly, chronic electrical stimulation models in rat skeletal muscle increase fatty acid transport proportionally to the increase in the amount of FAT/CD36 on the plasma membrane (100). Similarly, denervation decreases the capacity of skeletal muscle to transport fatty acids across the phospholipids bilayer in proportion to the reductions in plasma membrane associated FAT/CD36 (100). Interestingly, in some of these perturbations (denervation) whole muscle protein FAT/CD36 content did not change, indicating FAT/CD36 was redistributed from the plasma membrane. In humans, arterial-

venous sampling techniques have shown that chronic training can induce an increase in fatty acid uptake (174). However, these techniques do not directly measure transport, and therefore can be influenced by other metabolic processes (eg. increased fatty acid oxidation rates). Although the plasma membrane has not been isolated in humans following training interventions, mainly as a result of the tissue requirements, traditional exercise prescriptions have been shown to increase the total mRNA expression of FAT/CD36 (171). High intensity training has also been shown to increase fatty acid oxidation, and total expression of FAT/CD36 (Perry *et al.*, unpublished), however these findings are not universally reported (35) highlighting the need to measure plasma membrane content, which represents the metabolically relevant compartment for FAT/CD36.

With exercise, and the associated increase in FFA release from adipose tissue and FFA delivery to skeletal muscle, a greater demand on protein-mediated transport exists. Interestingly, transport can increase by; i) increasing the apparent intrinsic activity of FAT/CD36; and ii) by increasing the amount of transport proteins on the plasma membrane either through increasing *de novo* synthesis of FAT/CD36 and/or translocation of FAT/CD36 from an intracellular depot to the plasma membrane, in a similar fashion to GLUT-4. Over the past few years it has been shown that FAT/CD36 can increase on the plasma membrane of skeletal muscle (23) following as little as 30 minutes of muscle contraction. Techniques that separate intracellular organelles have revealed that increases in plasma membrane FAT/CD36 are associated with decreases in the intracellular content of this protein (23). Given these data, and the short duration of the protocol (unlikely to involve *de novo* FAT/CD36 synthesis) it has been suggested that FAT/CD36 can translocate to the plasma membrane during muscle contractions, thereby increasing the

rate of fatty acid transport. Although the exact mechanisms remain elusive, it is known that contraction and 5-aminoimidazole-4-imidazole-carboxamide-1- $\beta$ -D-ribose (AICAR) (39, 40, 113) can also induce this response, and therefore it is feasible to assume that AMP-activated protein kinase (AMPK) and the energy status of muscle cells contribute to this response. In addition, pharmacologically activating protein kinase C (PKC) isoforms with the phorbol ester phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes increases the rate of fatty acid transport [reviewed in (111)]. Presumably this occurred through FAT/CD36 translocation, as SSO administration prevented this response. Since PKC isoforms are activated by calcium, and cytosolic calcium levels are elevated 200-400 fold with exercise, PKC activation represents a feasible mechanism to induce the translocation of FAT/CD36 to the plasma membrane. Inhibiting extra cellular signal regulated kinase (ERK) signaling can prevent FAT/CD36 translocation, implicating an additional signaling pathway (173). In contrast, increasing cyclic AMP (cAMP) levels in cardiac myocytes did not induce FAT/CD36 translocation (119), suggesting catecholamine elevations associated with exercise are not responsible for the increase in fatty acid transport. Insulin has also been shown to induce translocation, and appears to involve phosphoinositol triphosphate kinase (PI3-K) signaling (38, 114, 116). Therefore, there seem to be similarities in the regulation of GLUT-4 and FAT/CD36 trafficking within skeletal muscle (Figure 1.4). However, while there is evidence to suggest an equal abundance of FAT/CD36 on the plasma membrane and in intracellular depots at rest (23), confocal microscopy found only negligible amounts of intracellular FAT/CD36 (184). A likely explanation for the discrepancy is the folding of the fluorescent probes, and the

ability to detect them in the interior of the cell. However, others using the same confocal procedures have observed an intracellular depot of FAT/CD36 (87).



**Figure 1.4. Schematic representation of the selected signaling pathways involved in the translocation of both GLUT-4 and FAT/CD36.** Short forms are the same as defined in text. Modified from (111). Note, GLUT-4 and FAT/CD36 may occupy different intracellular depots, and there may be separate insulin and contraction sensitive depots.

### *1.3.2 Plasma membrane associated fatty acid binding protein*

In 1985, Berk and colleagues identified FABPpm as the first fatty acid transport protein (167). Although originally found in the liver, FABPpm has now been reported in intestinal cells, adipocytes, cardiac and skeletal muscle of both rats and humans. Similar to FAT/CD36, FABPpm follows an abundance expression hierarchy, with heart >> red >> white muscle (25). In addition, FABPpm has been shown to directly bind fatty acids, and the amount of FABPpm located on the plasma membrane has been correlated with fatty acid transport. Antibodies specific for FABPpm drastically reduce fatty acid transport (although transport is not entirely inhibited) (25). Recently, transfection of FABPpm cDNA into 3T3-L1 fibroblasts demonstrated that enhanced expression of FABPpm can increase fatty acid uptake (82). Electrotransfection of FABPpm cDNA into rat skeletal muscle has also been shown to induce a pronounced increase in total FABPpm protein (+160%) and in the plasma membrane associated FABPpm (+173%). These changes were associated with a +79% increase in fatty acid transport into vesicles. Importantly FAT/CD36 expression and plasma membrane FAT/CD36 content were not altered, suggesting the increase in transport was related to the increase in FABPpm (43). However, given that the increase in plasma membrane FABPpm was not proportional to the increase in transport, and that inhibiting FABPpm only reduces transport by ~50% (25), it appears that FABPpm is not the only protein for fatty acids to gain entry into cells.

As with FAT/CD36, chronic electrical stimulation-induced increases in fatty acid transport are proportional (and strongly correlated) to increases in the amount of FABPpm on the plasma membrane. In contrast, denervation decreased the capacity of skeletal

muscle to transport fatty acids across the phospholipids bilayer, and subsequently reduced FABPpm on the plasma membrane (100). Interestingly, denervation did not change the whole muscle expression of FABPpm, indicating that a redistributed of FABPpm occurred. In humans, chronic exercise prescriptions have been shown to increase the total expression of FABPpm (94, 175), while short term, high intensity training has been shown to increase fatty acid oxidation, and FABPpm (169) in as little as 7 training sessions over 2 weeks. While training induced increases in FABPpm are not universally reported (35, 171), part of the discrepancy may be related to the different training protocols. In addition, total transport protein content does not represent the physiological relevant compartment. Recently in rat skeletal muscle, FABPpm content has been shown to increase on the plasma membrane, with concomitant reductions in the intracellular compartment, in response to both insulin and contraction (72). The increase in plasma membrane FABPpm suggests it is translocated in a similar fashion to FAT/CD36, given the short duration (60 minutes) of the protocol and the unaltered total FABPpm expression. Although less is known about the mechanisms responsible for FABPpm translocation, at least the contraction signals appear to be similar to FAT/CD36 as AICAR, an AMPK mimetic agent, translocates both proteins to the plasma membrane (39). However, in models of obesity, the ability to translocate FABPpm has been retained, while insulin induced FAT/CD36 translocation is lost, suggesting some divergence in their regulation (72). The importance of these findings could relate to differences in intracellular signaling, or possibly due to differences in the intracellular location of these transport proteins.

When SSO and a polyclonal antiserum against FABPpm are included in the surrounding medium transport into vesicles is inhibited by 20-80% (25, 176).

Surprisingly, whether these inhibitors are included independently, or in combination, fatty acid transport is reduced to a comparable level (118), suggesting that FABPpm and FAT/CD36 may work in a concerted effort to transport fatty acids.

### *1.3.3 Fatty acid transport proteins 1-6*

Less is known about the FATP family of transport proteins, because of inadequate probes and a lack of inhibitors. However, it does appear that at least FATP1 and 4 have transport roles in plasma membrane transport. As with FAT/CD36 and FABPpm, experiments overexpressing FATP1 and 4 have been conducted to determine the importance of these proteins. Originally found in adipocytes, stable transfection of FATP1 into 3T3-L1 fibroblasts increased the uptake of various fatty acids, without altering short chain fatty acid uptake (148). However, data in skeletal muscle has been slow to follow. Recently, the expression pattern of FATP4, but not FATP1, has been shown to follow a similar hierarchy to FAT/CD36 and FABPpm, heart>>red>>white muscle, suggesting a possible role for FATP4 in the regulation of fatty acid transport into skeletal muscle (Bonen *et al.*, unpublished). Data in support of this hypothesis have been generated using electrotransfection of both FATP1 and 4 cDNA into rat skeletal muscle (131). Increasing the expression of both FATP1 and 4 in skeletal muscle independently increases the transport of fatty acids into giant sarcolemmal vesicles, although FATP4 was twice as effective as FATP1 (131). In addition to their role in fatty acid transport, it appears that at least FATP1 and 4 contain an enzymatic function, catalyzing the activation of FFA to acyl-CoA thioesters (potentially trapping FFAs within the cell, and maintaining a concentration gradient). Although these observations have made it difficult to determine a direct role for the FATPs in FFA membrane transport, an elegant series of

experiments by DiRusso and colleagues have divorced the importance of the acyl-CoA synthetase enzymatic function from the FATP transport-mediated process, further implicating FATPs in fatty acid transport (49). These studies indicated that FATPs exhibit differential fatty acid transport effectiveness, with FATP1, 2 and 4 having the greatest impact on promoting fatty acid transport, while FATP3 and 5 have only a modest influence on fatty transport, and FATP6, despite being touted as a key cardiac fatty acid transport protein, had little effect on fatty acid transport. Recently, the possibility that FATPs can translocate in a similar fashion to FAT/CD36 and FABPpm has been postulated. In 3T3 L1 adipocytes, insulin administration for one hour increased fatty acid transport, and confocal immunofluorescence microscopy indicated that the plasma membrane FATP1 content increased during this time (160), however others have not been able to replicate these finding (107). In addition, it appears that insulin may increase FATP4 on the plasma membrane in human skeletal muscle. Importantly this was accomplished without altering total expression, and given the brevity of the protocol, translocation is a likely explanation (8).

#### **1.4 Lipolysis of Intramuscular Triacylglycerides**

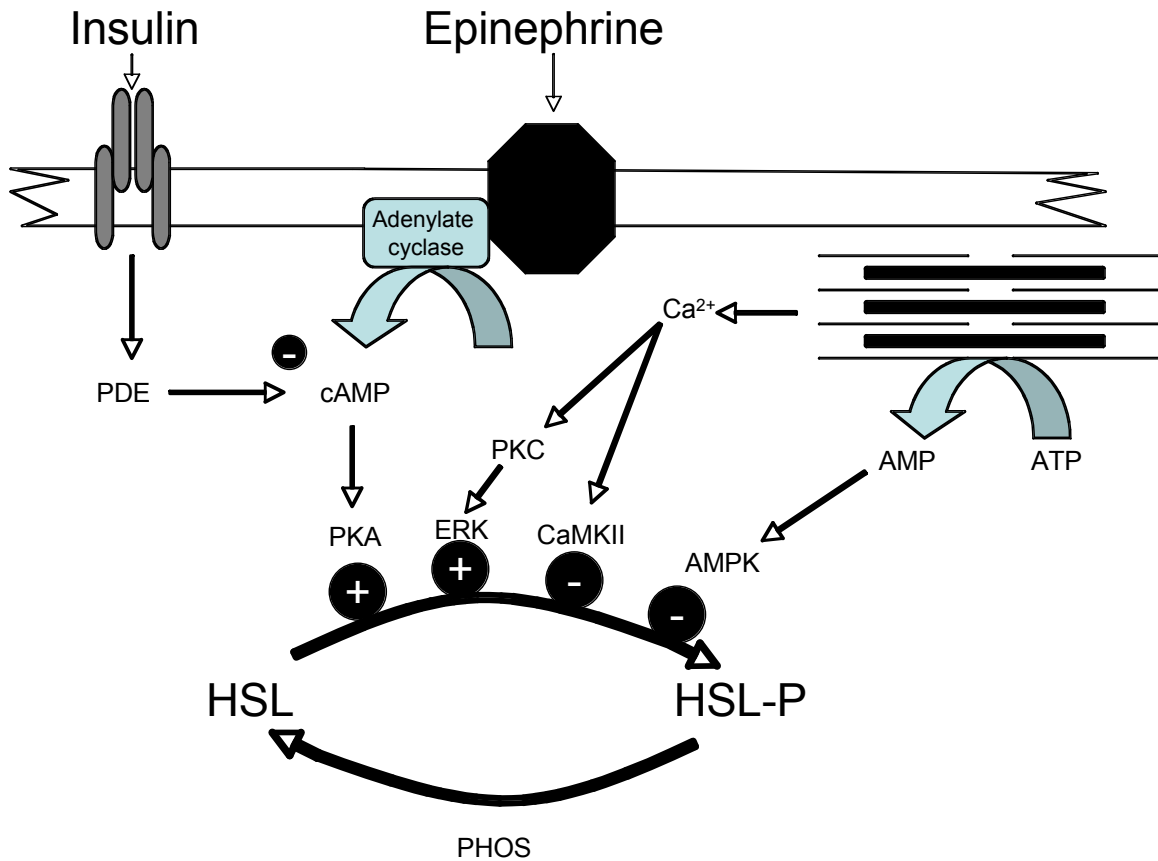
The lipolysis of IMTGs are regulated in a similar fashion to TAGs within adipose tissue, with hormone sensitive lipase (HSL), diglyceride (DG) and monoglyceride (MG) lipase catalyzing the sequential removal of FFAs. As in adipose tissue, HSL appears to regulate the level of lipolysis, as DG and MG lipases are near equilibrium enzymes. HSL is directly activated by phosphorylation, and inactivated by dephosphorylation. Resting lipolysis remains at a relatively low rate in response to several hormones, and the influence on protein kinase A (PKA). Plasma epinephrine concentration is low at rest, and

therefore cAMP and PKA levels are low. PKA phosphorylates and activates HSL, and therefore HSL activity and IMTG lipolysis will occur at a low rate. Exercise is associated with increases in both epinephrine and norepinephrine from the adrenal medulla and nerve-ending spillover, respectively. The rise in catecholamines is associated with increases in the activity of adenylate cyclase and the production of cAMP, PKA activation, phosphorylation of HSL and ultimately the lipolysis of IMTGs. HSL is additionally activated by ERK phosphorylation, which is stimulated by calcium activated PKC, which is increased with exercise. In contrast, HSL is inhibited by AMPK and calcium calmodulin dependent protein kinase (CaMKII), through phosphorylation of inhibitory sites, which are also activated during exercise. It appears that the balance between catecholamine, calcium levels, energy status and the circulating insulin levels dictates the overall lipolytic rate (Figure 1.5).

At the start of low intensity exercise, HSL activity increases in response to elevations in catecholamine, cytosolic calcium, PKC and ERK. In contrast, continued prolonged exercise induces a reduction in IMTG lipolysis, despite high levels of catecholamine, cytosolic calcium, PKC and ERK. This appears to occur in response to increases in AMP concentrations, activation of AMPK, and phosphorylation of inhibitory sites on HSL.

For years the activation of HSL was thought to be the only site of regulation for fatty acid oxidation; however migration of HSL to the lipid droplet, and removal of perilipin, a protective boundary around lipid droplets, may also be involved. In addition, a conundrum has always been the much higher affinity of HSL for DAGs compared to TAGs (105), suggesting additional enzymes might be involved in the lipolysis of TAGs. Recently such an enzyme was found in adipose tissue, although the significance and

regulation of adipose tissue triacylglyceride lipase (ATGL) (203) with respect to skeletal muscle lipolysis remains to be determined. These data might explain the high level of



HSL activity at rest, as further regulation both proximal and distal to HSL would regulate the overall lipolytic rate.

**Figure 1.5. Proposed regulation of HSL.** Phosphodiesterase (PDE); phosphorylase (PHOS); other acronyms are defined in text. Redrawn from (188).

Estimates of IMTGs range from 4-10 mmol/kg wet weight, a substantial amount that can provide 50-100% the energy stored as glycogen. Historically, there has been considerable controversy surrounding the ability of muscle to utilize IMTGs during exercise [for

review see (180)], although most researchers now believe that IMTGs represent a significant amount of energy during moderate, prolonged exercise. Regardless of the source, adipose tissue or IMTG, once FFA are located within the cytosol they are metabolized in a similar fashion, and since FFA are insoluble in an aqueous environment, they must be bound to proteins.

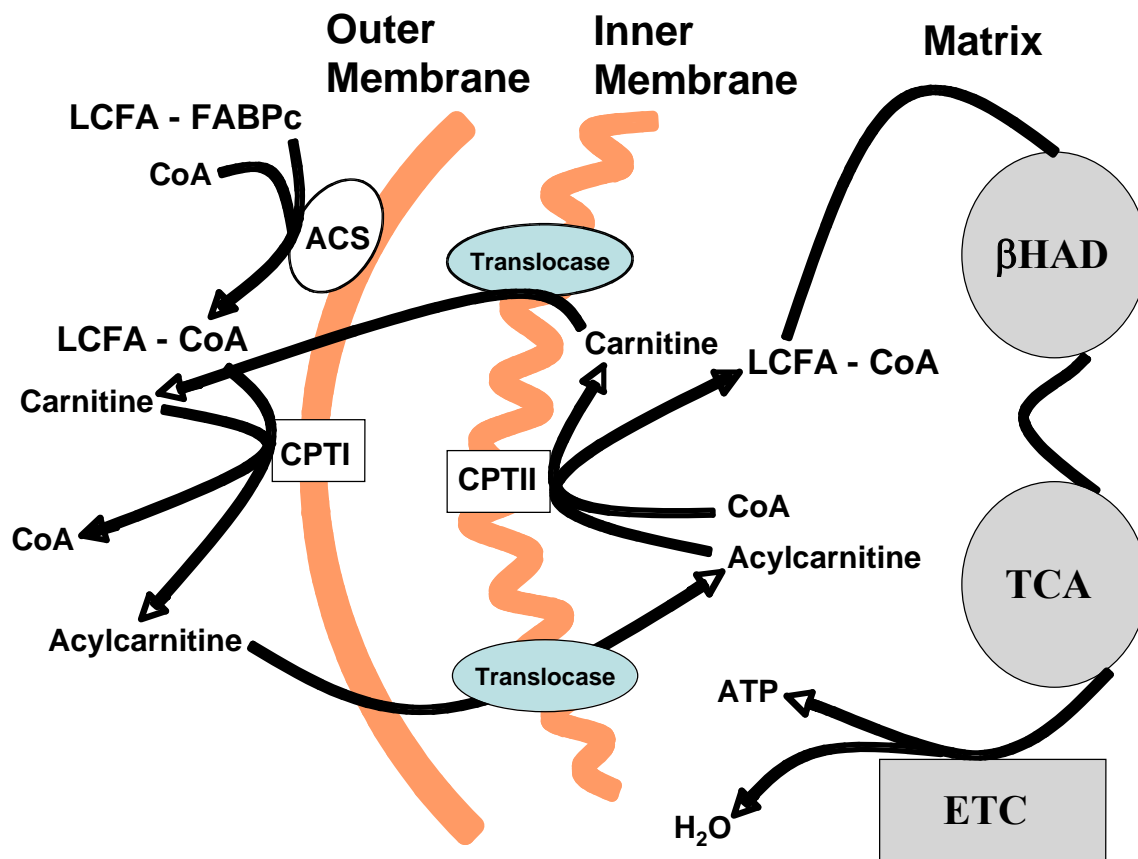
### **1.5 Transport of Fatty Acids Within Cytoplasm**

Once inside the cell fatty acids are bound to FABPc. Overexpression, knockout and knockdown studies have been utilized to determine the role of FABPc in fatty acid oxidation. FABPc heterozygous mice have an ~70% reduction in FABPc protein content, without alterations in fatty acid uptake (115). Although this suggests FABPc is not required for plasma membrane transport, FABPc null animals have a pronounced (~50%) reduction in LCFA transport. In addition, FABPc null animals have shown that skeletal muscle fatty acid oxidation is decreased by ~70% (115). However, quantitative estimates suggest that since FABPc is highly abundant, only ~2% of the total FABPc is bound to fatty acids at any particular time, indicating that under normal conditions this protein will not be limiting [for review see (63)]. Once bound, FABPc chaperones FFA, in an unknown manner, to various sites within the muscle cell. Thereafter, FFA are activated by acyl-CoA synthetase (ACS) and they can then either be esterified to IMTGs, or transported to the mitochondria for oxidation [for review see (80)].

### **1.6 Mitochondrial Transport of Acyl-CoAs**

Due to the polarity of acyl-CoAs they cannot passively cross the inner mitochondrial membrane and gain access to the matrix, the site of fatty acid oxidation. Therefore, prior to their entry into mitochondria, fatty acids undergo transesterification to

acyl-carnitine, a neutral intermediate, catalyzed by the enzyme carnitine palmitoyltransferase-I (CPTI) [reviewed in (123)] (Figure 1.6).



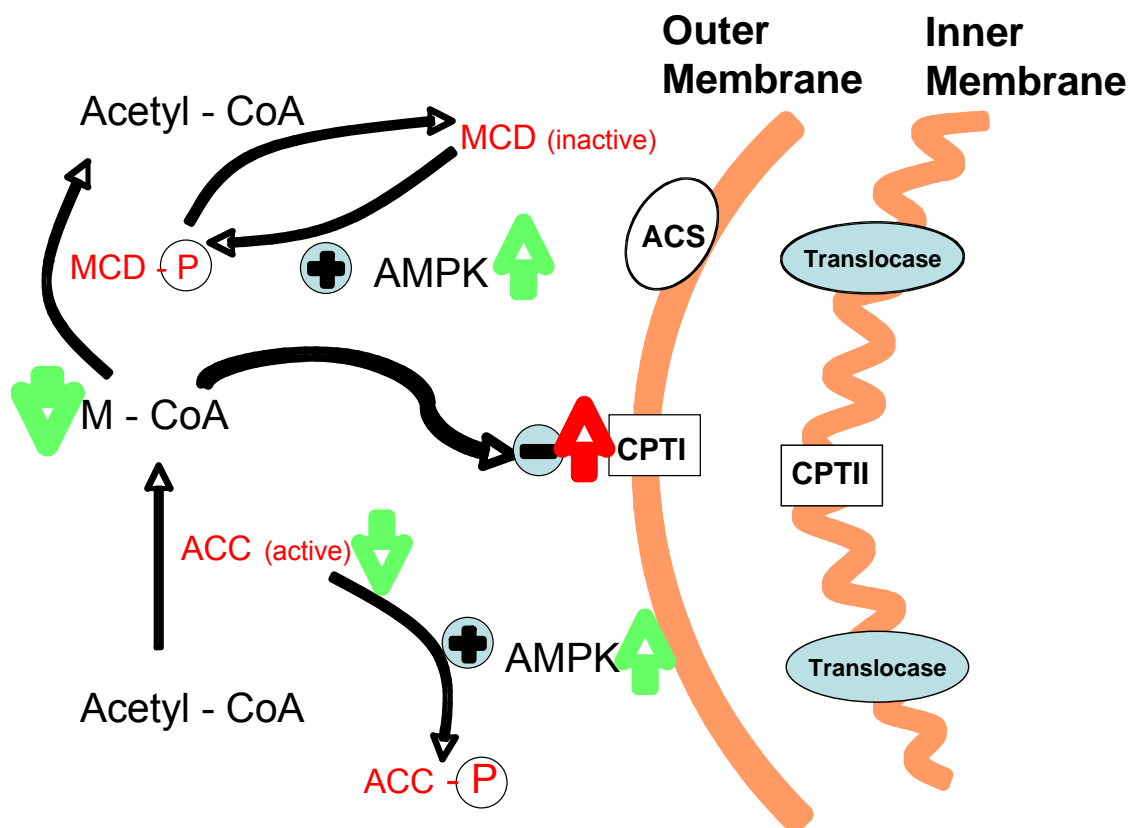
**Figure 1.6. Mitochondrial carnitine transport shuttle.** Tricarboxylic acid cycle (TCA); electron transport chain (ETC); Other acronyms are defined in the text. Redrawn from (93).

The CPT system was first conceptualized as a mechanism to transport LCFAs into mitochondria in the 1960s (30, 59), and since that time evidence has been generated in support of a pivotal role in fatty acid oxidation, and a potential site for linking the magnitude of LCFA oxidation reciprocally with CHO oxidation rates (31, 140). There are

two genes that regulate the expression of CPT1A and CPT1B, the liver and muscle CPTI isoforms respectively [reviewed in (27)]. These two isoforms are only ~60% homologous (199); however, both isoforms have a predicted molecular weight of 88 kDa, while the muscle isoform has an apparent size of 82 kDa when separated using SDS-page analysis (193). Although only 60% homologous, the isoforms have the same  $K_m$  for palmitoyl-CoA, while the  $K_m$  for carnitine in muscle is 10 fold higher (less sensitive) (124), which correlates with the high concentration of carnitine in muscle compared to liver (81, 136).

In the liver, malonyl-CoA (M-CoA) production is the first committed step in TAG *de novo* synthesis, and is regulated by acetyl-CoA carboxylase (ACC). ACC catalyzes the movement of a carbon from citrate to acetyl-CoA, producing M-CoA. M-CoA is a potent inhibitor of CPTI activity. It is easy to conceptualize the importance of this system during conditions of high CHO availability, as M-CoA production will decrease fatty oxidation, while promoting TAG synthesis. In the liver, ACC can be phosphorylated by several intermediates, including cAMP, PKA, AMPK and PKC. Phosphorylation has been shown to induce several changes, including a reduction in maximal activity, and an increase in the  $K_m$  for its substrates (74, 97). Skeletal muscle, which is a non-lipogenic tissue, surprisingly expresses ACC. While both CPTI isoforms can be inhibited by M-CoA, they display differential regulation, as liver CPTI sensitivity to M-CoA is attenuated during fasting (32), while the muscle isoform is unaffected. In addition, the isoforms have different  $IC_{50}$  for M-CoA, as liver is less sensitive by two orders of magnitude (124, 190). Moreover, the regulation of ACC appears to be different between these two tissues, as in skeletal muscle, AMPK phosphorylation has only been shown to induce functional effects in ACC activity (192). In skeletal muscle, AMPK responds to the energy status of the cell, as well as various hormones [for review see (163)]. AMPK activation has been

shown to induce GLUT-4 and FAT/CD36 translocation to the plasma membrane (112), in addition to ACC phosphorylated inhibition, and reductions in M-CoA (192). Therefore, it has been postulated that with exercise-induced perturbations in energy status, AMPK activation would subsequently reduce M-CoA levels by inactivating ACC. AMPK activity has been shown to increase in human skeletal muscle during exercise, and when muscle glycogen levels are low (143, 189, 194), providing a plausible mechanism to reduce M-CoA levels. In addition, M-CoA levels can also be reduced by activating malonyl-CoA decarboxylase (MCD), an enzyme responsible for decarboxylating M-CoA to acetyl-CoA. It has been shown that muscle contraction, and the AMPK activator



AICAR, can increase rat MCD activity in skeletal muscle (146); however this relationship remains untested in human muscle (Figure 1.7).



**Figure 1.7. Proposed regulation of carnitine palmitoyltransferase I during exercise.**

Acronyms are defined in the text.

In skeletal muscle, CPTI activity is inhibited by M-CoA, and therefore, reductions in M-CoA content can subsequently increase fatty acid oxidation by increasing CPTI activity. However, in humans it appears possible to alter fatty acid oxidation independent of M-CoA concentrations (134, 135), questioning the validity of this mechanism. In humans, M-CoA levels have been correlated with fatty acid oxidation rates, but insulin infusion at a low dose has been shown to increase whole body fatty acid oxidation by ~20%, without altering M-CoA levels (9). In addition, insulin at higher dosages can decrease M-CoA ~20%, but this is not proportional to explain the ~40% increase in fatty acid oxidation (9). This suggests that in humans, fatty acid oxidation is at least partially regulated independently of M-CoA. In addition, while exercise is known to induce large increases in fatty acid oxidation, the role of M-CoA in this response has been unclear. In rat skeletal muscle, M-CoA levels have been shown to decrease ~30-50% during exercise, relieving CPTI inhibition, and subsequently increasing fatty acid oxidation (191). However, studies in humans suggest that M-CoA levels may not sufficiently decrease during moderate intensity exercise to entirely explain the increases in fatty acid oxidation (134, 135, 143). While several studies have shown that continuous exercise at various intensities is associated with an increased reliance on fatty acid oxidation, this has not been associated with reductions in M-CoA content, despite increases in acetyl-CoA, the precursor to M-CoA (135). However, recently, small reductions in M-CoA (~13%) have been observed during exercise; however the authors concluded that these reductions could not entirely account for the increase (+122%) in fatty acid oxidation (143). In addition, at least one study has shown that at the onset of exercise M-CoA levels can be reduced,

potentially 'priming' the system for fatty acid oxidation, although the importance of this observation remains unknown and does not appear to occur consistently at various intensities of exercise (135). Also, it has previously been hypothesized that increases in M-CoA at higher exercise intensities may decrease CPTI activity and fatty acid oxidation, but the projected increase in M-CoA does not occur (135, 143). Since M-CoA concentrations are 2-10 fold lower in human muscles compared to rat skeletal muscle, detecting a ~30-50% reduction with exercise is more difficult (134, 143). In addition, it is possible that whole muscle M-CoA levels are not representative of the local environment around CPTI, and the subcellular distribution of M-CoA maybe more important, or additional regulation may exist for CPTI (92).

Originally it was reported that free CoA, acetyl-CoA and M-CoA all attenuated the activity of CPTI in human (202) and rat (125) skeletal muscle, and it was suggested that free CoA and acetyl-CoA inhibit the catalytic site which is different from M-CoA inhibition. It was also reported that free CoA and acetyl-CoA acted in a synergistic fashion to reduce the sensitivity of CPTI for M-CoA (202). However, it should be pointed out that this study was conducted in muscle homogenates following sonication, and therefore the mitochondria were not intact and metabolically viable. CPTII is a near equilibrium enzyme, and since homogenizing compromises the integrity of the mitochondrial membranes, measurements in homogenates represent the combined effect of both CPTI and CPTII, making interpretations difficult. In addition, the magnitude of the changes observed were not large enough to explain the increases in fatty acid oxidation that have been observed with exercise.

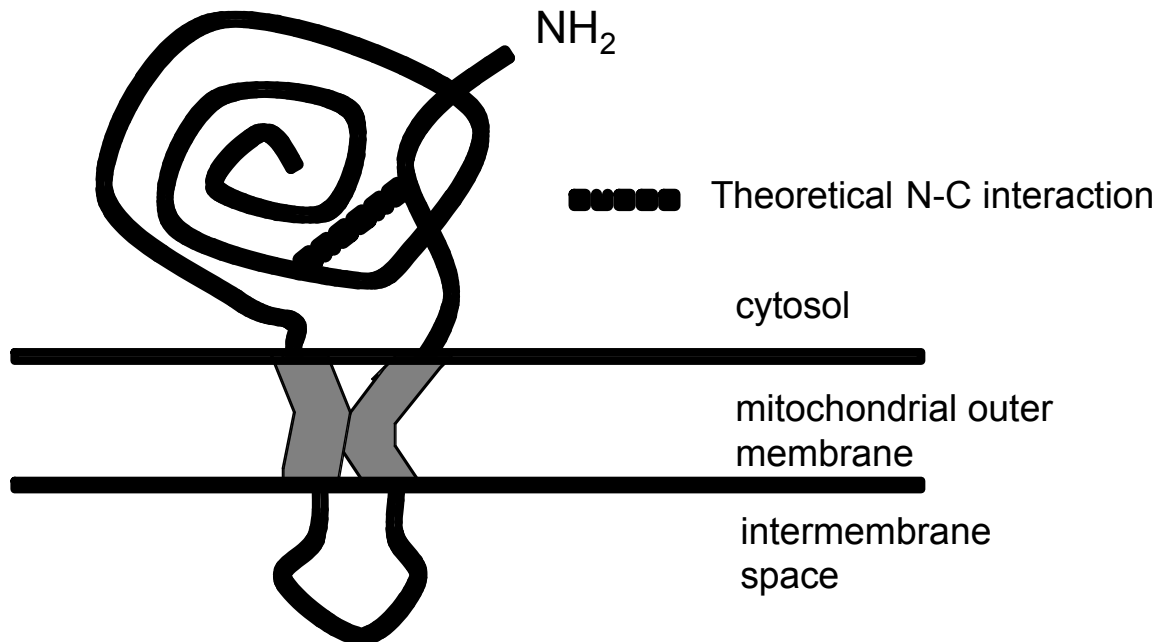
Isolating viable, intact mitochondria provides the ability to measure the influence of various factors on CPTI specifically. Using isolated mitochondria, Starritt and

colleagues (161) have shown that acetyl-CoA, free CoA and acetylcarnitine concentrations did not regulate CPTI activity, contradicting earlier reports. Further work *in vitro* has shown that concentrations of calcium, AMP, ADP and Pi, designed to mimic those in muscles at rest and during exercise, did not alter CPTI activity (16). These are important observations, as it had been hypothesized that exercise-associated regulators may override any M-CoA control during exercise, as is the case with many other important flux-generating enzymes in skeletal muscle [for review see (158)]. However, physiological reductions in pH (7.1 - 6.8) decreased CPTI activity in the presence of M-CoA, potentially explaining reductions in fatty acid oxidation with high intensity exercise (16, 161). Interestingly, cross-sectional examinations between trained and untrained individuals (161), and training (34) studies have found an increase in maximal CPTI activity ~100% with an apparent increase in the affinity of CPTI for M-CoA.

While M-CoA has been shown to inhibit CPTI activity, the exact mechanism (competitive or allosteric inhibition) remains debated. Originally, McGarry and colleagues proposed that the CPTI M-CoA binding site was located in the cytosol, while the catalytic site was located in the intermembrane space [for review see (123)]. With this model it was hypothesized that M-CoA allosterically regulated CPTI activity by inducing a conformational change in the enzyme. However, insights into the structure of CPTI suggest that both the catalytic and M-CoA binding sites are located in the cytosol (56). Current models of CPTI suggest a hairpin structure, with both N and C termini located in the cytosol, with two transmembrane domains (199) (Figure 1.8). The amino terminal consists of approximately 46 amino acids, while the carboxy terminal contains around 652 residues (198). Support for this topology comes from the observation that octanoyl-CoA and M-CoA when attached to agarose beads are impermeable to the outer mitochondrial

membrane; however they are still capable of acting as a substrate and inhibitor for CPTI (56).

Interaction of the two cytosolic loops (N and C termini) stabilizes the catalytic domain and modulates M-CoA sensitivity (57, 153). This has been demonstrated by work from Zammit's group using a set of six chimeric proteins, including all possible interactions between liver and muscle amino, carboxy and transmembrane sections. What is clear from their work is that the C-terminis contains both the catalytic core and the M-CoA binding site (83). However, it appears that modification of the N-terminis can alter the sensitivity of CPTI for M-CoA. In the liver isoform, deleting residues 1-18 in the N-terminal domain abolishes M-CoA inhibition (152), and altering even one amino acid within this region (glutamate to alanine) can reduce M-CoA sensitivity 100-fold (154). In contrast, deleting residues 19-30 has been shown to increase the CPTI M-CoA sensitivity 50 fold (84). The mechanism by which the N-terminal domain alters M-CoA sensitivity remains unknown, although Zammit's group has recently provided a plausible explanation. Specifically, in an elegant study they (53) found using a cross-linking technique, that alterations in the N-terminal domain attenuates M-CoA sensitivity and abolishes a physical/chemical interaction between the two cytoplasmic loops. In addition, they have provided evidence to suggest that the interaction likely occurs within residues 40-47. Although these data suggest CPTI M-CoA sensitivity can be altered, it is important to remember that all of these studies were conducted on the liver isoform which is only ~60% homologous to the muscle isoform. However recently, deletions in N-terminal residues 3-18 of the muscle CPTI isoform was shown to progressively attenuate M-CoA sensitivity (84), suggesting a similar mechanism may exist in skeletal muscle.



**Figure 1.8. Proposed CPTI topology.** Modified from (197).

In addition to acyl-CoA, carnitine is a substrate for CPTI, and therefore theoretically carnitine levels can limit fatty acid entry into mitochondria. However, exercise at a moderate-intensity (40-60%  $VO_{2peak}$ ) has been shown to decrease carnitine levels, despite pronounced increases in fatty acid oxidation [reviewed in (165)], suggesting that carnitine does not limit fatty acid oxidation at rest, and is in excess of that required by CPTI. However, exercising above moderate-intensity (~60 %  $VO_{2peak}$ ) further decreases carnitine levels, and is associated with decreases in the rate of fatty acid oxidation. Therefore, it is possible that if carnitine levels can be reduced below a critical point and decrease the rate of lipid oxidation (143). Carnitine can be acetylated by acetyl-

CoA moieties by a near equilibrium enzyme known as carnitine acetylcarnitine transferase (CAT), with the product being acetyl-carnitine (31). This enzyme is located within the mitochondria, and helps to buffer excess acetyl-CoA that can be produced at high exercise intensities. It has repeatedly been shown that myocellular concentrations of acetylcarnitine are elevated with increasing exercise intensities, potentially decreasing the available carnitine, and ultimately CPTI activity. This is a potential mechanism that links high pyruvate dehydrogenase activity, and CHO oxidation, with decreases in fatty acid oxidation (a reciprocal relationship previously described) (140, 143). It has also been shown that when exercise is initiated with low glycogen levels, less acetylcarnitine is produced, presumably because of a diminished capacity to generate acetyl-CoA. Although this was associated with increases in carnitine, and fatty acid oxidation, the regulatory mechanisms that initiate rapid increases in fatty acid oxidation at the onset of exercise remained undefined.

Recently, the possibility that mitochondrial fatty acid transport may involve other proteins in addition to CPTI has been proposed. FAT/CD36, as previously mentioned is a multi-ligand scavenger receptor with several functions, one of which is to interact with, and transport LCFAs across the plasma membrane phospholipid bilayer (reviewed in (102)). More recently, FAT/CD36 has been identified on the mitochondrial membrane of resting rat (36) and human (15, 149) skeletal muscle. Interestingly, the addition of SSO reduced the ability of isolated mitochondria to oxidize palmitate in a dose dependent manner, with maximal inhibition at ~80% (15). Importantly, SSO did not alter the ability of isolated mitochondria to oxidize shorter chain fatty acids (octanoate) (15, 36) or pyruvate (36), suggesting that SSO does not independently alter mitochondrial function. Interesting, although FAT/CD36 appears to be necessary for both rat and human

mitochondrial fatty acid oxidation, there appears to be species-specific differences. Specifically, SSO administration in rats has been shown to inhibit palmitate oxidation and CPTI activity, suggesting FAT/CD36 is located upstream, or at the level of CPTI (36). Importantly, binding of fatty acids to mitochondria was also inhibited with the administration of SSO, suggesting that FAT/CD36 might help trap acyl-CoAs and somehow hand them off to CPTI (36). However in human muscle, CPTI activity was not inhibited with SSO, but the oxidation of palmitoylcarnitine, the product of CPTI, was inhibited with SSO, suggesting that FAT/CD36 is located downstream of CPTI (15). Although FAT/CD36 appears to participate at some level in mitochondrial fatty acid transport, it may not be exclusive to CPTI. While FAT/CD36 protein and CPTI maximal activity cannot independently predict palmitate oxidation at rest, a multiple regression analysis significantly correlated these with mitochondrial palmitate oxidation ( $r=0.90$ ) (15). Moreover, FAT/CD36 has co-immunoprecipitated with CPTI in both rat (36) and human (149) skeletal muscle, suggesting a concerted effort to transport fatty acids into mitochondria. However, the exact mechanisms of action remain unknown.

As with the plasma membrane, alterations in mitochondrial FAT/CD36 protein content have been associated with a functional change in mitochondrial fatty acid oxidation. Chronic electrical stimulation in rats has been shown to increase the amount of mitochondrial FAT/CD36 by ~30%, and to subsequently increase palmitate oxidation in isolated mitochondria proportionally. This response was associated with an increase in total muscle FAT/CD36 content, and the increase in mitochondrial palmitate oxidation was blocked with SSO (36). Exercise training in combination with weight loss has also shown an increase in the amount of FAT/CD36 that coimmunoprecipitates with CPTI without altering total FAT/CD36 protein content, correlating with the increase in resting

fatty acid oxidation, suggesting that part of the exercise-induced increase in fatty acid oxidation relates to increases in the content of mitochondrial FAT/CD36 (149). Interestingly, acute electrical stimulation lasting only 30 minutes also results in a pronounced increase in the amount of mitochondrial FAT/CD36, and subsequently the ability of mitochondria to oxidize palmitate. This contraction-induced increase in oxidation can be blocked with the administration of SSO (36), again displaying that increases in fatty acid oxidation are at least partially mediated by FAT/CD36. The total muscle expression of FAT/CD36 was not altered in this short duration protocol, and therefore it is unlikely that *de novo* synthesis can account for these changes, and translocation remains a likely explanation.

Interestingly FABPpm has also been found on both plasma and mitochondrial membranes. FABPpm appears to have an identical amino acid sequence to mitochondrial aspartate aminotransferase (mAspAT) (168). In addition, transfection of cDNA into 3T3-L1 fibroblasts upregulated both plasma membrane FABPpm and mAspAT, and antibodies directed against mAspAT also detected FABPpm (82). Since it appears that FABPpm and FAT/CD36 alter plasma membrane fatty acid transport in a similar fashion, and the signals that induce FABPpm and FAT/CD36 translocation to the plasma membrane overlap, the observation that FAT/CD36 alters mitochondrial membrane fatty acid transport suggests that FABPpm may also have a role at this level.

### **1.7 Fatty Acid Oxidation in Skeletal Muscle From Lean and Obese Individuals**

Skeletal muscle metabolism represents a balance between CHO and fatty acid oxidation, and there appear to be regulatory mechanisms that link the utilization of these two substrates. Skeletal muscle accounts for ~80% of CHO disposal, and reductions in muscle glucose uptake are characteristic of insulin resistance [reviewed in ((21)]. While

we are only beginning to unravel the complexity of the development of insulin resistance, it has been known since the early 1990's that alterations in fatty acid oxidation are associated with obesity, insulin resistance, and ultimately type II diabetes (17). A hallmark of obesity is the elevation in circulating plasma NEFA, which have been negatively correlated with insulin sensitivity (138). A stronger negative relationship appears to exist between IMTGs and insulin sensitivity (104); however it is likely that more polar lipids species, namely DAGs and ceramides, are among the culprits in altering insulin signalling [for review see (177)]. Regardless of the primary lipid intermediate that is responsible for alterations in insulin sensitivity, mechanisms that increase FFA uptake can exacerbate this phenotype. Elevations in intracellular lipid species can result from increases in plasma membrane uptake, decreases in mitochondrial oxidation, increases in esterification rates or from any combination of these. Since FFA concentrations are typically elevated with obesity, this will increase the driving gradient into skeletal muscle, and transport by passive diffusion. However, since the majority of transport is protein mediated, it is important to understand how obesity alters the expression, and location of FAT/CD36, FABPpm and FATP1 and 4.

Rectus abdominus muscle taken from lean and obese women has revealed that the transport of fatty acids into giant sarcolemmal vesicles is increased ~4 fold with obesity (26). This could not be explained by increases in the total muscle expression of FAT/CD36 or FABPpm, however the plasma membrane content of FAT/CD36 was increased with obesity (26). This suggests a permanent redistribution of FAT/CD36 to the plasma membrane, providing a potential mechanism for elevating intramyocellular lipid species, and inducing insulin resistance. Obese Zucker rats are a common model for studying obesity related alterations in fatty acid oxidation because they share similarities

to insulin resistance in humans. Importantly, plasma membrane fatty acid transport is also increased in this model, and is also associated with increases in the plasma membrane content of FAT/CD36 without alterations in total muscle expression. In addition, neither insulin resistant humans (obese and type 2 diabetics) (26) or obese Zucker rats (109) are associated with increases in plasma membrane FABPpm, suggesting that the elevated transport is mediated by FAT/CD36.

Obese human and Zucker rat skeletal muscle have similar phenotypes, and therefore rat models of obesity have been used to gain insights into the regulatory differences with obesity. Since FAT/CD36 and FABPpm appear to play such prominent roles in regulating fatty acid oxidation, the ability of insulin and contraction to induce FAT/CD36 and FABPpm translocation to the plasma membrane in Zucker rats has been studied. Interestingly, obesity is associated with a higher basal palmitate uptake, and therefore the ability of insulin to elevate uptake is attenuated (72). FAT/CD36 plasma membrane content paralleled this trend, with higher basal content that was unaltered with insulin. Moreover, while the ability of insulin and contraction to induce FAT/CD36 translocation to the plasma membrane is lost in obese Zucker rats, FABPpm translocation in response to these stimuli is retained (72). Importantly these changes occurred without alterations in the total muscle expression of FAT/CD36 or FABPpm, suggesting a permanent redistribution of FAT/CD36 transport protein to the plasma membrane, at least partially explaining the hyper-lipid intramuscular environment associated with obese skeletal muscle. In contrast, FABPpm trafficking remains flexible even with obesity. These responses have not been studied in humans, and it will be interesting in the future to determine the flexibility of FAT/CD36 and FABPpm with obesity and insulin resistance.

In addition to increases in fatty acid transport, it has been suggested that fatty acid oxidation is compromised with obesity. Reductions in fatty acid oxidation cannot be explained by reductions in delivery, since obesity is associated with increases in plasma NEFA concentration, plasma membrane transport, and intracellular lipid species. Instead, alterations in mitochondrial fatty acid oxidative capacity have been proposed as a mechanism. Two plausible explanations exist for alterations in mitochondrial fatty acid oxidation, either mitochondrial content is decreased and/or there is a dysfunction in fatty acid oxidation within mitochondria. Work in skeletal muscle has indicated a reduction in mitochondrial content with obesity, along with a concomitant decrease in fatty acid oxidation (90). While ratios of electron transport chain capacity to mitochondrial DNA and mitochondrial size have been used to infer dysfunction (90, 142), recently the ability of mitochondria to oxidize fatty acids was directly measured in skeletal muscle from type 2 diabetics, and controversially shown to be increased, although the underlying mechanism remains unknown (8).

Although this most recent study suggests that mitochondrial oxidation is unaltered with obesity, there is still controversy regarding this issue. Recently, Muoio and colleagues have suggested that the relative proportion of fatty acids that are completely oxidized are decreased with a high fat diet, and speculated obesity may result in similar incomplete oxidation (103). Since beta-oxidation is regulated by substrates and products, an imbalance between beta-oxidation, tricarboxylic acid cycle (TCA) and electron transport chain, would increase the amount of acyl-CoAs located within the cell. This potentially has deleterious effects, and can induce oxidative damage in mitochondria, and induce apoptosis (5). In addition, since with obesity more FAT/CD36 has been located on the plasma membrane without total expression being altered, it is possible that less would

be available for mitochondria, potentially compromising oxidation and explaining the previously observed 'mitochondrial dysfunction' in the obese.

### **1.7 Summary**

Historically, fatty acid oxidation was considered entirely regulated by lipolysis of TAGs, and the FFA concentration in the plasma. However, it now appears that fatty acid oxidation is a highly regulated process, involving several sites of regulation. Although traditionally believed to only involve the release of FFA from adipose tissue (as regulated by HSL), recently the involvement of plasma membrane fatty acid transport proteins, FABPpm, FAT/CD36, and FATP1 and 4, have been shown to play a prominent role. In addition, the presence and significance of FAT/CD36 on mitochondrial membranes has revealed novel regulatory sites. While considerable progress has been made in the past 10 years with respect to understanding the regulation of fatty acid oxidation, future research is required to better understand the intracellular signals involved in FAT/CD36 mitochondrial translocation, the apparent interdependence of FABPpm and FAT/CD36, and the potential role of FATPs on mitochondrial membranes.

**CHAPTER TWO**  
**AIMS OF THE THESIS**

## 2.1 Aims of the thesis

The purpose of this thesis was to examine the regulation of mitochondrial long chain fatty acid (LCFA) oxidation in mammalian skeletal muscle. Specifically, the focus was to better understand the roles that CPTI, FAT/CD36, and FABPpm with respect to the regulation of mitochondrial LCFA transport and ultimately oxidation. In addition, given the prevalence of obesity and the severe consequences of insulin resistance, characterization of how these proteins are altered in these pathological conditions may help unravel the underlying causes.

Prior to this thesis, rodent models suggested that reductions in the content of M-CoA could account for the observed increase in mitochondrial fatty acid transport and oxidation during exercise. In contrast, this mechanism has not been observed in human muscle, and several other metabolic intermediates and regulators have been ineffective in altering CPTI activity in human skeletal muscle. Therefore, the conclusion is that some unknown and novel control mechanism exists to regulate CPTI activity and/or additional proteins contribute to regulation at this site. Recently, the FAT/CD36 protein has been identified on rat and human mitochondria membranes, and has been shown to influence the capacity of mitochondria to oxidize LCFA. Therefore, FAT/CD36 may represent a novel regulator of overall fatty acid oxidation rates during exercise in human skeletal muscle. FABPpm, another plasma membrane transport protein, has previously been identified on mitochondrial membranes, and may also influence mitochondrial LCFA membrane transport. However, the role of FABPpm with respect to LCFA oxidation has not previously been examined. In addition, obesity has been associated with an impaired ability to oxidize LCFA, and given the ability of FAT/CD36 to regulate the capacity of

mitochondria to oxidize LCFAs, may be associated with reductions in mitochondrial FAT/CD36 protein in this situation.

Therefore, it was hypothesized that:

- 1 – Exercise induced increases in fatty acid oxidation would be associated with reductions in the sensitivity of CPTI for the inhibitor M-CoA.
- 2 – Exercise induced increases in fatty acid oxidation would be associated with increases in mitochondrial FAT/CD36 content.
- 3 – Mitochondrial FABPpm would influence the ability of mitochondria to oxidize LCFA.
- 4 – Mitochondria isolated from the skeletal muscle of obese individuals would have a reduced capacity to oxidize LCFAs or a dysfunction in the ability to oxidize LCFAs per unit mitochondrial protein.
- 5 – Mitochondrial dysfunction would be associated with reductions in mitochondrial FAT/CD36 content.

**CHAPTER THREE**

**MITOCHONDRIAL LCFA OXIDATION, FAT/CD36 CONTENT AND**

**CPTI ACTIVITY IN HUMAN SKELETAL MUSCLE DURING**

**AEROBIC EXERCISE**

### 3.1 Abstract

Mitochondrial fatty acid transport is a rate-limiting step in long chain fatty acid (LCFA) oxidation. In rat skeletal muscle, the transport of LCFA at the level of mitochondria is regulated by carnitine palmitoyltransferase I (CPTI) activity and the content of malonyl-CoA (M-CoA), however this relationship is not consistently observed in humans. Recently, FAT/CD36 was identified on mitochondria isolated from rat and human skeletal muscle and found to be involved in LCFA oxidation. The present study investigated the effects of exercise (120 min of cycling at  $\sim 60\%$   $VO_{2\text{peak}}$ ) on CPTI palmitoyl-CoA and M-CoA kinetics, and on the presence and functional significance of FAT/CD36 on skeletal muscle mitochondria. Whole body fat oxidation rates progressively increased during exercise ( $P < 0.05$ ), and concomitantly M-CoA inhibition of CPTI was progressively attenuated. Compared to rest, 120 min of cycling reduced ( $P < 0.05$ ) the inhibition of 0.7, 2, 5 and 10  $\mu\text{M}$  M-CoA by 16%, 21%, 30% and 34%, respectively. Whole body fat oxidation and palmitate oxidation rates in isolated mitochondria progressively increased ( $P < 0.05$ ) during exercise, and were positively correlated ( $r = 0.78$ ). Mitochondrial FAT/CD36 protein increased by 59% ( $P < 0.05$ ) during exercise and was significantly ( $P < 0.05$ ) correlated with mitochondrial palmitate oxidation rates at all time points ( $r = 0.41$ ), However, the strongest ( $P < 0.05$ ) correlation was observed following 120 min of cycling ( $r = 0.63$ ). Importantly, the addition of sulfo-*N*-succinimidyl oleate, a specific inhibitor of FAT/CD36, reduced mitochondrial palmitate oxidation to  $\sim 20\%$ , indicating FAT/CD36 is functionally significant with respect to LCFA oxidation. We hypothesize that exercise induced increases in fatty acid oxidation occur as a result of an increased ability to transport LCFA into mitochondria. We further suggest that

decreased CPTI M-CoA sensitivity and increased mitochondrial FAT/CD36 protein are both important for increasing whole body fatty acid oxidation during prolonged exercise.

### 3.2 Introduction

As exercise duration increases there is a greater reliance on fatty acids to provide the reducing equivalents necessary for aerobic oxidation in the electron transport chain (186). Prior to oxidation, long chain fatty acids (LCFA) are activated by long-chain acyl-CoA synthetase. This molecule cannot passively cross into the mitochondrial inner matrix, where oxidation occurs, and therefore carnitine-dependent transport must precede oxidation. It is currently believed that the activity of carnitine palmitoyltransferase I (CPTI), which catalyzes the transesterification of LCFA-CoA to LCFA-carnitine, is the rate-limiting enzyme in the carnitine-dependent transport of LCFA [for review see (123)]. In rat skeletal muscle, CPTI activity is allosterically regulated by malonyl-CoA (M-CoA), and M-CoA levels have been shown to decrease during exercise permitting an increased rate of fatty acid oxidation. However, studies in humans suggest that M-CoA levels may not decrease sufficiently during moderate intensity exercise to explain the increases in fatty acid oxidation (48, 134, 135). Further studies have been conducted in search of other regulators of CPTI activity during exercise, but without success (16, 161). Although M-CoA levels may not change in human skeletal tissue during exercise, alterations in compartmentalization/cellular distribution or the sensitivity of CPTI to M-CoA may explain the changes in LCFA oxidation that have been observed.

Recently, it has been proposed that mitochondrial LCFA transport may involve other proteins in addition to CPTI (Campbell et al., 2004). Fatty acid translocase (FAT/CD36) is a multi-ligand scavenger receptor with several functions, one of which is to interact with, and transport LCFAs across phospholipids bilayers (3, 19, 24, 45, 122). A role for FAT/CD36 in LCFA transport has been identified in rat adipocytes (Abumrad et al., 1993) and skeletal muscle (Bonen et al., 2000). More recently, FAT/CD36 has

been identified on the mitochondrial membrane of resting rat (Campbell et al., 2004) and human (15) skeletal muscle and was shown to be required for LCFA oxidation. It has been shown that FAT/CD36 protein content correlates with oxidative capacity, and electrically stimulated muscle contraction increased FAT/CD36 in rat mitochondria without alterations in total muscle content (Campbell et al., 2004). Given the short duration of the exercise protocol used (30 min), and the unaltered total protein level of FAT/CD36, it has been proposed that FAT/CD36 translocates to the mitochondria in response to exercise (Campbell et al., 2004). This suggests that there is a level of regulation of fatty acid oxidation that has not previously been recognized. Whether a similar mechanism occurs in human muscle remains to be established.

The purpose of this study was to compare exercise-induced increases in whole body fatty acid oxidation, and isolated mitochondrial palmitate oxidation rates, to measurements associated with mitochondrial transport. We examined two regulators of mitochondrial LCFA transport, CPTI M-CoA sensitivity and the involvement of FAT/CD36 protein in fatty acid oxidation. We hypothesized that 120 min of exercise would reduce M-CoA inhibition of CPTI activity. In addition, we hypothesized that 120 min of exercise would increase the capacity of isolated mitochondria to oxidize palmitate in association with an increase in mitochondrial FAT/CD36 content.

### 3.3 Research Design and Methods

#### 3.3.1 Subjects

Fifteen healthy, recreationally active, individuals volunteered for this study ( $n = 10$  males;  $n = 5$  females (mean  $\pm$  S.E.); age:  $22 \pm 1$  yr, weight:  $76 \pm 3$  kg, BMI:  $24 \pm 1$  kg/m<sup>2</sup> and  $VO_{2peak}$ :  $48 \pm 2$  ml/min/kg body mass (bm). All subjects completed the same experimental protocol. Volunteers were randomly divided into two groups for the purposes of *in vitro* analysis as a result of the limitations in the amount of skeletal muscle tissue that can be obtained from each subject. There were no significant differences in the characteristics of the two groups so data was pooled for the analysis of whole body metabolic and blood measures, as well as for Western blot protein determination. Group A consisted of eight subjects (6 males, 2 females), from whom muscle samples were used for mitochondrial palmitate oxidation studies (age:  $22 \pm 1$  yr, weight:  $72 \pm 5$  kg, BMI:  $24 \pm 1$  kg/m<sup>2</sup> and  $VO_{2peak}$ :  $48 \pm 3$  ml/min/kg bm). Group B consisted of seven subjects (4 males, 3 females) from whom muscle samples were used for CPTI analysis ( $21 \pm 1$  yr, weight:  $80 \pm 4$  kg, BMI:  $25 \pm 1$  kg/m<sup>2</sup> and  $VO_{2peak}$ :  $49 \pm 4$  ml/min/kg bm). Female subjects were in the early follicular phase of their menstrual cycle phase at time of the experiments involving muscle biopsies. Subjects were fully informed of the purpose of the experiments and of any possible risk before giving written consent to participate. The study was approved by the University of Guelph Ethics Committee.

#### 3.3.2 Pre-experimental protocol

Subjects visited the laboratory on three occasions and were asked to refrain from exercise in the 48 hrs prior to each visit. On the first visit,  $VO_{2peak}$  was measured with a

metabolic cart (SensorMedics  $V_{\max}$  model, California, United States of America) during an incremental exercise test on a cycle ergometer (LODE Instrument, Groningen, The Netherlands). On the following two visits participants cycled for 2 hr at  $\sim 60\%$   $VO_{2\text{peak}}$  on the Lode cycle ergometer following a 12 hr overnight fast. The first visit was used to familiarize the subjects with the 2 hr procedure and to confirm the power output required to attain  $\sim 60\%$   $VO_{2\text{peak}}$ .

### *3.3.3 Experimental protocol*

On the third visit, subjects arrived at the laboratory following a twelve hour overnight fast. A catheter was inserted into a peripheral arm vein and both legs were prepared for muscle biopsies of the vastus lateralis muscle. Resting ventilatory and blood samples were obtained and a muscle sample was obtained under local anesthesia (2% lidocaine without epinephrine) using the percutaneous needle biopsy technique described by Bergstrom (12). Ventilatory and blood samples were obtained during exercise at 15, 30, 60, 90 and 120 min and additional biopsies were sampled at 30 and 120 min. In Group A, two muscle biopsies (total  $\sim 400$  mg) were sampled at each time point for the determination of mitochondrial palmitate oxidation. In Group B, one muscle biopsy ( $\sim 200$  mg) was sampled at each time point and for the determination of CPTI activity.

Immediately following tissue sampling visible fat and connective tissue were dissected free from the muscle and the samples were blotted to remove excess blood. The majority of the tissue was used for the immediate isolation of mitochondria for the determination of CPTI activity, palmitate oxidation rates and for the analysis of selected proteins with Western blotting. A second small section of the muscle biopsy sample ( $\sim 10$

mg), was homogenized and frozen in liquid N<sub>2</sub> for the subsequent analysis of citrate synthase (CS) activity.

#### 3.3.4 Determination of blood metabolites

Venous blood was sampled directly into vacutainers containing heparin, and partitioned into two fractions. An aliquot of 200 µl of whole blood was added to 1 ml of 0.6 M perchloric acid (PCA) and centrifuged. The deproteinized supernatant was stored at -80<sup>0</sup> C and later analyzed for glucose, lactate and glycerol (Bergmeyer,1974b). A second aliquot of whole blood was immediately centrifuged, the plasma was removed and stored at -80<sup>0</sup> C. The plasma was later analysed for free fatty acids (FFA) (Wako NEFA C test kit, Wako Chemicals, Richmond VA).

#### 3.3.5 Isolation of mitochondria from skeletal muscle

Differential centrifugation was used to obtain pure and intact mitochondria containing both intermyofibrillar (IMF) and subsarcelommal (SS) fractions (36). The IMF and SS mitochondria were pooled due to the limited amount of muscle tissue and the requirements for *in vitro* analysis. The mitochondria were resuspended in 1µl of medium III per initial mg of tissue and used for CPTI activity measurements. The remaining mitochondria were further diluted to a final volume of 500 µl for palmitate oxidation measurements.

For Western blotting analysis, mitochondria were further purified using a Percoll gradient (Sigma-Aldrich). Samples were centrifuged at 20,000 g for 1 hour and the mitochondrial layer was removed. The Percoll was removed from the sample by further

centrifuging at 20,000 g for 5 hours. At this point the mitochondria are no longer metabolically viable, but are suitable for Western blotting.

### *3.3.6 Mitochondrial lipid oxidation measurements*

Labeled CO<sub>2</sub> production and acid soluble trapped <sup>14</sup>C from palmitate oxidation were measured following a 30 min incubation of viable mitochondria in a sealed system as previously described (Campbell et al., 2004; Bezaire et al., 2006). Briefly, viable mitochondria (100 µl) were added to 900 µl aliquot of pre-gassed (5% CO<sub>2</sub> and 95% O<sub>2</sub>, constantly shaking at 37°C for 15 min) modified Krebs Ringer buffer, which was then sealed. The 20 ml glass scintillation vial contained a microcentrifuge tube with 500 µl of 1 M of benzethonium hydroxide inserted into a 1.5 ml centrifuge tube to capture <sup>14</sup>CO<sub>2</sub> produced during the oxidation reaction. The reaction was initiated by the addition of a 6:1 palmitate:BSA complex (containing 10 µCi of [1-<sup>14</sup>C] palmitate, for a final palmitate concentration of 77 µM) administered by syringe through the rubber cap. The reaction continued for 30 min at 37°C and was terminated with the addition of ice-cold 12 N PCA by syringe through the rubber cap.

A fraction of the reaction medium was removed through the cap and quantified by liquid scintillation to determine the isotopic fixation as previously described (Campbell et al., 2004; Bezaire et al., 2006). Gaseous CO<sub>2</sub> produced from oxidation of [1-<sup>14</sup>C] palmitate was measured by acidifying the remaining reaction mixture in the 20 ml glass scintillation vial with 1.0 ml of 1M H<sub>2</sub>SO<sub>4</sub>. Liberated <sup>14</sup>CO<sub>2</sub> was trapped by benzethonium hydroxide over a 90 min incubation period at room temperature. The microcentrifuge tube containing the <sup>14</sup>CO<sub>2</sub> was put in a scintillation vial, and radioactivity was counted.

Inhibition studies with sulfo-*N*-succinimidyl oleate (SSO) were performed by pre-incubating mitochondria with SSO dissolved in dimethylsulfoxide (DMSO) for 30 minutes as described by Bezaire et al. (2006). Based on dose-response experiments the final SSO concentration was set at 200  $\mu$ M (Bezaire et al., 2006). For control purposes, the same volume (1  $\mu$ l) of DMSO was added to vials that were not supplemented with SSO.

### *3.3.7 Carnitine palmitoyltransferase-I activity*

The forward radioisotope assay was used for the determination of CPTI activity as described by McGarry et al (124) with minor modifications. Briefly, the assay was conducted at 37°C and initiated by the addition of 10  $\mu$ l of mitochondrial suspension (1:3 dilution) to 10  $\mu$ l of varying palmitoyl-CoA concentrations (18.75, 37.5, 75, 150, and 300  $\mu$ M) and 80  $\mu$ l of a standard reaction medium (L-[<sup>3</sup>H]carnitine Amersham Bioscience, Buckinghamshire, England). The reaction containing 300  $\mu$ M palmitoyl-CoA was also carried out in the presence of various M-CoA concentrations (0.2, 0.7, 2, 5, and 10  $\mu$ M). The reaction was stopped after 6 min with the addition of ice-cold HCl. Palmitoyl-[<sup>3</sup>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.

Control studies were performed by the addition of SSO to the reaction mixture prior to initiation of the reaction. The final concentration of SSO was set at 200  $\mu$ M. As a control, the same volume of DMSO was added to the control tubes. Maximal CPTI activity was expressed in terms of the whole muscle ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g wet muscle}^{-1}$ ), and was normalized to the ratio of CS activity in intact mitochondrial suspensions to total muscle

CS activity to account for the quality of the mitochondrial preparation (see below). CPTI M-CoA and palmitoyl-CoA kinetic data were expressed as a percentage of maximal CPTI activity.

### *3.3.8 Citrate synthase activity*

Citrate synthase (CS) activity was determined in isolated mitochondria as well as in aliquots of homogenized whole muscle as previously described (Bezaire et al., 2006). The net difference between CS activity in intact mitochondria and mitochondria provided a measure of the viability of the mitochondria, as well when compared to the total muscle CS activity provided a measure of the mitochondria recovered during our isolation procedure (Bezaire et al., 2006). The CS activity was assayed spectrophotometrically at 37°C by measuring the disappearance of NADH (10, 159).

### *3.3.9 Western blotting*

Purified isolated mitochondrial fractions were analyzed for total protein (BCA protein assay), 25 µg of denatured protein from each sample were separated by electrophoresis on 8 % SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The monoclonal antibody MO25 was used to detect FAT/CD36 (Matsuno et al., 1996). Commercially available antibodies were used to detect cytochrome c oxidase IV (Cox-IV – Santa Cruz Biotechnology), Na<sup>+</sup>K<sup>+</sup> ATPase  $\alpha$  1 subunit (Upstate Biotechnology), and sarcoplasmic reticulum calcium ATPase (SERCA1) (Affinity Bioreagents Inc). An internal control of previously extracted human muscle crude membrane was used in each gel. Blots were quantified using

chemiluminescence and the ChemiGenius 2 Bioimaging system (SynGene, Cambridge, UK).

### *3.3.10 Calculation of whole body oxidation rates*

Whole body CHO and fat oxidation rates (g/min) were calculated according to the following equations (137, 159): CHO oxidation =  $4.585 \text{ VCO}_2 \text{ production (L/min)} - 3.226 \text{ O}_2 \text{ production (L/min)}$ , fat oxidation =  $1.695 \text{ O}_2 \text{ production (L/min)} - 1.701 \text{ CO}_2 \text{ production (L/min)}$ . To convert CHO and fat oxidation rates to kilojoules per minute, values were multiplied by 16.19 and 40.80, respectively.

### *3.3.11 Statistics*

All data are presented as the mean  $\pm$  S.E.. Differences between control and SSO treatments were analyzed with paired t-tests. One-way analysis of variance was used to determine significance between all other treatments. When significance was obtained, a Fisher's LSD post hoc analysis was completed. Associations between variables were investigated using Pearson correlation analyses, as appropriate. Statistical significance was accepted at  $P < 0.05$ .

### 3.4 Results

#### 3.4.1 Whole body metabolic measurements

VO<sub>2</sub> was greater (P<0.05) than rest at all time points during exercise, and progressively increased from 55% VO<sub>2peak</sub> at 15 min to 63% VO<sub>2peak</sub> at 120 min (Table 3.1). The VCO<sub>2</sub> and total oxidation rates increased with exercise by ~8% from 30 min to 120 min of cycling (Table 3.1).

**Table 3.1. Whole body metabolic measurements during 120 min of cycling at ~60%**

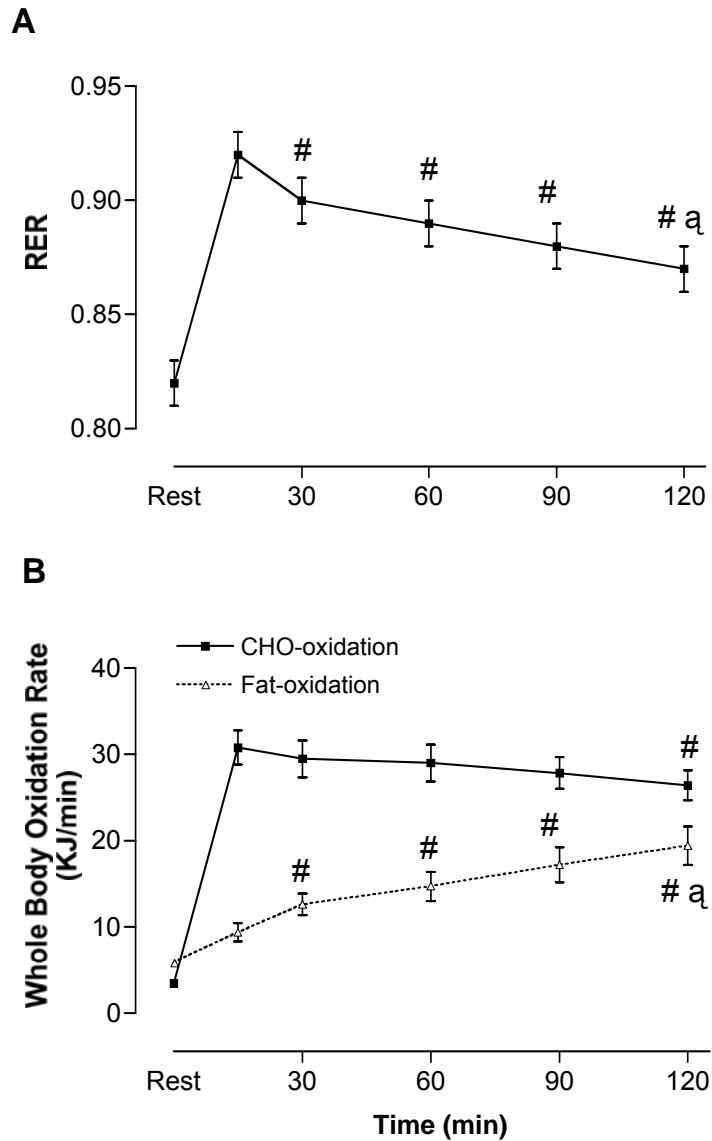
**VO<sub>2peak</sub>**

	Time					
	Rest	15 min	30 min	60 min	90 min	120 min
VO <sub>2</sub> (l/min)	0.40 ± 0.03	1.88 ± 0.10	1.98 ± 0.11	2.06 ± 0.11 #	2.13 ± 0.12 #†	2.17 ± 0.14 #†
% VO <sub>2max</sub>	11.6 ± 0.6	55.2 ± 3.0	57.7 ± 2.8	60.0 ± 2.9 #	61.8 ± 3.0 #†	62.7 ± 2.8 #†
VCO <sub>2</sub> (l/min)	0.33 ± 0.02	1.74 ± 0.09	1.79 ± 0.10	1.84 ± 0.10	1.87 ± 0.10 #	1.88 ± 0.11 #†
Calculated total oxidation (KJ/min)	9.4 ± 0.4	40.2 ± 2.1	42.1 ± 2.3 #	43.7 ± 2.4 #	45.0 ± 2.6 #†	45.8 ± 2.9 #††

Values are means ± S.E. (n=15). All time points are significantly (P<0.05) different from rest, # significantly different from 15 min, † significantly different from 30 min, and ‡ significantly different from 60 min.

The respiratory exchange ratio (RER) was greater than rest at all time points during exercise, and progressively decreased from 0.90 at 30 min to 0.87 at 120 min (Fig. 3.1A).

Whole body fat oxidation rates increased by 54% from 30 to 120 min of exercise (Fig. 3.1B). Whole body CHO oxidation rates decreased by 12% from 30 to 120 min of exercise (Fig. 3.1B).



**Figure 3.1. Whole body metabolic measures during 120 min of cycling at ~60%  $VO_{2peak}$ .** Values are means  $\pm$  S.E (n=15). A: Respiratory exchange ratio (RER). B: Calculated fat and carbohydrate (CHO) rates expressed in KJ/min. All time points are significantly ( $P < 0.05$ ) different from rest, # significantly different from 15 min, † significantly different from 30 min, and ‡ significantly different from 60 min.

### 3.4.2 Blood measurements

Blood glucose remained constant for the initial 30 min of exercise and progressively decreased ( $P<0.05$ ) following 60 min (Table 3.2). Blood lactate was greater than rest at all time points, and remained constant throughout exercise (Table 3.2). Plasma FFA and glycerol levels remained stable for the initial 30 min of exercise, and then significantly increased, during the final 90 min of exercise (Table 3.2).

**Table 3.2. Blood metabolites and plasma FFA concentrations during 120 min of cycling at ~60%  $\text{VO}_{2\text{Peak}}$**

	Time					
	Rest	15 min	30 min	60 min	90 min	120 min
Glucose (mM)	4.2 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	4.0 ± 0.1 *	3.8 ± 0.1 <sup>#</sup>	3.5 ± 0.1 <sup>##†‡Φ</sup>
Lactate (mM)	0.5 ± 0.1	2.0 ± 0.3 *	1.8 ± 0.3 *	1.5 ± 0.3 *	1.5 ± 0.3 *	1.5 ± 0.2 *
FFA (mM)	0.32 ± 0.03	0.31 ± 0.03	0.36 ± 0.05	0.59 ± 0.10 <sup>##†</sup>	0.71 ± 0.10 <sup>##†</sup>	0.89 ± 0.10 <sup>##†‡Φ</sup>
Glycerol (μM)	36 ± 7	47 ± 8	77 ± 26	145 ± 36 <sup>##†</sup>	180 ± 29 <sup>##†</sup>	234 ± 34 <sup>##†‡Φ</sup>

Values are means ± S.E. (n=15). \* significantly ( $P<0.05$ ) different from rest, <sup>#</sup> significantly different from 15 min, <sup>†</sup> significantly different from 30 min, <sup>‡</sup> significantly different from 60 min, and <sup>Φ</sup> significantly different from 90 min.

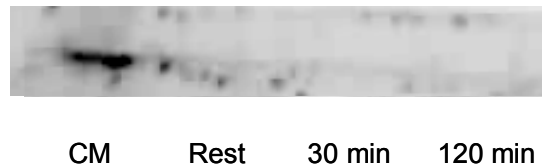
### 3.4.3 Mitochondrial purification

Across all experiments, mitochondrial recovery from skeletal muscle was  $26 \pm 2\%$ , while the quality of the preparation was  $88 \pm 2\%$  (see Methods). Western blotting demonstrated the isolation procedure successfully yielded highly purified mitochondria without contamination from other sources. Specifically, Western blotting demonstrated the absence of SERCA (110kDa), and  $\text{Na}^+\text{K}^+$  ATPase (112kDa) proteins, and the presence of COXIV (22kDa) on purified mitochondrial extracts from human skeletal muscle (Fig. 3.2).

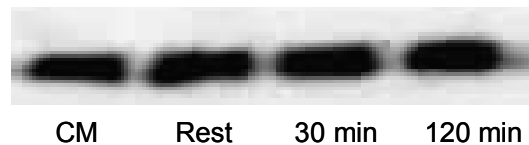
### A: Sarcoplasmic Reticulum Ca<sup>2+</sup>-ATPase



### B: Na<sup>+</sup>-K<sup>+</sup> ATPase



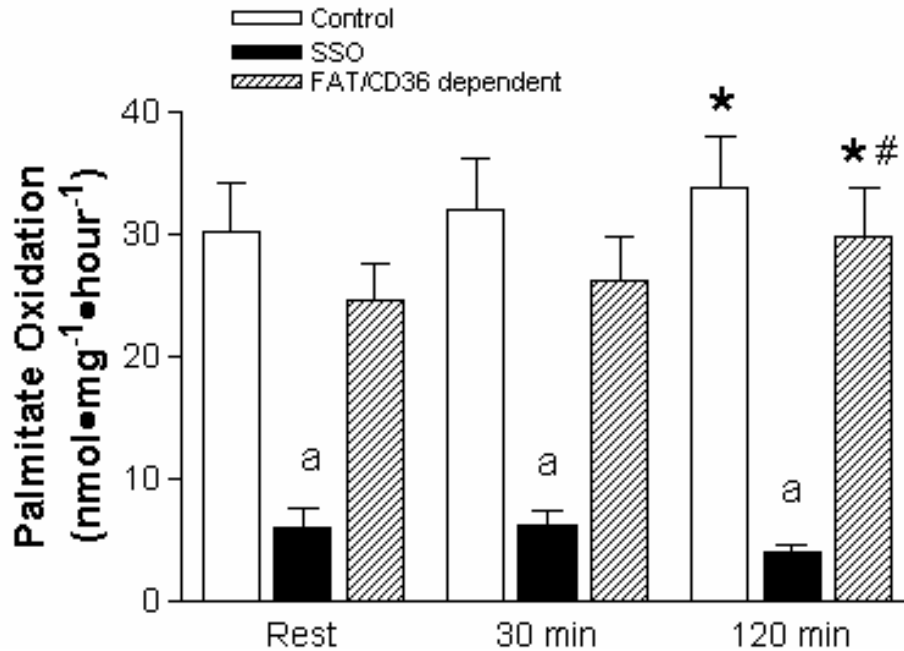
### C: Cytochrome c Oxidase IV



**Figure 3.2. Representative Western blots performed on purified isolated mitochondria from human skeletal muscle.** CM consisted of crude membranes obtained from human skeletal muscle. A, sarcoplasmic reticulum calcium ATPase. B, sodium potassium ATPase,  $\alpha$  1 subunit. C, cytochrome c oxidase IV.

#### 3.4.4 Mitochondrial palmitate oxidation

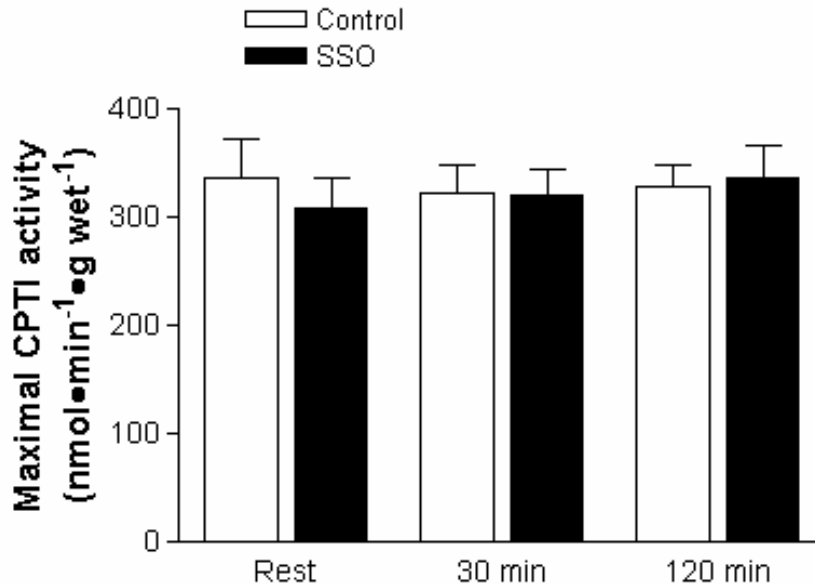
Isolated mitochondrial palmitate oxidation rates were higher ( $P < 0.05$ ) following 120 min of exercise compared to rest (Fig. 3.3). In the presence of SSO, a specific inhibitor of FAT/CD36 (Coort et al., 2002), palmitate oxidation was dramatically reduced at all time points (Fig. 3.3). Isolated mitochondrial FAT/CD36 dependent palmitate oxidation rates (subtraction of the 200  $\mu$ M SSO oxidation rates from control values) were also higher following 120 min of exercise compared to rest and 30 min (Fig. 3.3).



**Figure 3.3. In vitro palmitate oxidation rates during 120 min of cycling at ~60%  $VO_{2peak}$ .** Values are means  $\pm$  S.E. expressed in nmol/mg/hour (n=8). FAT/CD36 dependent palmitate oxidation was individually calculated as the difference between oxidation rates with and without 200  $\mu$ M SSO. \* significantly ( $P < 0.05$ ) different from rest, and # significantly different from 30 min, and <sup>a</sup> significantly different from control.

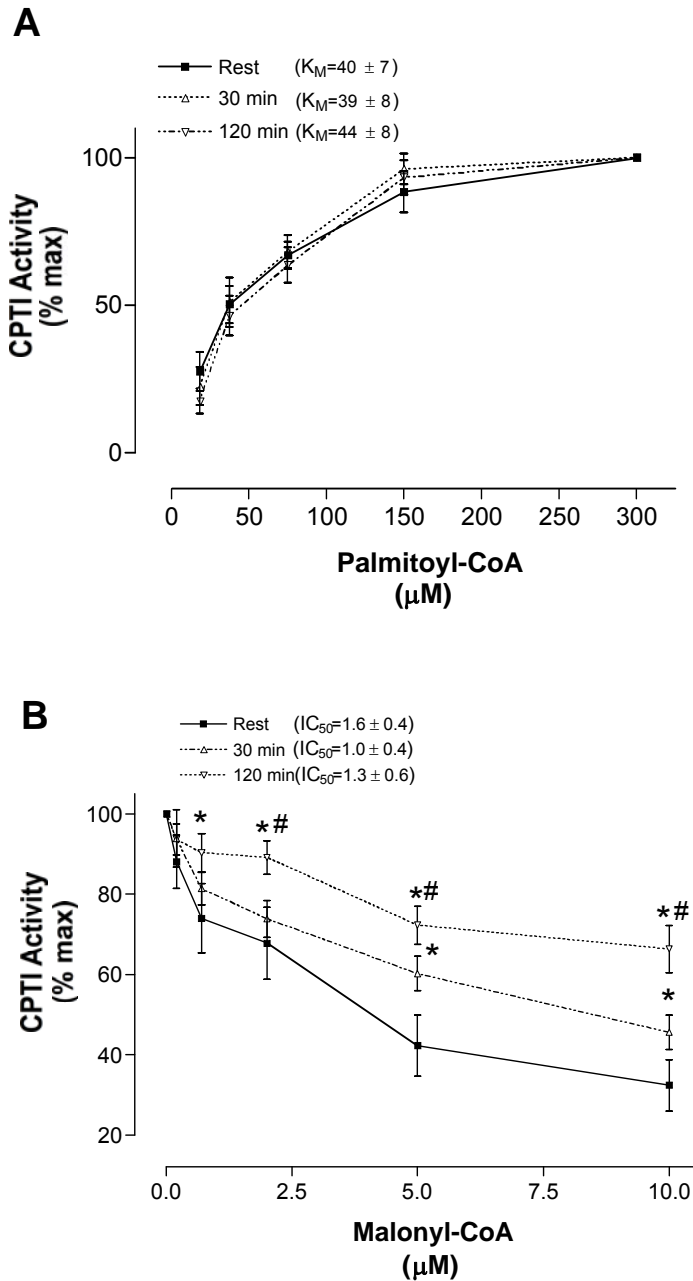
#### 3.4.5 CPTI activity

Maximal CPTI activity (at 300  $\mu$ M palmitoyl-CoA) was not altered during 120 min of exercise (Fig. 3.4), nor was it inhibited by the addition of 200  $\mu$ M SSO (Fig. 3.4).



**Figure 3.4. Maximal CPTI activity during 120 min of cycling at ~60%  $\text{VO}_{2\text{peak}}$ , and CPTI activity in the presence of 200  $\mu\text{mol}$  SSO. Values are means  $\pm$  S.E. expressed in nmol/min/g wet (n=7).**

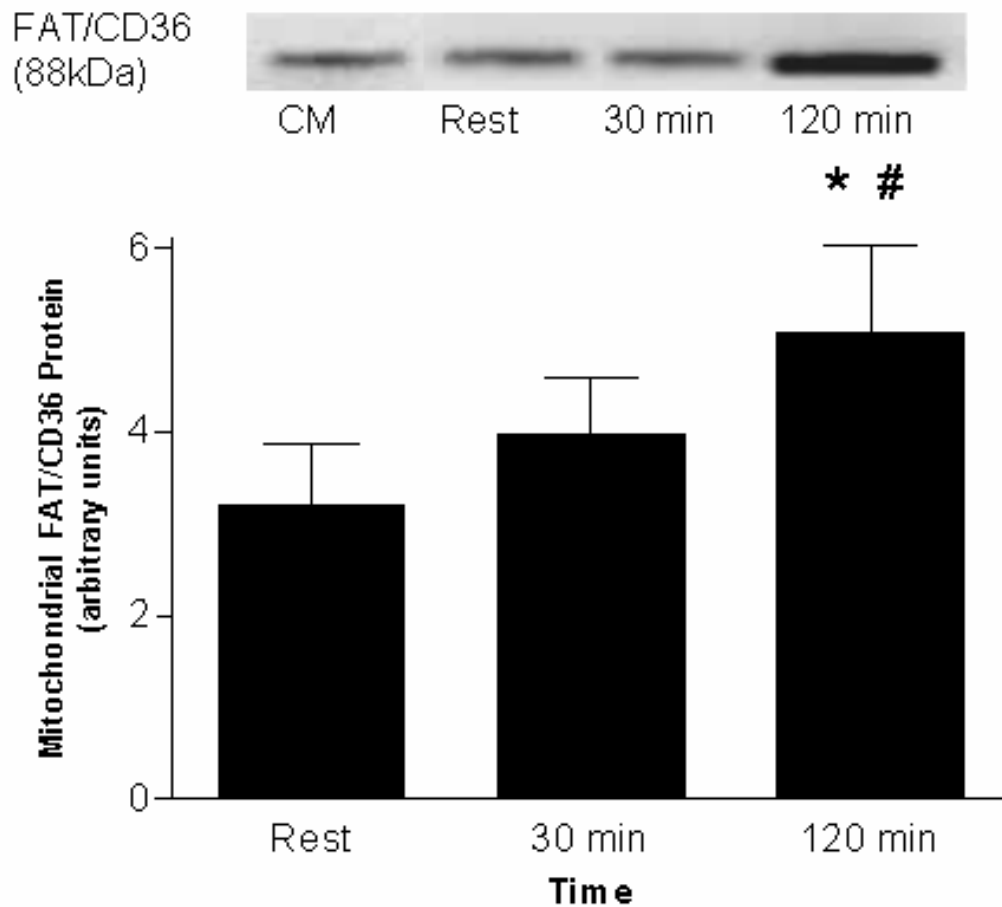
CPTI activity at various concentrations of palmitoyl-CoA was also not altered during exercise, nor was the palmitoyl-CoA Michaelis constant ( $K_M$ ) (Fig. 3.5A). However, the sensitivity of CPTI to the inhibitor M-CoA was progressively reduced following 120 min of exercise without altering the concentration required for 50% inhibition ( $\text{IC}_{50}$ ) (Fig. 3.5B). Compared to rest, CPTI activity following 30 and 120 min was higher in the presence of 5.0 and 10.0  $\mu\text{M}$  M-CoA (Fig. 3.5B). Also, following 120 min of exercise, CPTI activity was elevated in the presence of 0.7 and 2.0  $\mu\text{M}$  M-CoA.



**Figure 3.5. Carnitine palmitoyltransferase I (CPTI) kinetics during 120 min of cycling at  $\sim 60\%$   $\text{VO}_{2\text{peak}}$ .** Values are means  $\pm$  S.E., in % of maximal activity ( $n=7$ ). A: Palmitoyl-CoA kinetics. B: Malonyl-CoA kinetics at  $300 \mu\text{M}$  palmitoyl-CoA. \* significantly ( $P<0.05$ ) different from rest, and # significantly different from 30 min.

### 3.4.6 Mitochondrial FAT/CD36 protein content

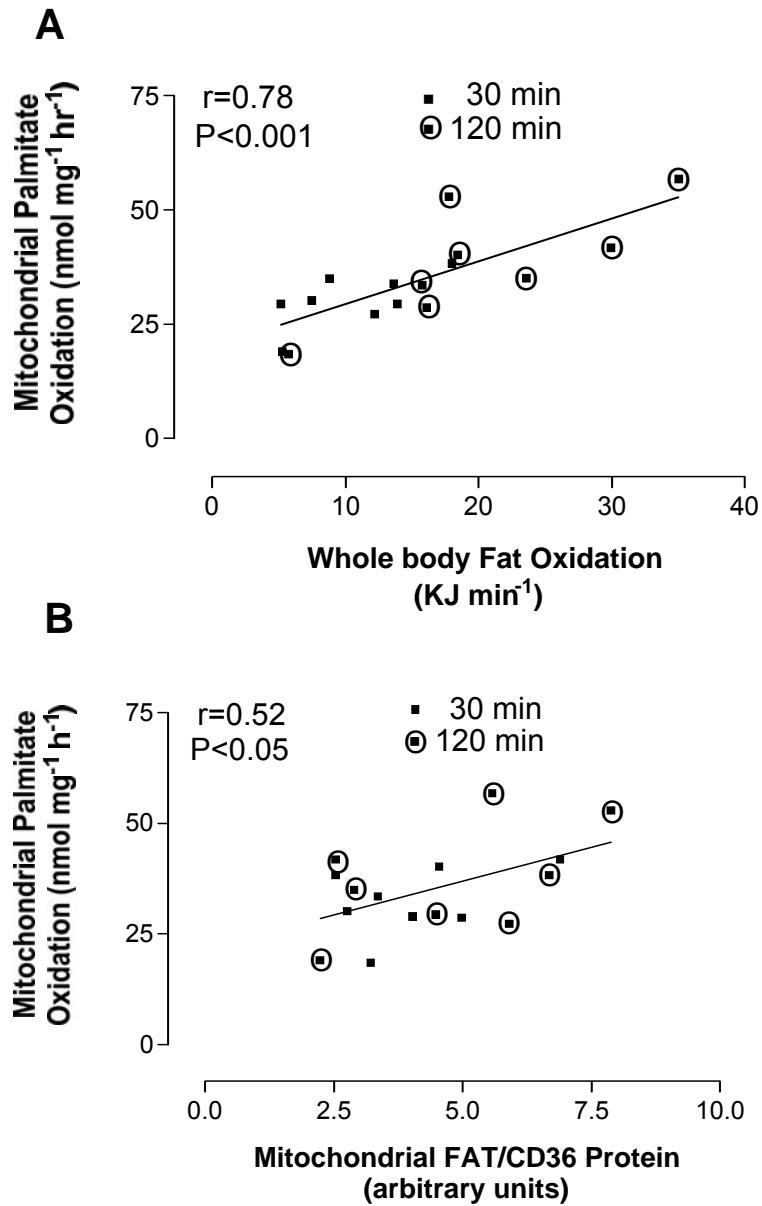
Western blots performed on all subjects (n=15) demonstrated a progressive increase ( $P<0.05$ ) in mitochondrial FAT/CD36 protein. Following 120 min of cycling the content of FAT/CD36 on the mitochondrial membrane increased by 59% (Fig. 3.6).



**Figure 3.6. Representative FAT/CD36 Western blot performed on isolated mitochondria following 120 min of cycling at ~60%  $VO_{2peak}$ . Values are means  $\pm$  S.E., in arbitrary units (n=15). \* significantly ( $P<0.05$ ) different from rest, and # significantly different from 30 min.**

### 3.4.7 Correlation analysis

A significant correlation ( $P < 0.001$ ) ( $r = 0.78$ ) existed between estimated whole body fat oxidation rates and mitochondrial palmitate oxidation rates during exercise (30 and 120 min) (Fig. 3.7A). Mitochondrial FAT/CD36 protein content and mitochondrial palmitate oxidation rates observed at all time points significantly correlated ( $P = 0.05$ ,  $r = 0.41$ ; data not shown). Mitochondrial FAT/CD36 protein content and palmitate oxidation rates analyzed in isolation at rest and 30 min, when fatty acid oxidation was the lowest, did not correlate ( $P = 0.17$ ,  $r = 0.39$  and  $P = 0.15$ ,  $r = 0.43$ , respectively; data not shown). However, a significant correlation was observed during exercise (30 and 120 min) ( $P < 0.05$ ,  $r = 0.52$ ) and following 120 min when fatty acid oxidation was greatest ( $P < 0.05$ ,  $r = 0.63$ ) (Fig. 3.7B).



**Figure 3.7. Relationships between mitochondrial palmitate oxidation, FAT/CD36 and whole body fat oxidation rates.** A: calculated correlation between estimated whole body fat oxidation rates and isolated mitochondrial palmitate oxidation rates at 30 and 120 min. B: Pearson correlation calculated between mitochondrial FAT/CD36 protein and isolated mitochondrial palmitate oxidation rates at 30 and 120 min.

### 3.5 Discussion

In the present study we examined the relationship between increases in whole body fat oxidation and two putative mitochondrial LCFA transport regulators in response to prolonged exercise. Specifically, we investigated palmitoyl-CoA and M-CoA CPTI sensitivity, and the presence and functional significance of FAT/CD36 on skeletal muscle mitochondria, during 120 min of cycling at  $\sim 60\%$   $VO_{2peak}$ . In accordance with our hypothesis, 120 min of cycling decreased the sensitivity of CPTI to M-CoA and increased FAT/CD36 mitochondrial protein content. Interestingly, the increased mitochondrial palmitate oxidation rates during exercise correlated with both whole body fat oxidation rates and FAT/CD36 mitochondrial protein content, suggesting an important role for mitochondrial FAT/CD36 in LCFA transport during exercise.

Generally, absolute fatty acid oxidation reaches maximal rates at  $\sim 64\%$   $VO_{2peak}$ , and about 50-70% of the total fat oxidized at this intensity is derived from plasma LCFA (for review see (180)). Although plasma LCFA have been reported to represent up to 70% of the fat oxidized during moderate intensity exercise, the mobilization of LCFA cannot entirely explain alterations in LCFA oxidation, as increases in whole body fat oxidation have been observed at the start of exercise without elevations in plasma FFA (133, 187). Regardless of the source, LCFA transport into mitochondria must precede its oxidation. In rat muscle it appears that CPTI activation is critical to increasing LCFA oxidation (42, 191), and recently involvement of FAT/CD36 in this process has also been implicated (Campbell et al., 2004). Thus we examined whether similar mechanisms contribute to increasing LCFA oxidation during exercise in isolated mitochondria.

### *3.5.1 CPTI activity*

The small reductions in M-CoA levels observed during prolonged exercise cannot fully explain the increases in fatty acid transport and oxidation that have been observed in humans during exercise (48, 134, 143). The results from this study are the first to show a decrease in the CPTI M-CoA sensitivity following exercise in human skeletal tissue. An alteration in total CPTI protein is not a likely explanation for the decreased M-CoA kinetics given the short duration of the exercise protocol, the unaltered maximal CPTI activity, and the constant palmitoyl-CoA sensitivity. Although the M-CoA sensitivity was altered following 30 min of exercise, importantly, the M-CoA sensitivity following 120 min of cycling was further attenuated. This study clearly depicts a situation where, despite constant or elevated M-CoA levels, CPTI activity can increase fatty acid transport into mitochondria as exercise duration increases beyond 30 min.

The CPTI enzyme, although anchored to the mitochondrial outer membrane possess two hydrophilic cytosolic domains. It has also been shown that changing the electrical charge of only a single amino acid in the C-terminus can drastically alter the sensitivity of CPTI for M-CoA (130). The existence of a cytosolic N/C interaction has been demonstrated to be required for M-CoA binding in rat liver tissue (53), but remains to be demonstrated in skeletal tissue. This interaction may create a plausible mechanism for the reduced M-CoA sensitivity observed in this study. Although this mechanism appears to exist in liver tissue, it has not been shown to occur in skeletal muscle, and currently the regulation of M-CoA kinetics in skeletal muscle remains speculative.

### *3.5.2 FAT/CD36 regulation*

FAT/CD36 protein has been isolated in adipocytes (Abumrad et al., 1993) and on plasma membranes (Bonen et al., 2000; Bonen et al., 2004; Coort et al., 2004), and been shown to influence the transport of LCFA across plasma membranes. Recently, it has also been demonstrated that the LCFA transport protein FAT/CD36 is present on mitochondrial membranes of rat (Campbell et al., 2005) and human (Bezaire et al., 2006) skeletal muscle. Mitochondrial FAT/CD36 was shown to be required for palmitate and palmitoylcarnitine oxidation in human skeletal muscle, and as a result it has been proposed that FAT/CD36 facilitates the transport of LCFA-carnitine to CPTII (Bezaire et al., 2006).

The palmitate oxidation results of the present study confirmed the findings of Bezaire et al. (2006) by demonstrating the necessary presence of FAT/CD36 for isolated mitochondria LCFA oxidation, and the inhibition of palmitate oxidation with the addition of SSO, a blocker of FAT/CD36. Importantly, it has previously been shown that the SSO concentration used in this study had no effect on either pyruvate or octanoate oxidation (Campbell et al., 2004; Bezaire et al., 2006). These observations demonstrate the specificity of SSO to inhibit FAT/CD36 and the importance of FAT/CD36 for the proper functioning of mitochondrial LCFA transport and oxidation during exercise. The importance of mitochondrial FAT/CD36 in a resting situation is still unclear. However, the present findings demonstrated that exercise induced an increase in mitochondrial FAT/CD36 protein content which correlated with rates of palmitate oxidation in isolated mitochondria, confirming previous results from our laboratory in rat skeletal muscle (Campbell et al., 2004). Since rat mitochondrial FAT/CD36 content increased in response

to acute (30 min) muscle contraction, and the observation that total muscle FAT/CD36 was unaltered, it has been proposed that FAT/CD36 translocated to the mitochondria during exercise (Campbell et al., 2004), similar to its apparent translocation to the sarcolemma (Bonen et al., 2000). While the feasibility of translocation from an intracellular pool appears plausible, the mechanisms responsible for increased mitochondrial FAT/CD36 protein during exercise remains to be identified.

A significant correlation existed between FAT/CD36 mitochondrial protein and mitochondrial palmitate oxidation rates following 120 min of cycling, as well as when data collected at 30 min and 120 min were combined. Although Campbell et al. (2004) previously reported that mitochondrial FAT/CD36 levels from several rat tissues followed an oxidative potential hierarchy, it has been reported that resting human mitochondrial FAT/CD36 protein does not correlate with palmitate oxidation on its own (Bezaire et al., 2006). This was also observed in the present study. Potentially this discrepancy can be explained as a result of variations in the training status, oxidative potential and fibre types between subjects. The implication is that at rest, as a result of the limited requirements for fatty acid transport and oxidation, mitochondrial FAT/CD36 may not be a limiting factor. However, mitochondrial FAT/CD36 appears to add flexibility to the regulatory system by increasing translocation to the mitochondrial membrane when LCFA transport and oxidation rates are increased. During prolonged exercise a greater reliance on FAT/CD36 mediated transport may develop, however the mechanism responsible for this alteration remains unknown.

It still remains to be shown how FAT/CD36 participates in transport at the mitochondrial membrane. In contrast to results in rat skeletal muscle, in which SSO

inhibited CPTI activity (Campbell et al., 2004), the present study and that of Bezaire et al. (2006) observed that SSO did not inhibit CPTI activity in human skeletal muscle. Moreover, since SSO inhibited palmitate oxidation in isolated mitochondria, the present study supports the interpretation of the role of mitochondrial FAT/CD36 originally proposed by Bezaire et al. (2006). They suggested that FAT/CD36 is located downstream of CPTI, facilitating the transport of LCFA-carnitine from CPTI to CPTII.

In summary, we are the first to demonstrate that the sensitivity of CPTI to M-CoA decreases, and mitochondrial FAT/CD36 content increases, in response to exercise-induced increases in whole body and isolated mitochondrial LCFA oxidation. Although decreased CPTI M-CoA sensitivity coincided with increased mitochondrial FAT/CD36 protein content, it is unclear at this time if they independently increase LCFA transport. We propose that CPTI M-CoA sensitivity and mitochondrial FAT/CD36 are not redundant but rather complementary mechanisms that influence mitochondrial LCFA transport and oxidation.

## **CHAPTER FOUR**

# **FATTY ACID BINDING PROTEIN FACILITATES SARCOLEMMA FATTY ACID TRANSPORT BUT NOT MITOCHONDRIAL OXIDATION IN RAT AND HUMAN SKELETAL MUSCLE**

#### 4.1 Abstract

The transport of long-chain fatty acids (LCFA) across mitochondrial membranes is regulated by carnitine palmitoyltransferase I (CPTI) activity. However, it appears that additional fatty acid transport proteins, such as fatty acid translocase (FAT)/CD36, influence not only LCFA transport across the plasma membrane, but also LCFA transport into mitochondria. Plasma membrane associated fatty acid binding protein (FABPpm) is also known to be involved in sarcolemmal LCFA transport, and it is also present at the mitochondria. At this location it has been identified as mitochondrial aspartate amino transferase (mAspAT), despite being structurally identical to FABPpm. Whether this protein is also involved with mitochondrial LCFA transport and oxidation remains unknown. Therefore, we have examined the ability of FABPpm/mAspAT to alter mitochondrial fatty acid oxidation. Muscle contraction increased ( $P < 0.05$ ) the mitochondrial FAT/CD36 content in rat (+22%) and human skeletal muscle (+33%). In contrast, muscle contraction did not alter the content of mitochondrial FABPpm/mAspAT protein in either rat or human muscles. Electrotransfecting rat soleus muscles, *in vivo*, with FABPpm cDNA increased FABPpm protein in whole muscle (+150%;  $P < 0.05$ ), at the plasma membrane (+117%;  $P < 0.05$ ) and in mitochondria (+80%;  $P < 0.05$ ). In these FABPpm-transfected muscles palmitate transport into giant vesicles was increased by +73% ( $P < 0.05$ ), and fatty acid oxidation in intact muscle was increased by +18% ( $P < 0.05$ ). In contrast, despite the marked increase in mitochondrial FABPpm/mAspAT protein content (+80%), the rate of mitochondrial palmitate oxidation was not altered ( $P > 0.05$ ). However, electrotransfection increased mAspAT activity by +90% ( $P < 0.05$ ), and the mitochondrial FABPpm/mAspAT protein content was significantly correlated with mAspAT activity ( $r = 0.75$ ). It is concluded that FABPpm has two distinct functions,

depending on its subcellular location, a) it contributes to increasing sarcolemmal LCFA transport while not contributing directly to LCFA transport into mitochondria, and b) its primary role at the level of mitochondria is to function as an enzyme involved in the transport reducing equivalents into the matrix.

## 4.2 Introduction

The cellular transport of long-chain fatty acids (LCFA) across the plasma membrane has long been thought to occur via passive diffusion. However, in recent years a substantial body of literature has emerged indicating that LCFA uptake into muscle cells occurs via a protein mediated process [for review see (18, 102, 109)]. A number of proteins have been shown to facilitate the uptake of LCFA into parenchymal cells, including fatty acid translocase, the homolog of human CD36 (FAT/CD36, 88kDa) (3), a family of fatty acid transport proteins (FATP1-6, 63-70 kDa) (62, 77, 148), and plasma membrane associated fatty acid binding protein (FABPpm, 43 kDa) (82, 151, 166). While little is known about the regulation of FATP1-6 in muscle tissue, considerable evidence has accumulated to indicate that FAT/CD36 and FABPpm are important in regulating the uptake of LCFAs into cardiac and skeletal muscle.

Recently, FAT/CD36 was also found to be present in skeletal muscle mitochondria membranes (15, 36). In rodents, electrically-induced muscle contraction (30 min) increased the mitochondrial content of FAT/CD36 (36). Similar results have been observed in mitochondria isolated from human muscle after 2 h of moderate intensity exercise ( $\sim 60\%$   $VO_{2max}$ ) (78). In both rodents and humans, the muscle contraction-induced increases in muscle mitochondrial FAT/CD36 were associated with increases in fatty acid oxidation in isolated mitochondria (36, 78). In addition, when FAT/CD36 was blocked by SSO, a specific inhibitor of FAT/CD36, mitochondrial fatty acid oxidation was almost completely inhibited ( $\sim 90\%$ ), either in mitochondria obtained from resting or exercised muscle (15, 36, 78). These studies do not negate the well-known role of carnitine palmitoyltransferase (CPTI) activity in mitochondrial fatty acid oxidation.

However, it appears that FAT/CD36 works in conjunction with CPTI, as together these two proteins predict rates of mitochondrial fatty acid oxidation ( $r = 0.90$ ; (15)), and FAT/CD36 and CPTI are co-localized in mitochondria (36, 149).

Whether FABPpm, another LCFA transport protein, also contributes to regulating mitochondrial LCFA oxidation is not known. However a series of studies have shown that FABPpm and mitochondrial aspartate aminotransferase (mAspAt) are identical proteins (14, 29, 168), and are found at the plasma membrane and the mitochondria, respectively (37). To date it is known that plasma membrane FABPpm contributes to regulating fatty acid transport (151, 168, 200) while in mitochondria mAspAt catalyzes the following reversible reaction:  $\text{glutamate} + \text{oxaloacetate} \leftrightarrow \text{aspartate} + 2\text{-oxoglutarate}$  (106). Transfecting 3T3 fibroblasts (82) or skeletal muscle (43) with mAspAt cDNA increased plasmalemmal FABPpm and increased the rates of LCFA transport (43, 82), as well as the rates of LCFA oxidation in muscle (43). Whether the upregulation of LCFA oxidation was due to the increased influx of LCFA into the muscle and/or to an increase in FABPpm/mAspAt at the mitochondria was not determined. Furthermore, there is strong evidence suggesting that FABPpm collaborates with FAT/CD36 to transport LCFAs across the plasma membrane (118), and a similar role for FABPpm/mASpAT could perhaps also occur in mitochondria where FAT/CD36 is also present. Thus, it is important to resolve whether FABPpm/mAspAt has a role in both plasma membrane LCFA transport and in mitochondrial LCFA oxidation.

Because we have shown that upregulation of LCFA oxidation in contracting muscles involves the translocation of FAT/CD36 to the mitochondria (36, 78) we first examined whether FABPpm/mAspAt was also translocated to the mitochondria during

electrically induced muscle contraction in a rat muscle and after 2 h of cycling exercise in human muscle. In addition, we also transfected rat muscle with mAspAt cDNA to examine the effects of FABPpm/mAspAt upregulation on the rates of plasma membrane LCFA transport, and on the rates of LCFA oxidation in intact muscle and in isolated mitochondria. Lastly, we also examined the effects of FABPpm/mAspAt upregulation on mAspAT activity in whole muscle and isolated mitochondria.

## 4.3 Research Design and Methods

### 4.3.1 *Electrical stimulation of rat muscle*

Female Sprague-Dawley rats (n=5, weighing ~175 g) were used in the contraction experiments. Animals were housed in a climate and temperature controlled room, on a 12:12 hour reverse light-dark cycle. Rat chow and water were provided *ad libitum*. The study was approved by the University of Guelph Animal Ethics Committee.

To ascertain if mitochondrial FABPpm/mAspAT content was altered in response to muscle contraction in a similar manner to FAT/CD36, rat hindlimbs were acutely (30 min) stimulated as described previously in our laboratory (23, 36). Briefly, while under anesthesia (intraperitoneal injections of sodium pentobarbital, 6 mg/100g body wt; MTC Pharmaceuticals, Cambridge, ON, Canada), a small incision was made in one hind limb, and stimulating electrodes placed on the exposed sciatic nerve. The contralateral muscle in the same animal acted as a resting control. Muscle contraction consisted of stimulating the sciatic nerve in one hind limb (10 V, 100 Hz, 100-ms trains, 20 tetani/min for 30 min) (Grass S48 Stimulator; Astro-Med Inc., Longueuil, QC, Canada), as we have reported previously (36). Immediately after stimulation the contracting soleus, and the contralateral resting soleus were removed for mitochondrial isolation as described below, and used for Wesern blotting analysis.

### 4.3.2 *Human exercise experiments*

Twelve healthy, recreationally active, individuals volunteered for this study (n = 9 males; n = 3 females (mean  $\pm$  S.E.); age:  $22 \pm 1$  yr, weight:  $77 \pm 4$  kg, BMI:  $24 \pm 1$  kg/m<sup>2</sup> and VO<sub>2</sub>peak:  $49 \pm 2$  ml/min/kg body mass. Subjects were fully informed of the purpose

of the experiments and of any possible risk before giving written consent to participate. The study was approved by the University of Guelph Ethics Committee.

Participants cycled for 2 hr at  $\sim 60\%$   $VO_{2peak}$  on a cycle ergometer (LODE Instrument, Groningen, The Netherlands), following a 12 hr overnight fast. Pulmonary  $O_2$  uptake was measured with a metabolic cart (SensorMedics  $V_{max}$  model, CA, USA). Prior to exercising, both legs were prepared for muscle biopsies of the vastus lateralis muscle. Ventilatory and muscle samples were obtained before (Pre) and after (Post) 2 hours of cycling. Muscle samples were obtained under local anesthesia (2% lidocaine without epinephrine) using the percutaneous needle biopsy technique described by Bergstrom (12). Immediately following tissue sampling, visible fat and connective tissue were dissected free from the muscle and the samples were blotted to remove excess blood. The tissue was used for the immediate isolation of mitochondria and for the determination of selected proteins with Western blotting.

#### *4.3.3 Rat solei electrotransfection*

Female Sprague-Dawley rats (weighing  $\sim 175$  g) were used in these experiments. Animals were housed in a climate and temperature controlled room, on a 12:12 hour reverse light-dark cycle. Rat chow and water were provided *ad libitum*. The study was approved by the University of Guelph Animal Ethics Committee.

During surgery, rats were maintained unconscious by inhalation of the anesthetic isoflurane (AErane; Baxter, Deerfield, IL, USA) at a range of 2 to 2.5% in pure oxygen at a flow rate of 2 L/min. Electrotransfection of FABPpm/mAspAt was performed as we have described previously (43). Briefly, the control limb was left 'intact' and the

experimental lower hindlimb was shaved and sterilized with iodine solution. A 1.5 cm incision was made laterally along the left hindlimb parallel to the tibia and extending to the Achilles tendon. The soleus muscle was exposed and 100  $\mu$ l of 0.45 % saline solution containing 250  $\mu$ g of plasmid cDNA (provided by Dr. A. Iriarte, University of Missouri, MO) was injected using a 27 gauge needle. The plasmid contained the open reading frame of FABPpm under the control of the CMV promotor as described previously (43), with the addition of a FLAG epitope (Sigma, St. Louis, MO, USA), inserted at the C-terminus by PCR methods. The modified FABPpm-FLAG was subcloned into pcDNA3.1 between *EcoRI* and *XbaI* sites. Immediately following the cDNA injection, electroporation of the intact soleus muscle was performed as previously described (43) (8 electric pulses: 200 V/cm, 1 Hz, 20 ms in duration) (ECM 830 Square Wave Electroporator; BTX, Holliston, MA, USA) using Tweezertrodes (BTX, Holliston, MA, USA). After the pulse was delivered, the overlying superficial muscle was sutured, and the skin incision was closed. Seven days following this procedure the animals were randomly divided into 3 groups. The first group was used for the analysis of whole muscle measurements (Westerns, palmitate transport, oxidation, and incorporation in diacylglycerol (DAG) and triacylglycerol (TAG) pools) (n=12). The second group was used to isolate vesicles (n=12). Due to tissue requirements the solei of 8 animals were pooled to create one sample for each independent FA transport assay. The third group was used for isolating mitochondria (n=5). Due to tissue requirements, the solei of 2 animals were pooled to create one sample for mitochondrial analysis (ie. n=1=2 rats), thus 10 rats were used to create 5 data points. However, prior to pooling for mitochondrial isolations, a small section of muscle (~10 mg) was removed for determining the enzymatic characteristics of

each animal (see below). Additional experiments were conducted with an empty vector to ensure that the transfection procedures did not alter any of the measured parameters.

#### *4.3.4 Preparation of giant vesicles*

Giant vesicles from pooled control and electrotransfected solei muscles were generated as previously described (23, 25, 26, 101, 109, 110, 162). Briefly, the tissues were cut into thin layers (1-3 mm thick) and incubated for 1 h at 34°C in 140 mM KCl/10 mM MOPS (pH 7.4), aprotinin (30 µg/ml; Sigma, . Louis, MO, USA) and collagenase (type VII, 150 units/ml) in a shaking water bath. At the end of the incubation, the supernatant fraction was collected and the remaining tissue was washed with KCl/MOPS and 10 mM EDTA which resulted in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (G.E. Healthcare, Aurora, OH, USA), and aprotinin were added to a final concentrations of 3.5% (v/v) and 10 µg/ml, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (w/v) and a 1 ml KCl/MOPS upper layer. This sample was centrifuged at 60 x g for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in KCl/MOPS and recentrifuged at 12 000 g for 5 min. The pellet was resuspended in KCl/MOPS.

#### *4.3.5 Palmitate uptake by giant vesicles*

Palmitate uptake studies were performed as we have previously described (25, 26, 101, 109, 116, 162). Fatty acid free BSA (MP Biomedicals, Solon, OH, USA), [9,10-<sup>3</sup>H]-palmitate (Perkin Elmer, Wellesley, MA, USA) and [<sup>14</sup>C]-mannitol (Perkin Elmer, Wellesley, MA, USA) were purchased from commercial sources. For palmitate transport

measurements, 40  $\mu$ l 0.1% BSA in KCl/MOPS, containing unlabeled (15  $\mu$ M) and radiolabeled 0.3  $\mu$ Ci [ $^3$ H]-palmitate and 0.06  $\mu$ Ci [ $^{14}$ C]-mannitol, was added to 40  $\mu$ l of vesicle suspension. The incubation was carried out for 15 s. Palmitate uptake was terminated by addition of 1.4 ml ice-cold KCl/MOPS, 2.5 mM HgCl<sub>2</sub> and 0.1% BSA. The sample was then centrifuged in a microfuge at 12 000 rpm for 2 min. The supernatant was discarded, and radioactivity was determined in the tip of the tube.

#### *4.3.6 Whole muscle palmitate metabolism*

In control and electrotransfected soleus muscle we examined the metabolism of palmitate. For these measurements we used methods that our group has previously published (50-52). Briefly, soleus muscle strips were preincubated for 20 min (30<sup>0</sup>C), followed by a 60 min incubation period (30<sup>0</sup>C). The incubation medium contained 0.5 mM palmitate, and 5mM glucose, as well as 2 $\mu$ Ci of [ $^{14}$ C]palmitate (G.E. healthcare, Aurora, OH, USA). Palmitate oxidation was determined by acidifying the incubation to release the <sup>14</sup>CO<sub>2</sub>, which was trapped by benzethonium hydroxide (Sigma, St. Louis, MO, USA). To determine the incorporation of palmitate into DAG and TAG pools, muscle was homogenized, spotted on silica gel plates (Silica Gel GF, 250 mm; Analtech, Newark, DE), and resolved in solvent (60:40:4 heptane-isopopylether-acetic acid) for 45 min. Plates were air dried, sprayed with dichlorofluorescein dye and visualized under long-wave ultraviolet light. Individual lipid bands were quantified against known standards, and scraped into vials for liquid scintillation counting.

#### *4.3.7 Isolation of mitochondria from skeletal muscle*

Differential centrifugation was used to obtain pure and intact mitochondria containing both intermyofibrillar (IMF) and subsarcelommal (SS) fractions (36). All procedures were identical to those previously published by our group (15, 78). Briefly, muscle (~300 mg) was homogenized with a tight fitting Teflon pestle. The homogenate was centrifuged at 800 g for 10 min, to separate the SS and IMF mitochondria. The IMF mitochondria were treated with a protease (0.025 ml/g; Sigma, St. Louis, MO, USA) for exactly 5 minutes to digest the myofibrils. Further centrifugation was used to remove the myofibrils, and recombine the IMF with the SS mitochondria. The combined samples were centrifuged twice at 10 000 g for 10 min. The pellet was resuspended in 1 µl of buffer per mg of tissue. Following the oxidation measurements, the remaining mitochondria were further purified using a Percoll gradient for Western blotting analysis. Samples were centrifuged at 20 000 g for 1 hour and the mitochondrial layer was removed. The Percoll was removed from the sample by further centrifuging at 20 000 g for 5 hours.

#### *4.3.8 Mitochondrial palmitate oxidation*

Labeled CO<sub>2</sub> production from palmitate oxidation and acid soluble trapped <sup>14</sup>C were measured during state 4 respiration following a 30 min incubation of viable mitochondria in a sealed system, as previously described by our laboratory (15, 36, 78). Briefly, viable mitochondria (100 µl) were added to a system containing a pre-gassed modified Krebs Ringer buffer supplemented with 5 mM ATP, 1 mM NAD<sup>+</sup>, 0.5 mM DL-carnitine, 0.1 mM coenzyme A, 25 µM cytochrome C, and 0.5 mM malate. A microcentrifuge tube containing 500 µl of 1 M of benzethonium hydroxide inserted into a

1.5 ml centrifuge tube, was placed in the system to capture  $^{14}\text{CO}_2$  produced during the oxidation reaction. The system was then sealed with a rubber cap and further sealed with parafilm. The reaction was initiated by the addition of a 6:1 palmitate:BSA complex (containing 10  $\mu\text{Ci}$  of  $[1-^{14}\text{C}]$  palmitate, for a final palmitate concentration of 77  $\mu\text{M}$ ) administered by syringe through the rubber cap. The reaction continued for 30 min at 37°C and was terminated with the addition of ice-cold 12 N perchloric acid by syringe through the rubber cap.

A fraction of the reaction medium was removed through the cap and analyzed for isotopic fixation. Gaseous  $\text{CO}_2$  produced from oxidation of  $[1-^{14}\text{C}]$  palmitate was measured by acidifying the remaining reaction mixture. Liberated  $^{14}\text{CO}_2$  was trapped by the benzethonium hydroxide over a 90 min incubation period at room temperature. The microcentrifuge tube containing the  $^{14}\text{CO}_2$  was put in a scintillation vial, and radioactivity was determined.

#### *4.3.9 Mitochondrial viability*

Electrotransfection was done on 5 rats (weighing  $\sim 250$  g), and mitochondria isolated from both electrotransfected and contralateral control limbs as outlined above. Using a Clark-type electrode, mitochondrial respiration rates were measured in state 3 and state 4 conditions, and the respiratory control ratios (RCR) were calculated for both control and electrotransfected limbs to ensure the isolation procedure yielded intact mitochondria, and that electrotransfection did not alter the viability. Briefly, following the isolation procedure outlined above, 500  $\mu\text{g}$  ( $\sim 50$   $\mu\text{l}$ ) of mitochondria were added to 1 500  $\mu\text{l}$  of oxygraph medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-base, 10 mM  $\text{K}_2\text{HPO}_4$ , 0.1 mM EDTA and 0.8 mM  $\text{MgCl}_2$  (pH 7.0). State 3 respiration

was measured in the presence of 5 mM pyruvate, 2mM malate and 300  $\mu$ M ADP, and subsequently, state 4 respiration was measured following the consumption of the ADP. The RCR was calculated as the ratio between state 3 and state 4 respiration rates (41, 127, 147).

#### *4.3.10 $\beta$ -HAD, citrate synthase and aspartate aminotransferase activities*

A portion of the soleus muscle (~10 mg) was immediately homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer (10) and used for the measurements of  $\beta$ -hydroxyacyl-CoA dehydrogenase ( $\beta$ -HAD), citrate synthase (CS) and mAspAT activities. Total muscle  $\beta$ -HAD activity was measured in Tris-HCl buffer (50 mM Tris-HCl, 2 mM EDTA, 250  $\mu$ M NADH, pH 7.0) and 0.04% Triton-X. The reaction was started by addition of 100  $\mu$ M acetoacetyl-CoA and absorbance was measured at 340 nm over a 2 min period (37°C) (10).

Citrate synthase activity was determined in isolated mitochondria as well as in aliquots of homogenized whole muscle. Citrate synthase activity in intact mitochondria was determined by first assaying the extramitochondrial fraction in the suspension (1:20 dilution) and then assaying the total CS activity of the suspension (1:20 dilution) after lysing the mitochondria with 0.04% Triton X-100 and repeated freeze-thawing. The net difference provided a measure of the viability of the mitochondria, as well when compared to the total muscle CS activity provided a measure of the mitochondria recovered during our isolation procedure. The CS activity was assayed spectrophotometrically at 37°C by measuring the disappearance of NADH at 412 nm (159).

Mitochondrial aspartate aminotransferase activity was also measured in isolated mitochondria and in aliquots of homogenized whole muscle. Mitochondria and homogenate

were repeatedly freeze thawed prior to analysis. Maximal measurements were determined in a 75 mM phosphate buffer (75 mM phosphate, 60 mM 2-oxoglutarate, 6 U malate dehydrogenase, 0.25 % Triton-X, and excess NADH (adjusted to optimize spectrophotometer sensitivity range)). The reaction was started by addition of 400 mM aspartate and absorbance was measured at 340 nm over a 2 min period (25<sup>0</sup>C) (11).

#### *4.3.11 Western blotting*

Whole muscle crude membranes were generated as previously described (23, 25, 109), analyzed for total protein (BCA protein assay), and 25 µg of denatured protein was loaded for Western blotting. The plasma membrane content of fatty acid transporters were determined by generating vesicles (as described above), analyzing for total protein (BCA protein assay), and loading 10 µg of denatured protein for Western blotting. Purified isolated mitochondrial fractions were analyzed for total protein (BCA protein assay) and 25 µg of denatured protein from each sample were loaded for Western blotting. All proteins were separated by electrophoresis on an 8 % SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The FABPpm polyclonal antibody used was produced in our laboratories and has been used previously in our work (23, 25, 109). The MO-25 antibody used to detect FAT/CD36 was produced in N.N. Tandon's laboratory. Commercially available antibodies were used to detect cytochrome c oxidase IV (Cox-IV; Invitrogen, Burlington, ON, Canada), sarcoplasmic reticulum calcium ATPase (SERCA2) (Sigma, St. Louis, MO, USA) and Glut-4 (Chemicon International Inc., Temecula, CA, USA). In addition, an epitopic FLAG-tag (Sigma, St. Louis, MO, USA), specific to a 6 amino acid sequence inserted at the N-terminus of FABPpm, was used to detect *de novo* synthesis as a result of the cDNA electrotransfected

into soleus muscles. An internal control of previously extracted crude membranes from rat was used in each gel. Blots were quantified using chemiluminescence and the Syngene 2 ChemiGenius bioimaging system (Syngene, Cambridge, UK).

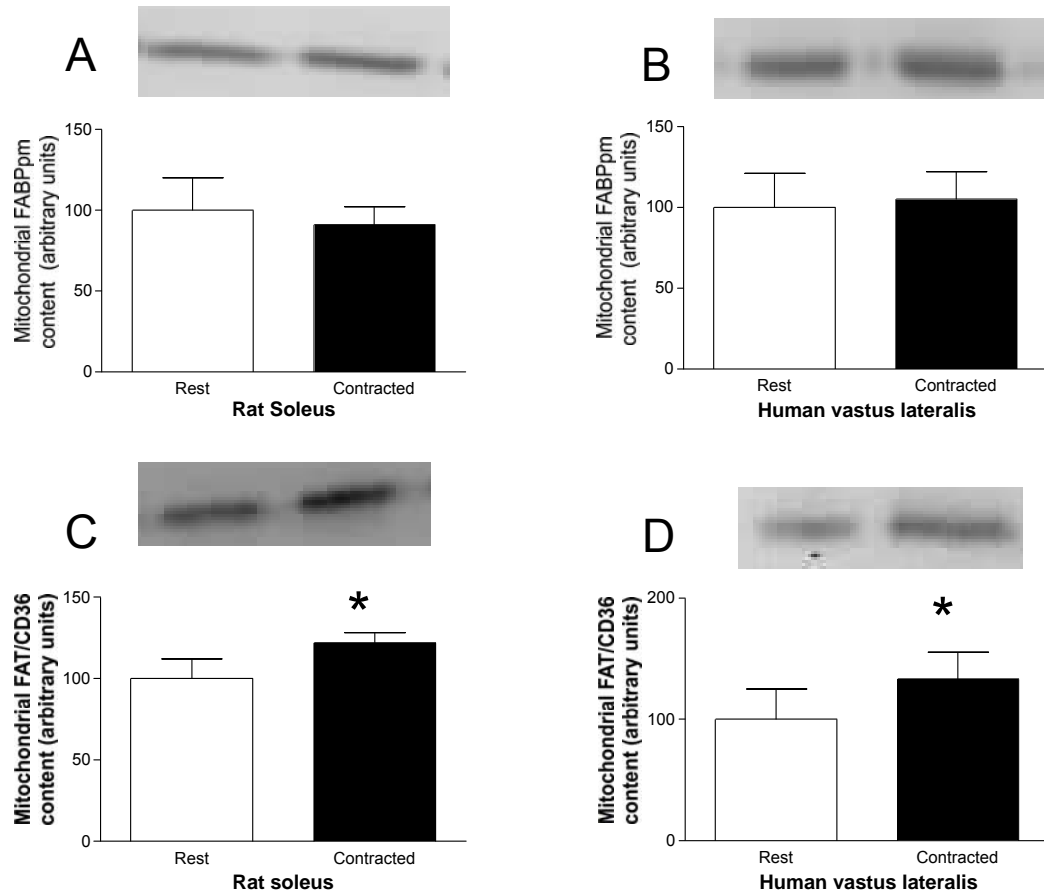
#### *4.3.12 Statistics*

All data are presented as the mean  $\pm$  S.E. All data were compared using a paired t-test. Associations between variables were investigated using Pearson correlation analyses. Statistical significance was accepted at  $P < 0.05$ .

## 4.4 Results

### *4.4.1 Effects of contraction on mitochondrial FABPpm content*

Initially we examined whether muscle contraction or exercise increased the mitochondrial content of FABPpm/mAspAt, as we have previously shown for FAT/CD36 in both rat (36) and human muscle (78). In accordance with our previous work (36), electrical stimulation increased FAT/CD36 on rat mitochondria by +22% ( $P < 0.05$ , Fig. 4.1C). In contrast, electrical stimulation did not alter rat mitochondrial FABPpm content (Fig. 4.1A). Similarly, 2 hours of cycling at  $\sim 60\%$   $VO_{2peak}$ , increased mitochondrial FAT/CD36 in human muscle by +33% ( $P < 0.05$ , Fig. 4.1D), but did not alter mitochondrial FABPpm/mAspAt content (Fig. 4.1B).



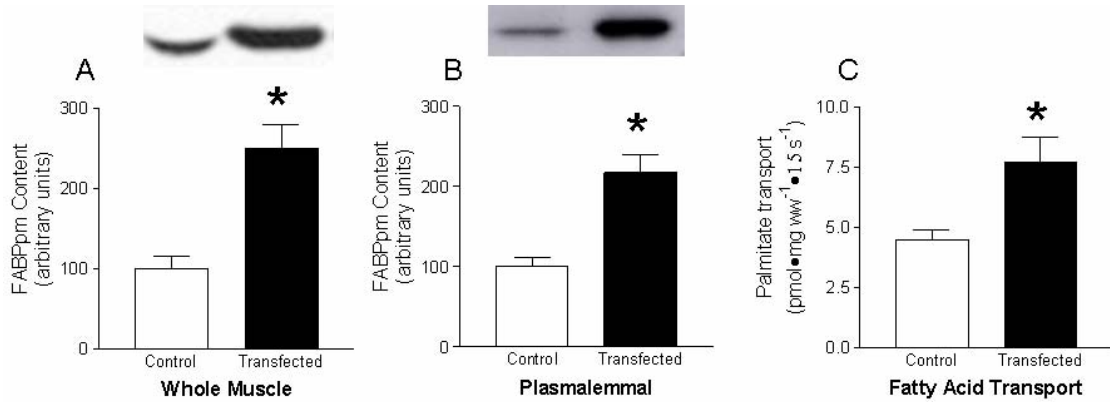
**Figure 4.1. Effects of muscle contraction on the content of mitochondrial FABPpm/mAspAT and FAT/CD36.** Values are means  $\pm$  S.E., expressed in arbitrary units. A: Mitochondrial FABPpm/mAspAT content in rat soleus muscle at rest, and following 30 min of electrical stimulation (n=5). B: Mitochondrial FABPpm/mAspAT content in human vastus lateralis muscle at rest, and following 2 hours of cycling at  $\sim 60\%$   $VO_{2peak}$  (n=12). C: Mitochondrial FAT/CD36 content in rat soleus muscle at rest, and following 30 min of electrical stimulation (n=5). D: Mitochondrial FAT/CD36 content in human vastus lateralis muscle at rest, and following 2 hours of cycling at  $\sim 60\%$   $VO_{2peak}$  (n=7). \* significantly different ( $P < 0.05$ ) from rest.

*4.4.2 Effects of FABPpm/mAspAT electrotransfection on plasma membrane proteins, palmitate transport into giant vesicles and palmitate oxidation in intact soleus muscle*

While the contraction-induced upregulation of FAT/CD36 has been linked to increased rates of mitochondrial LCFA oxidation (36, 78), the lack of change in mitochondrial FABPpm/mAspAt, during muscle contraction begins to suggest that this protein is not involved with fatty acid oxidation in mitochondria. To examine this more definitively we examined the effects of upregulating FABPpm/mAspAt on rates of LCFA transport and fatty acid oxidation in intact muscle and in isolated mitochondria.

When FABPpm/mAspAt was transfected into soleus muscles, there was a +150% increase in muscle FABPpm/mAspAT ( $P < 0.05$ , Fig. 4.2A), and a +117% increase in the plasmalemmal FABPpm content ( $P < 0.05$ , Fig. 4.2B). The content of FAT/CD36, another fatty acid transporter, was not altered with transfection in either whole muscle or at the plasma membrane (data not shown).

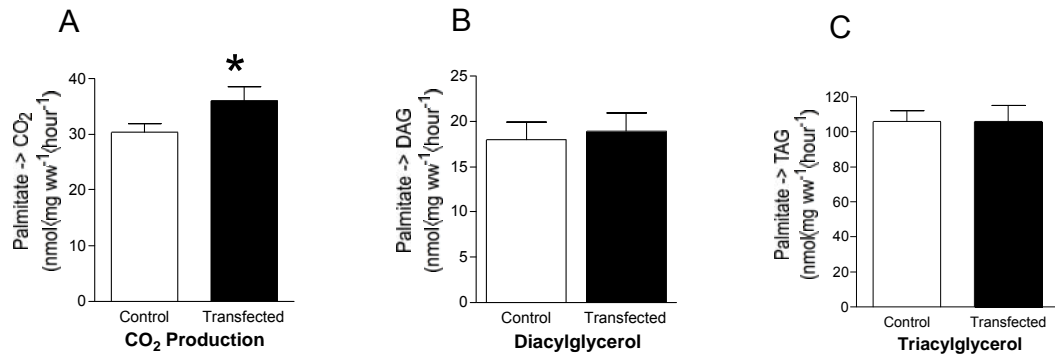
Transfection increased ( $P < 0.05$ ) the rate of palmitate transport into giant sarcolemmal vesicles by +73% (Fig. 4.2C).



**Figure 4.2. Effects of electrotransfection with FABPpm cDNA on soleus muscle.**

Values are means  $\pm$  S.E., protein contents expressed in arbitrary units, and transport expressed in  $\text{pmol}\cdot\text{mg wet weight (ww)}^{-1}\cdot 15 \text{ s}^{-1}$  ( $n=12$ ). A: Whole muscle FABPpm protein. B: Plasmalemmal FABPpm protein. C: Palmitate transport into giant sarcolemmal vesicles. \* significantly different ( $P<0.05$ ) from control.

In intact soleus muscle, there was a somewhat smaller increase in the rate of palmitate oxidation following transfection (+18 %,  $P<0.05$ , Fig. 4.3A), while the rates of palmitate incorporation into DAGs (Fig. 4.3B) or TAGs were not altered (Fig. 4.3C).

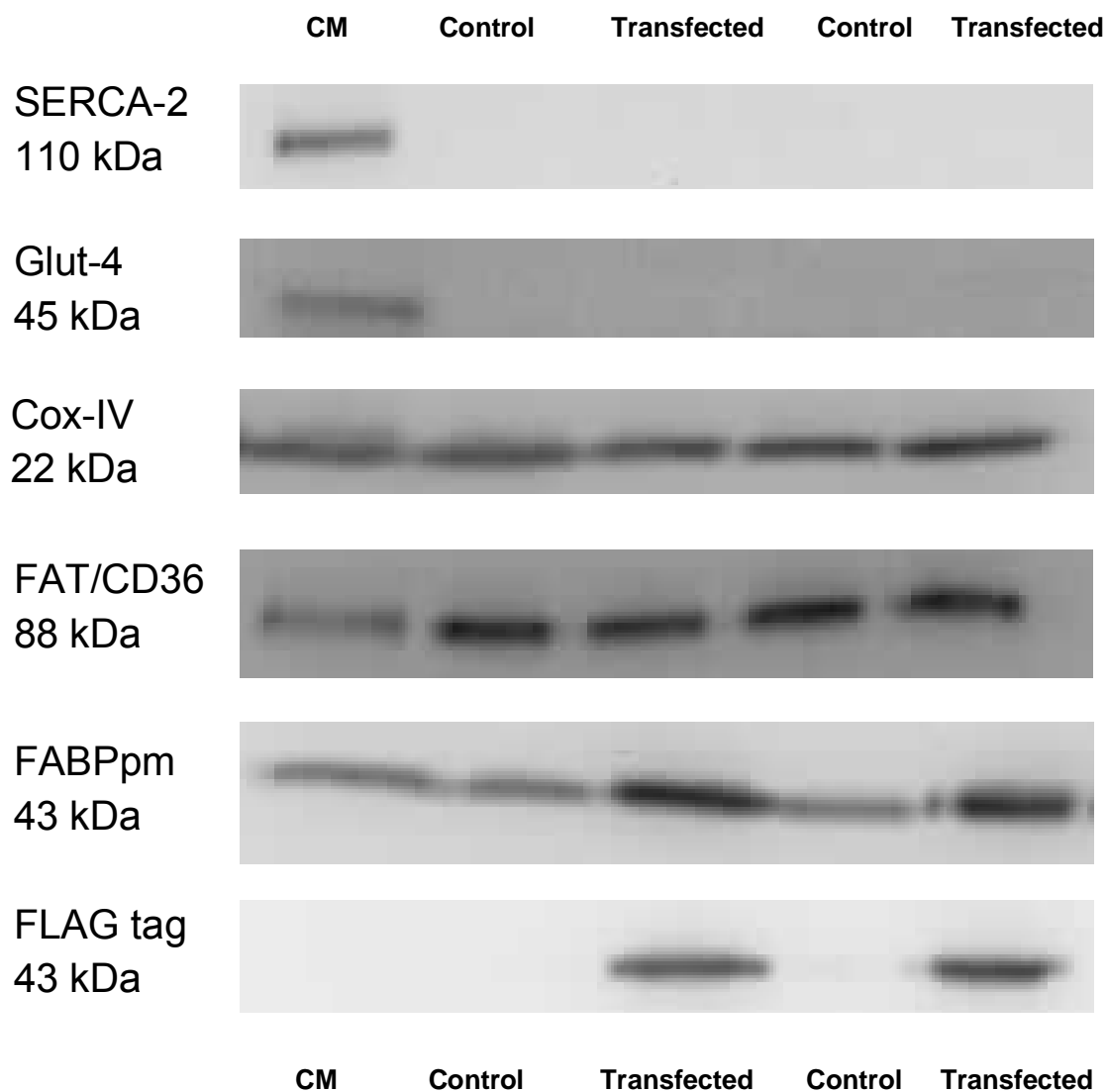


**Figure 4.3. Effects of electrotransfection with FABPpm cDNA on the fate of palmitate in soleus muscle.** Values are means  $\pm$  S.E., expressed in nmol·mg wet weight (ww)<sup>-1</sup>·hour<sup>-1</sup> (n=12). A; Palmitate incorporation into diacylglycerides. B; Palmitate incorporation into triacylglycerides. C; Palmitate oxidation. \* significantly different (P<0.05) from control.

#### 4.4.3 Electrotransfecting FABPpm/mAspAT in mitochondria

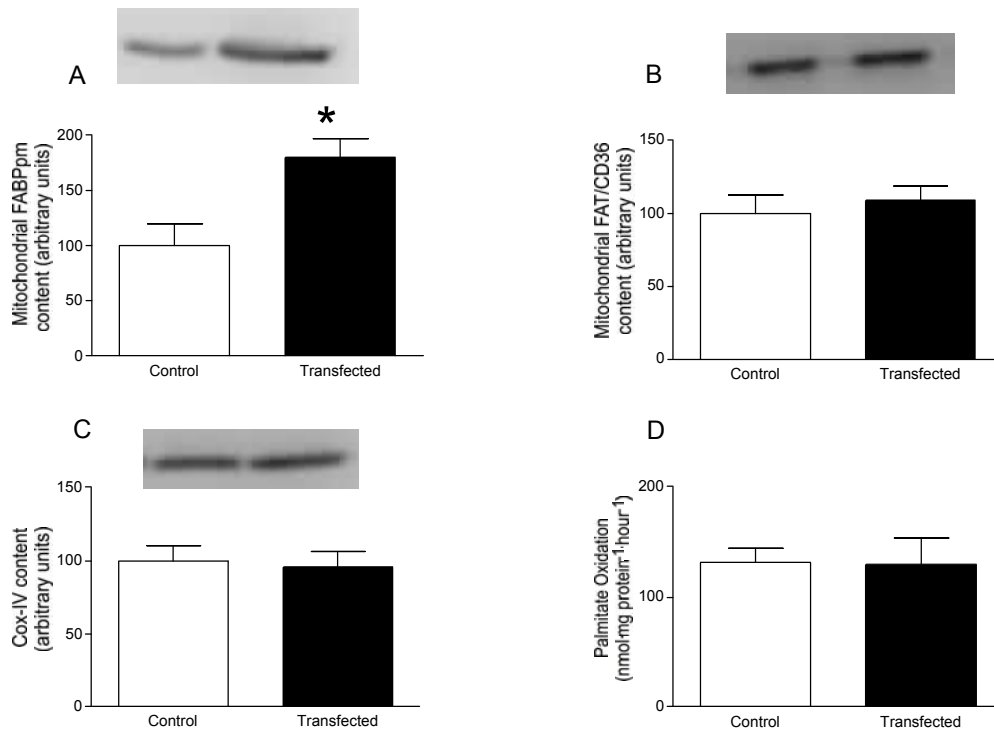
Next we determined whether the electrotransfection of FABPpm/mAspAt into muscle increased FABPpm/mAspAt in mitochondria, and whether this altered the rates of palmitate oxidation and/or aspartate aminotransferase activities in these organelles. The mitochondrial isolation procedure yielded intact mitochondria as the RCR values were similar to previous publications (41, 127, 147), as were the mitochondrial recovery and viability as calculated from CS values (see methods) (15, 34, 78). In addition, control and electrotransfection RCR values were not different ( $10.4 \pm 1.9$  vs  $10.0 \pm 1.4$ , respectively; P=0.84). The absence of SERCA (110kDa) and Glut-4 (45kDa) proteins, and the presence of Cox-IV (22kDa), FAT/CD36 (88kDa), and FABPpm (43kDa) (Fig. 4.4)

indicated that the isolation procedures successfully yielded highly purified mitochondria without contamination from other sources.



**Figure 4.4. Representative Western blots performed on mitochondria isolated from control and electrotransfected solei.**

A FLAG tag specific for the exogenous expression of FABPpm (43kDa) was only found on the purified mitochondrial extracts obtained from the FABPpm/mAspAT electrotransfected muscles (Fig. 4.4). Electrotransfection of FABPpm/mAspAT into soleus muscle increased the mitochondrial FABPpm/mAspAT protein by +80% ( $P < 0.05$ , Fig. 4.5A), without altering mitochondrial FAT/CD36 (Fig. 4.5B), or Cox-IV (Fig. 4.5C) contents. In addition, control experiments with an empty vector did not alter mitochondrial FABPpm/mAspAT, FAT/CD36 or Cox-IV contents (data not shown).

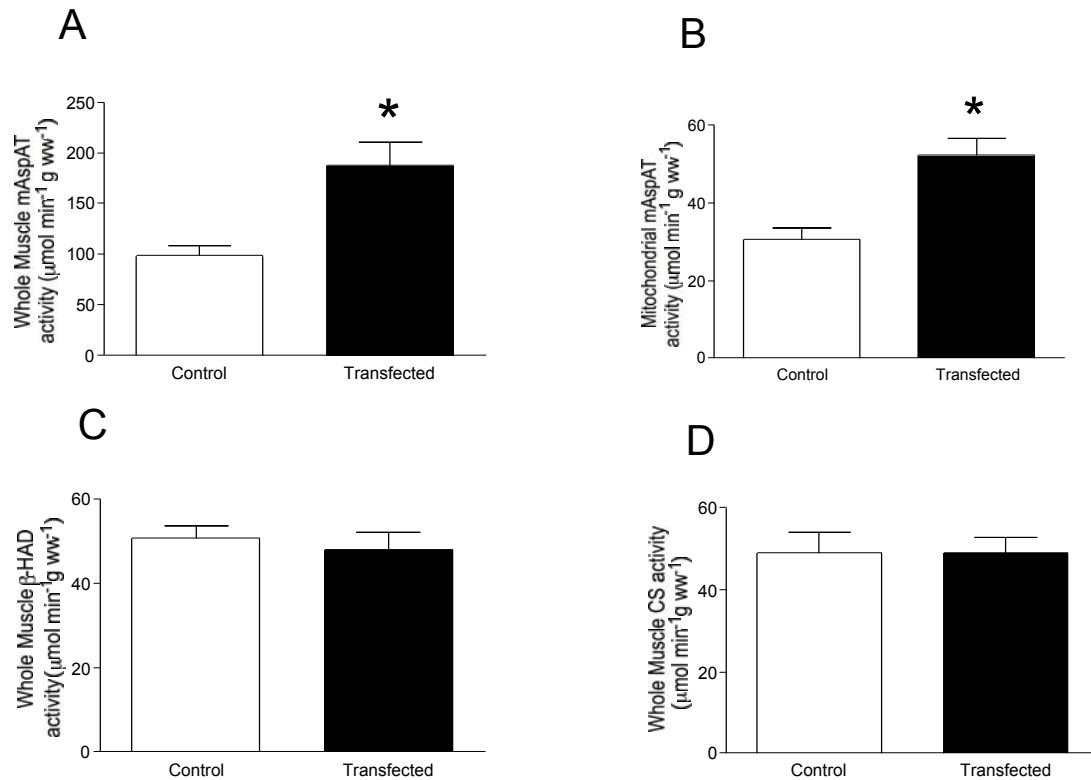


**Figure 4.5. Effects of electrotransfection with FABPpm cDNA on the content of soleus muscle mitochondrial proteins, and the ability of mitochondria to oxidize palmitate.** Values are means  $\pm$  S.E., expressed in arbitrary units (n=5). A: Mitochondrial FABPpm/mAspAT protein content. B: Mitochondrial FAT/CD36 protein content. C: Cox-IV protein content. D: Isolated mitochondrial palmitate oxidation, expressed in  $\text{nmol}\cdot\text{mg protein}^{-1}\cdot\text{hour}^{-1}$ . \* significantly different ( $P<0.05$ ) from control.

#### 4.4.4 Mitochondrial palmitate oxidation and enzymatic activity of FABPpm/mAspAT

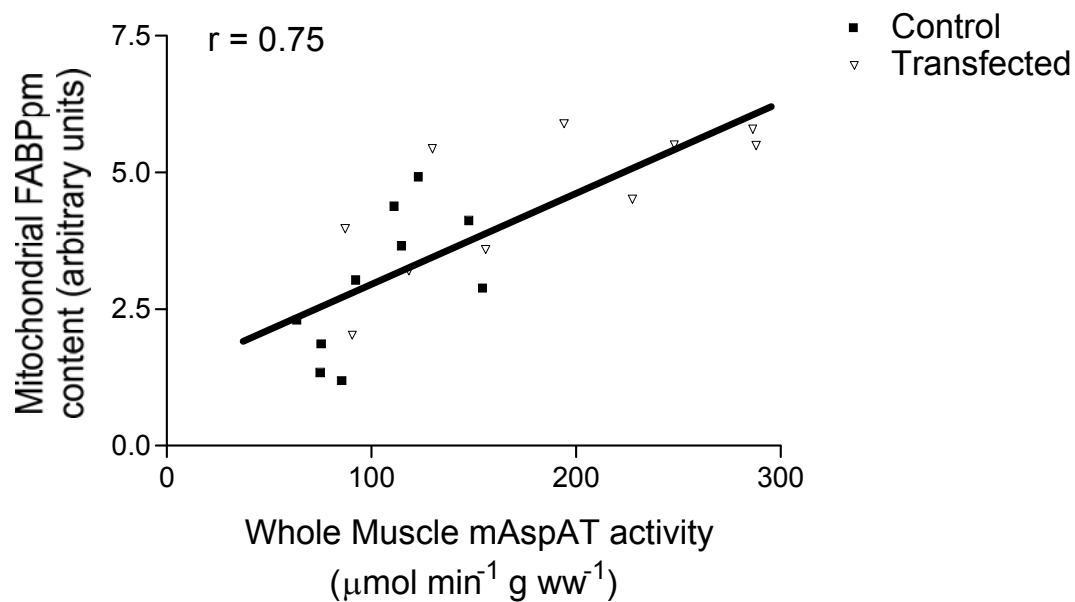
Although electrotransfection increased FABPpm/mAspAT content on mitochondria by +80%, isolated mitochondrial palmitate oxidation rates were not altered (Fig. 4.5D). In contrast, FABPpm/mAspAT electrotransfection resulted in a +90% increase ( $P<0.001$ ) in whole muscle mAspAT activity (Fig. 4.6A), and a +70% increase

( $P < 0.001$ ) in isolated mitochondrial mAspAT activity (Fig. 4.6B). Whole muscle  $\beta$ -HAD activity (Fig. 4.6C) and whole muscle CS activity (Fig. 4.6D) were not altered.



**Figure 4.6. Effects of electrotransfection with FABPpm cDNA on mitochondrial enzymatic activity.** Values are means  $\pm$  S.E., expressed in  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}$  wet weight (ww)<sup>-1</sup>. A: Mitochondrial aspartate aminotransferase (mAspAT) activity in homogenates (n=10). B: Mitochondrial aspartate aminotransferase activity (mAspAT) in isolated mitochondria (n=5). C: Homogenate  $\beta$  hydroxyacyl-CoA dehydrogenase ( $\beta$ -HAD) activity (n=10) D: Homogenate citrate synthase (CS) activity (n=10). \* significantly different ( $P < 0.05$ ) from control.

In control experiments with an empty vector, mAspAT activity was not altered (data not shown). There was a significant correlation ( $r=0.75$ ) between mAspAT activity and mitochondrial FABPpm protein levels (Fig. 4.7).



**Figure 4.7. Relationship between mitochondrial FABPpm/mAspAT content and mAspAT activity in muscle homogenates.** (n=20, 10 control and 10 transfected muscles).

## 4.5 Discussion

Studies by Berk and colleagues demonstrated that FABPpm and mAspAt are identical proteins (14, 29, 168), that are located at the plasma membrane and the mitochondria, respectively. We previously reported that FABPpm/mAspAT overexpression increased not only the rates of LCFA transport into muscle but it also increased the rates of palmitate oxidation in whole muscle (43). This raised the spectre that FABPpm might have a role in regulating fatty acid oxidation at the level of the mitochondria, as we have repeatedly demonstrated for FAT/CD36 (15, 36, 78). The present work has shown that: i) unlike FAT/CD36, muscle contraction in rats and exercise in humans does not increase mitochondrial FABPpm/mAspAT, while ii) the upregulation of this protein in muscle stimulates the rate of fatty acid transport but iii) fails to alter the rate of fatty acid oxidation in isolated mitochondria. Hence, iv) the FABPpm-induced upregulation of fatty acid oxidation in whole muscle would seem to be solely related to the increased rate of plasmalemmal fatty acid transport. Finally, v) overexpression of FABPpm/mAspAT markedly increased the activity of aspartate aminotransferase. Thus, these studies have shown that despite the fact that FABPpm and mAspAt are identical proteins (14, 168), they have quite distinct metabolic functions depending on their subcellular location. Specifically, the protein appears to function as a LCFA transport protein on the plasma membrane, and as an enzyme associated with the transport of reducing equivalents into mitochondria.

Although LCFA transport was originally viewed as a purely passive process, it has become increasingly apparent that LCFA transport proteins are involved in the regulation of cellular fatty acid uptake and metabolism. Research at the level of the plasma membrane, specifically pertaining to FAT/CD36 and FABPpm proteins, has provided

insight into the regulation of LCFA transport. It has been shown that FAT/CD36 and FABPpm proteins are upregulated whenever fatty acid oxidation is chronically increased, such as during 7-day low frequency muscle stimulation in rodents (22, 100) and exercise training in humans (171), or in insulin deficient animals (108). In these studies, the rates of fatty acid transport are concomitantly altered as a result of changes in FAT/CD36 and FABPpm protein expression and their appearance at the plasma membrane (22, 100, 108). In addition, rates of fatty acid transport can also be altered when these transporter proteins are redistributed to the plasma membrane, in the absence of any change in their protein expression which not only occurs acutely but also chronically. A translocation of these LCFA transporter proteins from a low density microsomal pool to the plasma membrane has been observed following muscle contraction (23) and AMP kinase activation (39, 113), thereby facilitating a very rapid upregulation of fatty acid transport into muscle cells. A similar chronic translocation ('relocation') was found in muscle from obese Zucker rats (109) and in obese and type 2 diabetic humans (26). Taken altogether it is apparent that FAT/CD36 and FABPpm can influence fatty acid metabolism both acutely and chronically by altering the rate of LCFA entry into parenchymal cells.

Recently, FAT/CD36 has been suggested to translocate to the mitochondria during exercise, in both rat and human skeletal muscle, increasing the ability of mitochondria to oxidize palmitate (36, 78), and hence providing another level of regulation in fatty acid oxidation. At the level of the plasma membrane the acute regulation of FAT/CD36 and FABPpm has in the past appeared similar (39, 40). However, the current work suggests that the acute regulation of these proteins appears to be different at the level of the mitochondria. The present study supports the belief that FAT/CD36 content on

mitochondria increases following muscle contraction (36, 78), while contraction does not alter mitochondrial FABPpm protein content, in either rats or in human skeletal muscle, and represents a much less flexible system.

Since fatty acid oxidation appeared to increase during exercise without alterations in mitochondrial FABPpm/mAspAT content, a series of studies were designed to determine the role of FABPpm/mAspAT at the level of mitochondria. Electrotransfection of FABPpm cDNA into soleus muscle has previously been shown to increase rates of plasma membrane palmitate transport and whole muscle palmitate oxidation, independent of changes in FAT/CD36 (43). In the present study we have replicated these findings, and investigated the role of mitochondrial FABPpm/mAspAT. Since FABPpm is located both on the plasma membrane and mitochondria (37, 168), it was not known if the increase in palmitate oxidation following electrotransfection of FABPpm resulted solely from enhanced rates of fatty acid transport across the plasma membrane, or whether FABPpm/mAspAT also played a role in fatty acid oxidation in mitochondria. The novel finding in the current study is that an +80% increase in mitochondrial FABPpm/mAspAT protein failed to alter mitochondrial palmitate oxidation. This indicates that FABPpm/mAspAT, unlike FAT/CD36 (15, 36, 78, 149), is not involved in regulating fatty acid oxidation in mitochondria. Therefore, since whole muscle oxidation was increased, without an increase in the ability of mitochondria to oxidize palmitate, increases in plasma membrane fatty acid transport and mass action are responsible for the observed increase in whole muscle oxidation.

While others have suggested that FABPpm and mAspAT are structurally identical proteins, but responsible for different functions as a result of their subcellular

compartmentation (82, 168), we are the first to directly establish this. The increase in both the content of FABPpm at the plasma membrane and rate of fatty acid transport across the plasma membrane following electrotransfection, further supports the belief that FABPpm functions as a fatty acid transporter at this level (43, 82, 118, 175, 176). Altering the mitochondrial FABPpm/mAspAT protein presumably did not affect transport into mitochondria, since oxidation remained constant. However, increasing the mitochondrial content of FABPpm/mAspAT increased mAspAT enzymatic activity. This evidence strongly suggests that FABPpm contributes to transporting fatty acids across plasma membranes, as well as enzymatically regulating the rate of the following reaction (glutamate + oxaloacetate  $\leftrightarrow$  aspartate + 2-oxoglutarate) in mitochondria (106). These different functions for the same protein may be explained by a differential post-translational modification of the protein depending on the subcellular location, or a co-functioning with other proteins (such as FAT/CD36 at the plasma membrane as previously suggested (118)).

In summary, we measured mitochondrial FABPpm/mAspAT content in contracting rat and human skeletal muscle, and have demonstrated an inability of FABPpm to translocate to mitochondria during contraction, suggesting it does not regulate mitochondrial fatty acid oxidation. In addition, we have used electrotransfection to independently alter the content of FABPpm in rat soleus muscle on both the plasma membrane and mitochondria. We propose that the FABPpm-mediated upregulation of fatty acid oxidation, occurs via an increased rate of fatty acid transport across the plasma membrane, and thus mass action accounts for their increased mitochondrial oxidation. It is well known that another fatty acid transport protein, FAT/CD36, can have different functions in different tissues [for review see (55)], and the current novel finding is that

FABPpm has diverse metabolic functions depending on its subcellular compartmentalization. Functionally, in muscle, it appears that FABPpm is a fatty acid transport protein on the plasma membrane, and FABPpm/mAspAT is a functional enzyme at the level of the mitochondria, facilitating the transport of reducing equivalents into the mitochondrial matrix.

**CHAPTER FIVE**

**SKELETAL MUSCLE MITOCHONDRIAL FAT/CD36 CONTENT AND  
PALMITATE OXIDATION ARE NOT DECREASED IN OBESE  
WOMEN**

## 5.1 Abstract

A reduction in fatty acid oxidation has been associated with lipid accumulation and insulin resistance in the skeletal muscle of obese individuals. We examined whether this decrease in fatty acid oxidation was attributable to a reduction in muscle mitochondrial content and/or a dysfunction in fatty acid oxidation within mitochondria obtained from skeletal muscle of age-matched, lean (BMI =  $23.3 \pm 0.7 \text{ kg}\cdot\text{m}^{-2}$ ) and obese women (BMI =  $37.6 \pm 2.2 \text{ kg}\cdot\text{m}^{-2}$ ). The mitochondrial marker enzymes, citrate synthase (-34%),  $\beta$ -hydroxyacyl-CoA dehydrogenase (-17%) and cytochrome c oxidase (-32%) were reduced ( $P < 0.05$ ) in obese participants, indicating mitochondrial content was diminished. Obesity did not alter the ability of isolated mitochondria to oxidize palmitate, however fatty acid oxidation was reduced at the whole muscle level by 28% ( $P < 0.05$ ) in the obese. Mitochondrial FAT/CD36 did not differ in lean and obese individuals, but mitochondrial FAT/CD36 was correlated with mitochondrial fatty acid oxidation ( $r = 0.67$ ,  $P < 0.05$ ). It is concluded that the reduction in fatty acid oxidation in obese individuals is attributable to a decrease in mitochondrial content, not to an intrinsic defect in the mitochondria obtained from skeletal muscle of obese individuals. In addition, it appears that mitochondrial FAT/CD36 may be involved in regulating fatty acid oxidation in human skeletal muscle.

## 5.2 Introduction

It has been demonstrated that obese individuals have increases in intramuscular triacylglycerol (IMTG) storage, and this has been associated with insulin resistance (64-66, 89, 155). However, it appears unlikely that increases in IMTG levels represent a dysfunction in lipid metabolism (for review see (178)), and more reactive lipids, namely diacylglycerol (DAG) and ceramides, may be important for the development of insulin resistance (69, 129, 155). It has been suggested that reductions in fatty acid oxidation in obese individuals contribute to the intramuscular lipid accumulation (79, 88, 90, 95, 142, 170). Based on reductions in carnitine palmitoyltransferase I (CPTI) activity in obese skeletal muscle, and increased M-CoA content in obese models (145), it has been proposed that fatty acid transport into the mitochondria is diminished. This may account for the observed reduction in fatty acid oxidation (88, 95), although other proteins may also be involved in this process.

The transport of fatty acids across the plasma membrane is generally believed to be largely mediated by fatty acid transporters, among which fatty acid translocase (FAT/CD36) appears to have a prominent role. This protein is located in several subcellular domains (23), and recently FAT/CD36 has also been found on rat and human muscle mitochondria (15, 36, 78). At this subcellular location, FAT/CD36, along with CPTI, contributes to regulating mitochondrial fatty acid oxidation in skeletal muscle at rest, as blocking mitochondrial FAT/CD36 has been shown to almost completely inhibit fatty acid oxidation (15, 36, 78). During exercise mitochondrial fatty acid oxidation is upregulated, and is accompanied by an increase in mitochondrial FAT/CD36 content (36, 78). The exercise-induced increase in fatty acid oxidation is also completely inhibited when FAT/CD36 is pharmacologically blocked (36, 78). In other work, an increase in mitochondrial FAT/CD36 has been associated with exercise-induced weight loss and an improvement in whole body fatty acid oxidation (149).

Thus, it appears that changes in mitochondrial FAT/CD36 are associated with changes in mitochondrial fatty acid oxidation.

Fatty acid binding protein (FABPpm) is another well recognized plasma membrane fatty acid transport protein that has also been found on mitochondria (14, 168). However, the role of FABPpm with respect to mitochondrial fatty acid oxidation remains unknown.

The net accumulation of lipids in obese skeletal muscle may result from a combination of increased whole muscle fatty acid uptake and/or decreased whole muscle oxidation. While the concept of impaired fatty acid oxidation as a mechanism to increase intramuscular lipid species has gained attention in recent years, the exact mechanism remains unknown. However, two plausible explanations exist, either mitochondrial content is decreased and/or there is a dysfunction in fatty acid oxidation within mitochondria. Work in skeletal muscle has indicated a reduction in mitochondrial content with obesity, along with a concomitant decrease in fatty acid oxidation (90). While ratios of electron transport chain capacity to mitochondrial DNA and mitochondrial size have been used to infer dysfunction (90, 142), recently the ability of mitochondria to oxidize fatty acids was directly measured in skeletal muscle from type 2 diabetics, and controversially shown to be increased, although the underlying mechanism remains unknown (8).

The focus of the present study was to examine if the obesity-related decreases in skeletal muscle lipid oxidation are attributable to: a) a reduction in mitochondrial content and/or; b) to an intrinsic defect in mitochondria; and also c) whether there are reductions in the content of mitochondrial fatty acid transport proteins. We hypothesized that obesity would be associated with decreases in skeletal muscle mitochondrial content, as well as decreases in the ability of mitochondria to oxidize fatty acids. In addition, we speculated that

impairments in mitochondrial oxidation would be associated with reductions in the content of mitochondrial FAT/CD36 and FABPpm.

### 5.3 Research Design and Methods

The participants were nine lean (BMI < 27 kg·m<sup>-2</sup>; mean 23.3 ± 0.7 kg·m<sup>-2</sup>) and nine obese (BMI > 30 kg·m<sup>-2</sup>; mean 37.6 ± 2.2 kg·m<sup>-2</sup>) non-diabetic women (Table 4.1). Subjects were admitted to McMaster Health Sciences Centre for abdominal surgery and gave informed written consent prior to participating in the study. The University of Guelph and McMaster University Ethical Committees approved the experimental procedures. Prior to participation, individuals were screened and excluded from the study if any known diseases or medications, as well as if weight fluctuations in the 6 months prior to surgery were reported, similar to that previously reported (26, 33).

Following an overnight fast (12-18 hours) general anesthesia was induced with a short acting barbiturate, and maintained as required by a fentanyl and rocuronium volatile anesthetic mixture. A venous blood sample was sampled directly into a 5 ml heparinized tube, inverted, and placed on ice for future processing. A portion of the rectus abdominus muscle was sampled (~350 mg), and immediately placed in ice cold oxygenated buffer (modified Krebs Henseleit buffer, containing 8mM glucose) for transport to the laboratory.

#### 5.3.1 Mitochondrial enzyme activities

A portion of the muscle (~10 mg) was immediately homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer and used for the measurements of maximal β-hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (CS) activities. Total muscle β-HAD activity was measured in Tris-HCl buffer (50 mM Tris-HCl, 2 mM EDTA, 250 μM NADH, pH 7.0) and 0.04% Triton-X. The reaction was started by addition of 100 μM acetoacetyl-CoA and absorbance was measured at 340 nm over a 2 min period (37°C) (10).

The CS activity was assayed spectrophotometrically at 37°C by measuring the disappearance of NADH @ 412 nm (159).

Citrate synthase activity was also determined in the isolated mitochondrial preparations. Intact mitochondrial CS activity was determined by first assaying the extramitochondrial fraction in the suspension (1:20 dilution) and then assaying the total CS activity of the suspension (1:20 dilution) after lysing the mitochondria with 0.04% Triton X-100 and repeated freeze-thawing. The net difference provided a measure of the viability of the mitochondrial preparation, as well as a measure of the mitochondria recovered during the isolation procedure (when compared to the total muscle CS activity) (15). The mitochondrial recovery and viability were comparable to those reported previously from our group (15, 78).

### *5.3.2 Isolation of mitochondria from skeletal muscle*

Differential centrifugation was used to obtain pure and intact mitochondria containing both intermyofibrillar (IMF) and subsarcolemmal (SS) fractions (36). All procedures were identical to those previously published by us (15, 36, 78). Briefly, muscle (~350 mg) was homogenized with a tight fitting Teflon pestle. The homogenate was centrifuged at 800 g for 10 min, to separate the SS and IMF mitochondria. The IMF mitochondria were treated with a protease (0.025 ml/g; Sigma, St. Louis, MO, USA) for exactly 5 minutes to digest the myofibrils. Further centrifugation was used to remove the myofibrils, and recombine the IMF with the SS mitochondria. The combined samples were centrifuged twice at 10 000 g for 10 min. The pellet was resuspended in 1 µl of buffer/mg of tissue. Following the oxidation measurements, the remaining mitochondria were further purified using a Percoll gradient for Western blot analyses. Samples were

centrifuged at 20 000 g for 1 hour and the mitochondrial layer was removed. The Percoll was removed from the sample by further centrifuging at 20 000 g for 5 hours.

### 5.3.3 Mitochondrial palmitate oxidation

Labeled CO<sub>2</sub> production and acid soluble trapped <sup>14</sup>C from palmitate oxidation were measured following a 30 min incubation of viable mitochondria in a sealed system, and expressed per mg mitochondrial protein as described previously (15, 36, 78). Briefly, viable mitochondria (100 µl) were added to a system containing a pre-gassed modified Krebs Ringer buffer supplemented with 5 mM ATP, 1 mM NAD<sup>+</sup>, 0.5 mM DL-carnitine, 0.1 mM coenzyme A, 25 µM cytochrome C, and 0.5 mM malate. A microcentrifuge tube, containing 500 µl of 1 M of benzethonium hydroxide inserted into a 1.5 ml centrifuge tube, was placed in the system to capture <sup>14</sup>CO<sub>2</sub> produced during the oxidation reaction. The system was then sealed with a rubber cap and further sealed with parafilm. The reaction was initiated by the addition of a 6:1 palmitate:BSA complex (containing 10 µCi of [1-<sup>14</sup>C] palmitate, for a final palmitate concentration of 77 µM) administered by syringe through the rubber cap. The reaction continued for 30 min at 37°C and was terminated with the addition of ice-cold 12 M perchloric acid (PCA) by syringe through the rubber cap.

A fraction of the reaction medium was removed through the cap and analyzed for isotopic fixation. Gaseous CO<sub>2</sub> produced from oxidation of [1-<sup>14</sup>C] palmitate was measured by acidifying the remaining reaction mixture. Liberated <sup>14</sup>CO<sub>2</sub> was trapped by the benzethonium hydroxide over a 90 min incubation period at room temperature. The tube containing the <sup>14</sup>CO<sub>2</sub> was put in a scintillation vial, and radioactivity was counted.

### *5.3.3 Whole muscle palmitate oxidation*

Whole muscle palmitate oxidation rates, expressed per g wet weight, were calculated from mitochondrial palmitate oxidation rates expressed per mg mitochondrial protein. To accomplish this, the mitochondrial recovery was first applied to the mitochondrial oxidation value, and subsequently this value was divided by the starting wet weight value.

### *5.3.4 Determination of blood metabolites*

Venous blood was sampled directly into vacutainers containing heparin, and partitioned into two fractions. An aliquot of 200 µl of whole blood was added to 1 ml of 0.6 M PCA and centrifuged. The deproteinized supernatant was stored at -80<sup>0</sup> C and later analyzed for glucose (10). A second aliquot of whole blood was immediately centrifuged and the plasma was removed and stored at -80<sup>0</sup> C. The plasma was later analysed for free fatty acids (Wako NEFA C test kit, Wako Chemicals, Richmond, VA, USA) and insulin (RIA insulin kit, Cedarlane Laboratories, Hornby, ON, Canada).

### *5.3.5 Western blotting*

Whole muscle crude membranes were generated as previously described (23, 25, 109), analyzed for total protein (BCA protein assay), and 35 µg of denatured protein was loaded for Western blotting. Purified isolated mitochondrial fractions were analyzed for total protein (BCA protein assay) and 25 µg of denatured protein from each sample was loaded for Western blotting. All samples were separated by electrophoresis on 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The FABPpm polyclonal antibody used was produced in the laboratory of Dr. J. Calles-Escandon (43, 100). The MO-25 antibody used to detect FAT/CD36 was produced in the laboratory of Dr. N.N. Tandon

(122). This antibody has also been used previously in our work (23, 36, 78). Commercially available antibodies were used to detect cytochrome c oxidase IV (Cox-IV – Invitrogen, Burlington, ON, Canada), GLUT-4 (Chemicon, International Inc., Temecula, CA, USA), caveolin-3 (cav-3 - BD Biosciences, Mississauga, ON, Canada), sarcoplasmic reticulum calcium ATPase (SERCA) (Affinity Bioreagents Inc., Golden, CO, USA), fatty acid transport protein 4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and hormone sensitive lipase (HSL – ProSci Incorporated, Poway, CA, USA). An internal control of previously extracted human muscle crude membrane was used in each gel. Blots were quantified using chemiluminescence and the ChemiGenius 2 Bioimaging system (SynGene, Cambridge, UK).

#### *5.3.6 Statistics*

All data are presented as the mean  $\pm$  S.E. Differences between lean and obese participants were analyzed with an unpaired, 2-tailed t-test. Associations between variables were investigated using Pearson correlation analyses, as appropriate. Correlations were done by including both lean and obese individuals. Statistical significance was accepted at  $P < 0.05$ .

## 5.4 Results

### 5.4.1 Subject characteristics

There were no differences between lean and obese participants in mean age, fasting blood glucose or fasting free fatty acid concentrations (Table 5.1). In contrast, there was a significant ( $P<0.05$ ) increase in body mass, BMI and fasting insulin concentrations in the obese participants (Table 5.1).

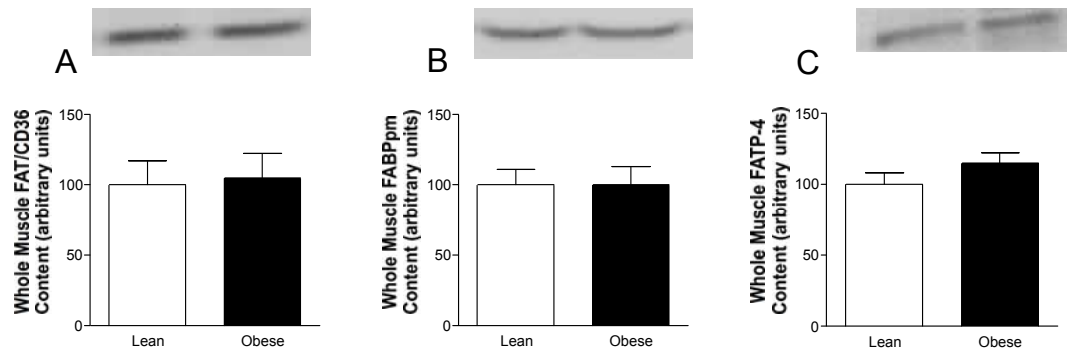
**Table 5.1. Subject Characteristics**

Characteristics	Lean	Obese
Age (years)	47 ± 3	45 ± 3
Body mass (kg)	59.7 ± 2.0	99.2 ± 6.8 *
BMI ( $\text{kg}\cdot\text{m}^{-2}$ )	23.3 ± 0.7	37.6 ± 2.2 *
Fasting blood glucose ( $\text{mmol}\cdot\text{L}^{-1}$ )	4.1 ± 0.1	4.5 ± 0.4
Fasting plasma insulin ( $\text{pmol}\cdot\text{L}^{-1}$ )	24.8 ± 1.9	35.9 ± 4.9 *
Fasting free fatty acids ( $\text{mmol}\cdot\text{L}^{-1}$ )	0.87 ± 0.09	0.86 ± 0.06

Values are presented as means ± S.E. (n=9), except for blood measurements (n=6). \* significantly different from lean ( $P<0.05$ ).

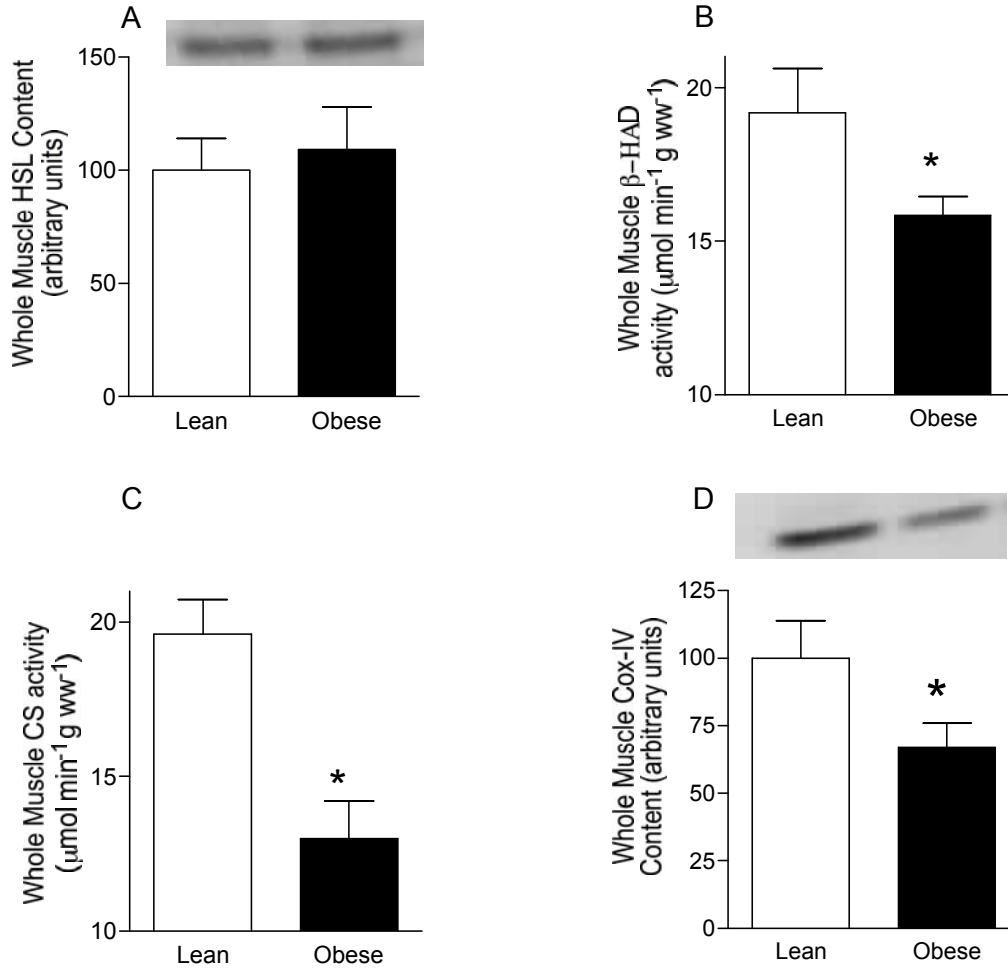
### 5.4.2 Effect of obesity on whole muscle fatty acid transport protein contents and fatty acid oxidation enzymes

Obesity did not alter the whole muscle protein expression of FAT/CD36, FABPpm, fatty acid transport protein 4 (FATP-4; Fig. 5.1A-C, respectively).



**Figure 5.1. Representative Western blots displaying the effects of obesity on the expression of fatty acid transporters.** Values are means  $\pm$  S.E., expressed in arbitrary units (n=9). A: Whole muscle FAT/CD36 content. B: Whole muscle FABPpm content. C: Whole muscle FATP-4 content.

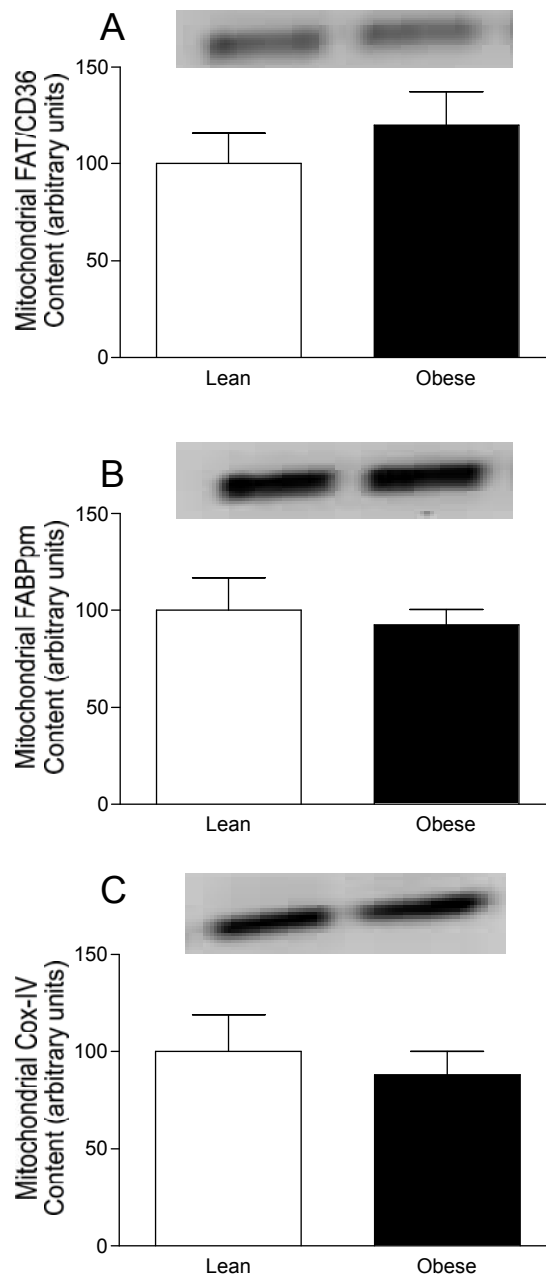
Obesity did not alter HSL protein (Fig. 5.2A). In contrast, the whole muscle activity of  $\beta$ -HAD and CS, as well as Cox-IV protein content, were reduced ( $P < 0.05$ ) by 17%, 34% and 32% with obesity (Fig. 5.2B-D, respectively). CS activity negatively correlated with BMI ( $r = -0.53$ ;  $P < 0.05$ ), however  $\beta$ -HAD ( $r = -0.31$ ), HSL ( $r = 0.38$ ) and Cox-IV ( $r = -0.26$ ) did not.



**Figure 5.2. The effects of obesity on the expression of enzymes involved in the lipolysis and oxidation of fatty acids.** Values are means  $\pm$  S.E (n=9). A: Representative Western blot of whole muscle hormone sensitive lipase (HSL) content (expressed in arbitrary units). B: Homogenate  $\beta$  hydroxyacyl-CoA dehydrogenase ( $\beta$ -HAD) activity (expressed in  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}$  wet weight ( $\text{ww}$ ) $^{-1}$ ). C: Homogenate citrate synthase (CS) activity (expressed in  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}$  wet weight ( $\text{ww}$ ) $^{-1}$ ). D: Representative Western blot of whole muscle cytochrome c oxidase complex 4 (Cox-IV) content (expressed in arbitrary units). \* significantly ( $P < 0.05$ ) different from lean.

#### *5.4.3 Effect of obesity on skeletal muscle mitochondrial proteins*

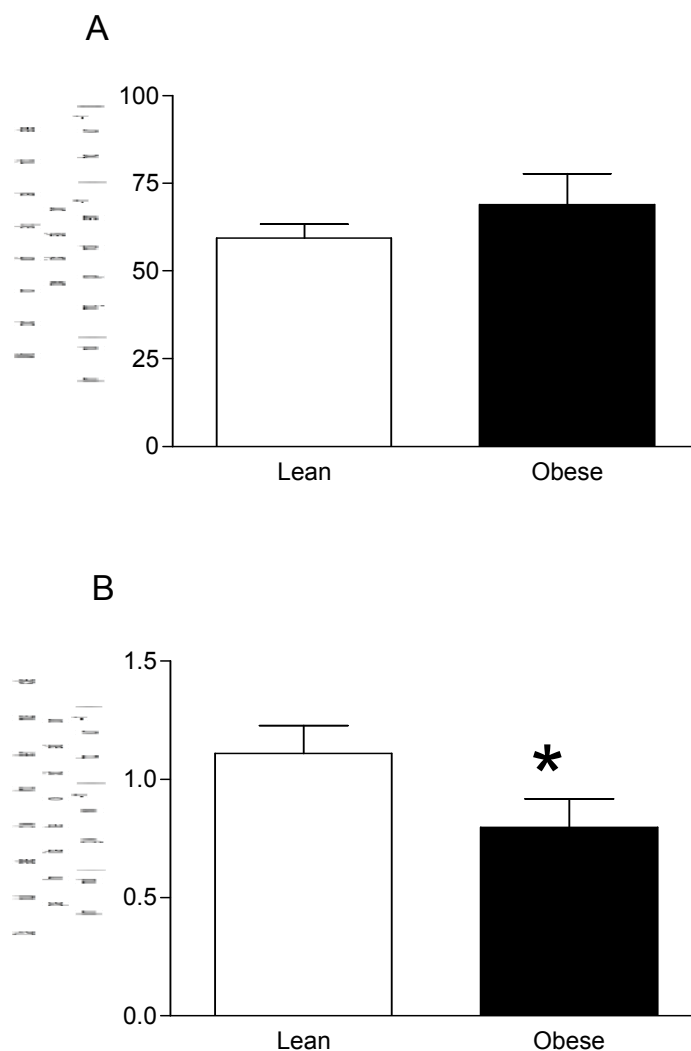
The mitochondrial recovery was not different between lean and obese participants ( $21 \pm 1\%$  vs.  $23 \pm 1\%$ ), nor was the viability of the mitochondrial preparation ( $92 \pm 1\%$  vs.  $94 \pm 1\%$ ). The absence of SERCA (110 kDa), Glut-4 (45 kDa) and Cav-3 (23 kDa) proteins, indicated that the isolation procedures successfully yielded highly purified mitochondria without contamination from other membrane or intracellular sources. In addition, the isolated mitochondria exhibited significant expression of FAT/CD36, FABPpm and Cox-IV contents (Fig. 5.3A, B and C, respectively) that did not differ with obesity.



**Figure 5.3. Representative Western blots displaying the effects of obesity on the expression of mitochondrial proteins.** Values are means  $\pm$  S.E., expressed in arbitrary units (n=9). A: Mitochondrial FAT/CD36 content. B: Mitochondrial FABPpm content. C: Mitochondrial Cox-IV content.

#### 5.4.4 Effect of obesity on whole muscle and mitochondrial fatty acid oxidation

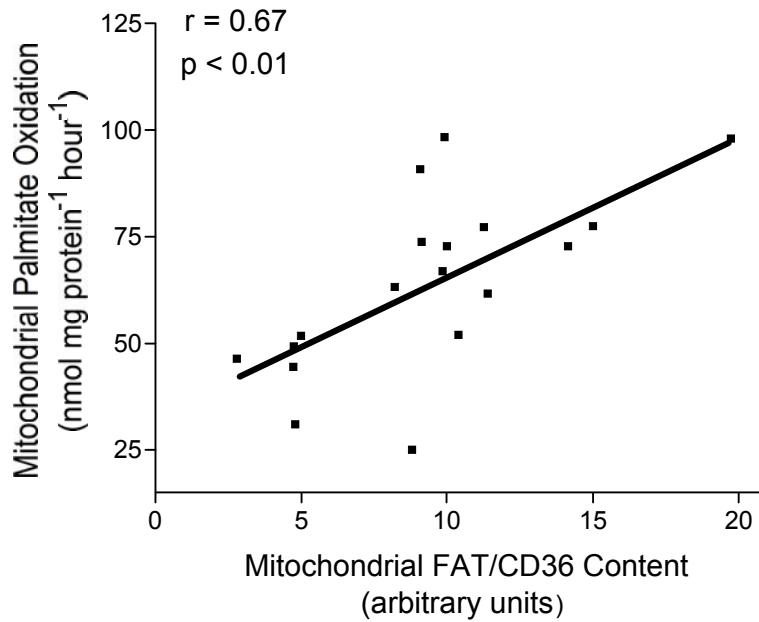
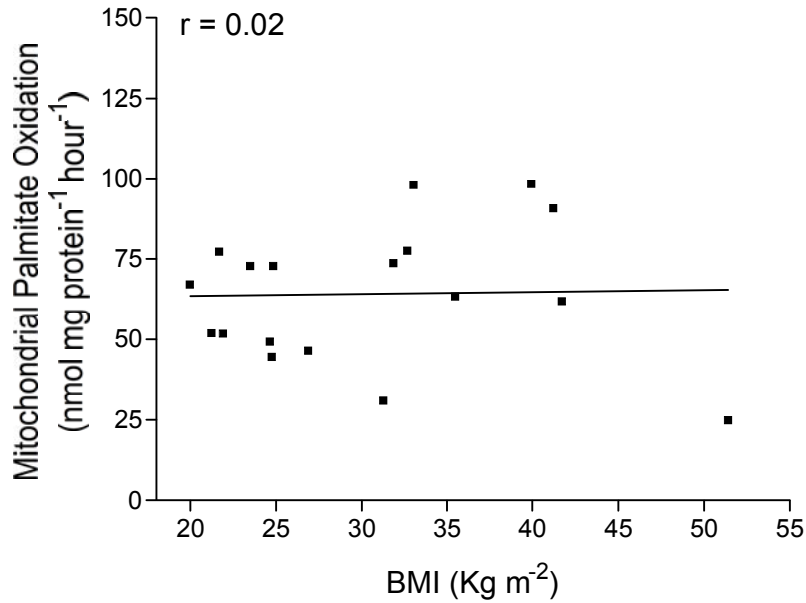
Obesity did not alter the rate of palmitate oxidation in isolated mitochondria (Fig. 5.4A). In contrast, palmitate oxidation calculated in the whole muscle was reduced in obese individuals (Fig. 5.4B).



**Figure 5.4. The effects of obesity on the ability of skeletal muscle to oxidize palmitate.**

Values are means  $\pm$  S.E. (n=9). A: Isolated mitochondrial palmitate oxidation (expressed in  $\text{nmol}\cdot\text{mg protein}^{-1}\cdot\text{hour}^{-1}$ ). B: Calculated whole muscle palmitate oxidation (expressed in  $\text{nmol}\cdot\text{g wet weight (ww)}^{-1}\cdot\text{hour}^{-1}$ ). \* significantly ( $P<0.05$ ) different from lean.

The rate of palmitate oxidation in isolated mitochondria did not correlate with BMI ( $r=0.02$ ; Fig. 5.5A). However, independent of BMI, palmitate oxidation in isolated mitochondria significantly ( $P<0.05$ ) correlated with mitochondrial FAT/CD36 content ( $r=0.67$ ; Fig. 5.5B), but not with mitochondrial FABPpm content ( $r=0.42$ ). In addition, calculated whole muscle palmitate oxidation was significantly correlated ( $P<0.05$ ) with whole muscle CS activity ( $r=0.53$ ) and  $\beta$ -HAD activities ( $r=0.50$ ).



**Figure 5.5. Relationships between mitochondrial palmitate oxidation, FAT/CD36 and BMI.** (n=18, 9 lean and 9 obese individuals). A: Correlation between mitochondrial palmitate oxidation and BMI. B: Correlation between mitochondrial palmitate oxidation and the mitochondrial content of FAT/CD36.

## 5.5 Discussion

Mitochondrial dysfunction has been proposed to explain reductions in fatty acid oxidation in skeletal muscle of obese individuals, leading to elevations in fatty acid storage and ultimately insulin resistance (64-66, 89, 90, 95, 155). While there is evidence in support of this hypothesis (88, 90, 95, 142, 156), these findings are not uniformly supported (26, 33, 139, 164, 179). The main novel findings of the present study were: i) mitochondrial fatty acid oxidation rates were not different in obese vs. lean individuals; ii) the mitochondrial contents of FAT/CD36 and FABPpm were similar in the two groups; iii) whole muscle fatty acid oxidation rates and mitochondrial content (whole muscle CS,  $\beta$ -HAD and Cox-IV) were reduced in obese individuals; iv) total muscle expression of FAT/CD36, FABPpm and FATP-4 were not altered with obesity; and v) mitochondrial fatty acid oxidation rates were highly associated with the content of FAT/CD36 on mitochondrial membranes. Therefore, we suggest that obesity-related impairments in fatty acid oxidation are a result of reductions in mitochondrial content, and not from an inherent dysfunction of the mitochondria to oxidize fatty acids. In addition, at the level of the mitochondria, the capacity to oxidize fatty acid is related to the availability of FAT/CD36.

### 5.5.1 Whole muscle protein content

In the present study we found that whole muscle FAT/CD36 and FABPpm are unaltered with obesity. This corroborates our earlier finding that whole muscle FAT/CD36 is not decreased with obesity (26). However, we previously reported that obesity resulted in a small (~25%) reduction in whole muscle FABPpm, and increased with type 2 diabetes (26). This discrepancy might have arisen due to sample population

variation as a result of small sample sizes, and the current data suggesting obesity does not alter homogenate FABPpm fits with the literature pertaining to other fatty acid transporter proteins (8, 26). In addition, for the first time we report that whole muscle FATP-4 in lean and obese individuals does not differ, and this is in agreement with Bandyopadhyay et al. (8). Previously, we reported that obese individuals repartition FAT/CD36 to the plasma membrane, thereby increasing fatty acid transport into the muscle (26). While Bandyopadhyay et al. (8) also report an increased amount of FAT/CD36 on plasma membranes in obese type 2 diabetics, their results are more difficult to interpret, as they also show a proportional increase in FAT/CD36 in the intracellular compartment. This inconsistency might result from the different genders studied, or the skeletal muscle sampled. In the present study females were exclusively used and the rectus abdominus was sampled, while Bandyopadhyay et al. (8) primarily used male participants (20 male and 1 female) and sampled from an unspecified muscle biopsy (probably the vastus lateralis) (8). Regardless of these discrepancies, it is becomingly increasingly clear that the transport of fatty acids into skeletal muscle is increased with obesity and type 2 diabetes. Interestingly, FATP-4 appears to follow a similar trend, as type 2 diabetics also have increased levels of FATP-4 on the plasma membrane fraction (8).

### *5.5.2 Whole muscle fatty acid oxidation, enzymes and mitochondrial volume*

The current data suggests that obese individuals have an impairment at the whole muscle level to oxidize fatty acids, when plasma membrane LCFA transport is removed, corroborating the findings of others (79, 95, 170). In addition, our whole muscle palmitate oxidation values correlated with whole muscle CS and  $\beta$ -HAD activities, further

suggesting mitochondrial content determines the rate of whole muscle fatty acid oxidation. For fatty acid oxidation to be impaired in obese individuals, either mitochondrial content must be substantially reduced and/or the ability of the mitochondria to oxidize fatty acids must be impaired. We corroborate the findings of others that obesity is related to reductions in CS and  $\beta$ -HAD activities, as well as a decrease in Cox-IV content (90, 95, 142, 156). Although various co-factors, and covalent and allosteric regulators can influence the kinetics of enzymes, the maximal activity of an enzyme is indicative of the total amount of the protein. Therefore, reductions in CS and  $\beta$ -HAD maximal activities indicate a loss of the mitochondrial enzymes in skeletal muscle of obese individuals. Collectively, these data indicate that the mitochondrial content in skeletal muscle of obese individuals is reduced (86). Although a decrease in mitochondrial content can explain the observed reduction in whole muscle fatty acid oxidation, further measurements were required to determine if this mechanism was solely responsible, or if mitochondrial dysfunction further exacerbated the impairment in obese individuals' skeletal muscle lipid oxidation.

### *5.5.3 Mitochondrial fatty acid oxidation and transport proteins*

Contrary to our hypothesis, there were no differences in the ability of mitochondria isolated from obese individuals to oxidize fatty acids. This suggests that mitochondrial dysfunction is not a mechanism for the observed reduction in homogenate fatty acid oxidation, and a decrease in mitochondrial content is primarily responsible. We originally hypothesized that mitochondria isolated from obese participants would have a diminished ability to oxidize fatty acids, possibly as a result of a decrease in the content of FAT/CD36 (15, 36, 78), and perhaps FABPpm. Since mitochondria isolated from the

obese did not differ in the ability to oxidize fatty acids, one would not expect the content of both transporters to be different. In agreement, the content of both mitochondrial FAT/CD36 and FABPpm did not differ with obesity. In addition, although mitochondrial palmitate oxidation rates in lean and obese individuals did not correlate with BMI, the rates did correlate highly with FAT/CD36 mitochondrial content. Interestingly, FABPpm did not correlate with mitochondrial oxidation, suggesting it is not rate limiting for the transport of LCFA into mitochondria. Although this needs further investigation, the current belief is that the main function of FABPpm is to catalyze a reaction involved in transporting reducing equivalents into the mitochondria, since it is identical to mitochondrial aspartate amino transferase (14, 168).

Previous work from our group using a specific inhibitor of FAT/CD36, suggests that FAT/CD36 is necessary for fatty acid oxidation at the level of the mitochondria (15, 36, 78). In addition, we have previously shown that the content of FAT/CD36 on mitochondria can predict the ability of mitochondria to oxidize fatty acids during exercise (78). Others have also shown that mitochondrial FAT/CD36 can be altered with training, and the training-induced increases in mitochondrial FAT/CD36 correlated significantly with changes in resting fatty acid oxidation (149). The current work further strengthens the evidence that FAT/CD36 has a vital role in the transport of fatty acids into mitochondria. We recognize that CPTI also has an important role in this level of regulation (15, 78), and further research is required to determine exactly how CPT-I and FAT/CD36 may interact to facilitate LCFA transport.

Previous data suggests that there may be a threshold required for impairments in whole muscle fatty acid oxidation to be manifested i.e. BMI between 40 and 50 (79, 170). In the current study our BMI in the obese group was relatively high (mean of  $37.6 \text{ kg}\cdot\text{m}^{-2}$ )

but since they did not display mitochondrial dysfunction, it is possible that they were below this threshold. However, it was not directly determined in these previous studies whether mitochondrial function was impaired. In our study, the obese group displayed reductions in whole muscle CS and  $\beta$ -HAD activities, reduced Cox-IV content and reduced whole muscle fatty acid oxidation rates, all of which indicate lipid oxidation was compromised. Thus, the current data suggests that the reductions in fatty acid oxidation that have been associated with obesity result from reductions in mitochondrial content, and not from intrinsic mitochondrial alterations. This is an important distinction, because it suggests that interventions are required to increase mitochondrial biogenesis, but these do not have to ‘remodel’ existing mitochondria. Indeed, previous work has shown that regular exercise can increase mitochondria volume in the obese population, as shown by increases in CS,  $\beta$ -HAD and CPTI activity (34). In addition, associated with these changes in obese skeletal muscle, is an increase in fatty acid oxidation, and subsequently reductions in total DAG and total ceramides contents, as well as improved insulin sensitivity (34).

In the current study, obese participants displayed a trend ( $P=0.12$ ) towards an increase in the capacity of mitochondria to oxidize fatty acids. Previously, Bandyopadhyay et al. (8) showed that the capacity of mitochondria to oxidize fatty acids in obese type 2 diabetics ( $BMI = 36.93 \text{ kg}\cdot\text{m}^{-2}$ ) was actually increased. Together, these studies suggest that a ‘reverse-continuum’ may exist in mitochondrial oxidation with obesity. That is, while whole muscle fatty acid oxidation may decrease with increasing BMI as a result of reductions in mitochondrial content, the capacity of the remaining mitochondria to oxidize fatty acids might actually compensate by increasing oxidation in an undetermined way to counteract this affect. Interestingly, the BMI of our obese

participants was almost identical to that of Bandyopadhyay and colleagues, suggesting the continuum might be based on insulin sensitivity, and not BMI (8). Importantly, it remains to be determined if the diabetic increase in mitochondrial capacity to oxidize fatty acids is a beneficial response, or if in combination with a decrease in electron transport chain capacity, will lead to increases in lipid peroxide species, and subsequently oxidative damage that could exacerbate the diabetic phenotype (61, 67, 150).

We originally hypothesized that mitochondria from obese individuals would display an impaired ability to oxidize fatty acids. It has been suggested that the SS mitochondria from obese individuals may display a disproportionate impairment in fatty acid oxidation, as a greater reduction in the ratio of electron transport chain activity to mitochondrial DNA was observed in this mitochondrial fraction (142). The SS fraction only represents ~25% of the total mitochondrion in skeletal muscle (47). In the current study we pooled the SS mitochondria with the IMF mitochondria to ensure adequate protein was recovered for our functional assays. Although this approach limited our ability to detect impairments in the SS mitochondria, in the previously mentioned study a significant reduction in the ratio of electron transport chain activity to mitochondrial DNA was also observed in IMF mitochondria, and at the whole muscle level (142). While this suggested dysfunction in all mitochondria, the present data suggests that mitochondrial dysfunction in fatty acid oxidation is not required for reductions in whole muscle fatty acid oxidation. It should be noted that only a small fraction of mitochondria were recovered during the isolation procedures (~20%), and therefore it is possible that a dysfunction is present in the mitochondria not recovered. Since the various sub cellular mitochondria were pooled in the current study, future research should focus on examining the potential differences in the SS and IMF mitochondria of obese individuals.

In conclusion, we have shown that obesity does not alter the ability of skeletal muscle mitochondria to oxidize fatty acids. We observed that obesity related reductions in skeletal muscle fatty acid oxidation are attributable to reductions in mitochondrial content, and not to intrinsic alterations, or dysfunction within the mitochondria. We further show that the content of FAT/CD36 on mitochondria is not different in obese muscle. However, FAT/CD36 significantly predicts the ability of mitochondria to oxidize LCFA, independent of BMI status.

**CHAPTER SIX**  
**INTEGRATIVE DISCUSSION**

## 6.1 Discussion

Fatty acids play essential roles in cell signaling, membrane composition and energy provision in a number of tissues. However, due to its mass (~28% of total body weight), skeletal muscle LCFA oxidation represents a large portion of whole muscle fatty acid oxidation both at rest, and during exercise. One of the key features of skeletal muscle is the ability to rapidly increase LCFA oxidation during exercise to rates several fold above resting levels. Although calculations from RER values suggest that 40-80% of resting whole body energy is derived from fatty acids (depending on diet), this represents a very low fatty acid oxidation rate in absolute terms (~0.10 g/min), since the energy demands are relatively low (180). However, during moderate-intensity exercise, the fatty acid oxidation rate can increase several fold in a matter of minutes. While rapid increases in fatty acid oxidation could result from elevations in intramuscular acyl-CoA levels, and mass action overriding the inhibition of M-CoA, acyl-CoA levels have not been shown to increase within 10 minutes of exercise despite pronounced elevations in fatty acid oxidation (186). This suggests that oxidative flux is matched to fatty acid delivery, and not regulated by mass action and the content of acyl-CoA, and additional mechanisms must regulate the rapid increase in LCFA oxidation at the onset of exercise. While this thesis supports the existence of two novel mechanisms that regulate LCFA oxidation during prolonged exercise, neither the attenuation in CPTI M-CoA sensitivity nor the increase in mitochondrial FAT/CD36 content can account for the rapid increase in fatty acid oxidation at the start of exercise. In addition, mitochondria isolated from obese individuals did not differ in FAT/CD36 content or in the ability to oxidize palmitate. Instead, reductions in mitochondrial content accounted for the observed reductions in whole muscle fatty acid oxidation rates.

## 6.2 Regulation of CPTI

CPTI forms acylcarnitines by catalyzing the trans-esterification of activated LCFA and carnitine. This commits acyl-CoA moieties to oxidation, preventing them from undergoing other cellular metabolic fates. It has long been known that CPTI can be inhibited by M-CoA, and therefore CPTI has been viewed as a key regulator of overall LCFA oxidation in both liver and skeletal muscle. In the liver, M-CoA concentrations are reduced, and the sensitivity of CPTI for M-CoA is attenuated, in fasted conditions known to promote LCFA oxidation (32, 53). In skeletal muscle, intralipid infusion increases LCFA oxidation, while concomitantly reducing M-CoA concentrations at rest, suggesting a potential mechanism to regulate LCFA oxidation rates during exercise (9). While exercise has been shown to reduce M-CoA content in rat skeletal muscle (191), this finding has not been reported in humans to sufficiently account for the increase in LCFA oxidation (134, 135, 143). Therefore, either current methodologies and smaller M-CoA contents in human skeletal muscle (134, 143) make it difficult to accurately determine exercise induced reductions in human muscle M-CoA content, or additional mechanisms account for the increase in LCFA oxidation that is associated with exercise.

### 6.2.1 Direct alterations in CPTI

The current thesis provides a plausible alternative mechanism to account for increases in LCFA transport during exercise. Specifically, two hours of cycling at ~60%  $\text{VO}_{2\text{peak}}$  was shown to progressively attenuate the ability of M-CoA to inhibit CPTI activity. This trend was only observed at supra-physiological concentration of M-CoA after 30 minutes of exercise, however a more pronounced effect was observed following 120 minutes of exercise when M-CoA sensitivity was attenuated within the physiological

range. Thus, even if M-CoA concentrations are maintained in contracting human skeletal muscle, CPTI activity can increase during exercise, and ultimately regulate LCFA oxidation rates. However, given the time course of the attenuation in M-CoA sensitivity, the mechanisms involved must be gradually increased, and do not respond rapidly. Although the mechanisms responsible for this observed trend are unknown, knowledge gained from studies on the liver CPTI isoform enables speculation.

Although CPTI topology was originally proposed to contain a M-CoA binding site on the outer leaflet of the outer mitochondrial membrane, and a catalytic core in the intermembrane space (123), evidence now suggests that both the M-CoA binding site and the catalytic core are exposed to the cytosol (83). Although this work was conducted on the liver isoform of CPTI, amino acid sequence alignment between liver and muscle CPTI isoforms has revealed several conserved regions, including both transmembrane domains and a 124 amino acid residue in the N-terminus, suggesting that the muscle isoform will adopt a similar topology [reviewed in (92)].

The N-terminal domain has been previously shown to dictate the sensitivity of CPTI for its inhibitor, M-CoA. Single point mutations within the N-terminus have pronounced effects, attenuating the ability of M-CoA to inhibit CPTI activity (154). It has also been suggested that a chemical link between the N and the C-termini is essential in maintaining M-CoA sensitivity within the liver isoform (53). Again, given the similar topology between the liver and muscle isoforms, and the observation that in human skeletal muscle the removal of the distal 18 amino acids in the N-terminal domain attenuated CPTI M-CoA sensitivity (153), alterations in the N-termini might explain the observations of the current thesis. Although this is plausible, it is uncertain what the mechanism(s) responsible for breaking the link between the N and C-termini would be.

Interestingly, increases in skeletal muscle acyl-CoA levels follow a similar time course as the observed reduction in M-CoA inhibition; moderate increases following an hour of exercise (2 fold), and a more pronounced increase following 2 hours of exercise (4 fold) (186). Due to the polarity of acyl-CoA, progressive increases could gradually alter the structure of CPTI, and subsequently the sensitivity of CPTI for M-CoA. In addition to acyl-CoA levels, several other possibilities exist to explain alterations in M-CoA sensitivity, including reactive oxidative damage, mitochondrial swelling, interactions with other proteins and/or direct phosphorylation (91, 92). However, given the pronounced time required to alter M-CoA sensitivity, it is unlikely that these mechanisms are sufficient to independently alter fatty acid oxidation at the onset of exercise.

#### *6.2.2 Indirect CPTI regulation*

It was suggested in the mid 1980s that CPTI could be directly phosphorylated (73), although this has not been consistently reported (68). In neonatal rat cardiac myocytes, which express both liver and muscle isoforms, hypoxia has been shown to stimulate CPTI activity, while a tyrosine phosphatase inhibitor inhibited CPTI activity (185). This suggests that either the liver and/or muscle isoforms can be directly phosphorylated. Subsequent measurements, using a specific inhibitor for the liver isoform, suggested that phosphorylation was not mediated by the muscle isoform. However, the effect of exercise on CPTI phosphorylation was not studied, and remains a possible mechanism that should be examined in the future.

Although independent of M-CoA, phosphorylation of the cytoskeletal proteins has also been reported to alter CPTI activity (181). This has been accomplished by using permeabilized hepatocytes and various effectors that increased CPTI activity. As a result

of cell permeabilization, M-CoA concentrations are negligible, and therefore the mechanism altering CPTI activity is most likely M-CoA independent. In addition, the ability to alter CPTI activity was lost when isolated mitochondria were used, suggesting a non-mitochondrial component (181). In addition, reconstitution of cytoskeletal fractions decreased CPTI activity in isolated mitochondria in a dose dependent manner, and this was attenuated with the addition of CaMKII. Recent work from the same laboratory also found support for an AMPK-dependent activation of CPTI, also mediated by phosphorylation of cytoskeletal compounds (182, 183). CaMKII (reviewed in (121)) and AMPK [reviewed in (163)] are known to increase with exercise, providing additional M-CoA independent mechanisms to account for increases in CPTI activity and LCFA oxidation during exercise. Although these remain untested but plausible mechanisms, cytoskeletal compounds would have to chronically alter the N-terminal of CPTI to explain the attenuated M-CoA sensitivity observed in the current thesis, given that measurements were made with isolated mitochondria. To date this has not been examined, and therefore it remains speculative, that cytoskeletal compound phosphorylation can alter CPTI M-CoA sensitivity, although clearly it can induce LCFA oxidation. Instead, phosphorylation of cytoskeletal components could increase CPTI early in exercise as a result of the rapid stimulation of AMPK and CaMKII, and alterations in M-CoA sensitivity could account for increases in LCFA oxidation during prolonged exercise.

### *6.2.3 CPTI sensitivity*

Recently, two novel muscle CPTI mRNA species have been described which result from alternative splicing of the CPTI transcript (195, 196). Amino acid sequencing has revealed deletions within the typically conserved N-terminal domain. The predicted

sizes of these two novel CPTI isoforms are 78 and 83 kDa, which are smaller than the traditional 88 kDa protein. Both novel CPTI isoforms contain the same leader sequence as the mature muscle isoform, and therefore should be targeted to the mitochondria (reviewed in (92)). However, given the previously described importance of the N-terminal domain, they are likely to have different kinetic characteristics. Recently, it has been suggested that a M-CoA insensitive pool of CPTI exists (96), and may reflect these new isoforms. A muscle hierarchy appears to exist in the ability of M-CoA to inhibit CPTI, as in the presence of a constant concentration of M-CoA, LCFA oxidation was inhibited more in white muscle compared to red (white gastrocnemius>>soleus>>red gastrocnemius) (96). Interestingly FAT/CD36 follows an inverse expression hierarchy, and may represent an alternative mechanism to induce similar LCFA oxidation trends (see section 6.4). In addition, 100  $\mu$ M M-CoA, which exceeds the  $IC_{50}$  by several orders of magnitude, inhibited LCFA oxidation by only ~60% in red muscle. Interestingly aerobically trained individuals have a higher maximal CPTI activity, suggesting a greater protein content of CPTI. Moreover, supra-physiological concentrations of M-CoA inhibit CPTI activity to the same absolute level in trained and untrained individuals (161). These data suggests that training preferentially increases the expression of the M-CoA sensitive CPTI isoform. Although this may account for the observed chronic training effects, the possibility that alterations in CPTI expression can account for the observations in the current thesis is unlikely, given: 1) the short 2 hour duration of the exercise protocol; 2) the unaltered maximal CPTI activity; and 3) the unaltered palmitoyl-CoA kinetics.

#### *6.2.4 CPTI location within mitochondrial membranes*

Within the plasma membrane, preferential locations for LCFA transport have been identified, including lipid rafts high in caveolin. Mitochondrial membranes have also been suggested to have similar locations dedicated to LCFA transport. The regulation of LCFA oxidation distal to CPTI is purported to occur through substrate-driven mass action. However, the current model of carnitine-dependent transport would therefore predict a substantial amount of acyl-carnitine in the intermembrane space. However, this has not been reported, suggesting that mass action alone does not account for transport of acyl groups distal to CPTI. The concept of a physical interaction, or at least a close proximity, between the outer and inner mitochondrial membranes forming what are known as contact sites, provides a plausible explanation [reviewed in (92)]. These contact sites have now been proposed to represent a microenvironment conducive to LCFA oxidation, by bringing enzymes that facilitate LCFA transport into close proximity. Fraser and Zammit utilized a swell/shrink technique and sonication to sub-fractionate mitochondrial membranes to study the subcellular distribution of various enzymes involved in LCFA transport (58). Interestingly, CPTI and CPTII proteins were both concentrated within the contact sites. Utilizing similar techniques, Kerner and Hoppel recently reported that porin and 3 unidentified proteins (53 kDa, 27 kDa and 19 kDa) were contained within these contact sites (92). Based on the observations that a polyanion inhibitor drastically reduced palmitoyl-CoA oxidation, but not palmitoyl-carnitine oxidation, Kerner and Hoppel suggested a role for porin in mediating the transport of activated LCFA to CPTI (92). They proposed a transport mechanism, whereby porin transported activated LCFAs into the intermembrane space, where CPTI catalytically

converted the compound to acyl-carnitine. However, as previously mentioned it now appears that the catalytic core of CPTI is located within the C-terminal loop in the cytoplasm. Given the discrepancies between their data and the known topology of CPTI, and the potential promiscuity of the polyanion inhibitor, at present it is difficult to put their observations into context.

However, given the previous observation that CPTI and FAT/CD36 coimmunoprecipitate in both rat (36) and human (149) skeletal muscle, it appears that these two proteins contain a physical link, or at least are in close proximity. FAT/CD36 is an 88 kDa protein when highly glycosylated. However, in the unglycosylated form, FAT/CD36 is a 53 kDa protein (70). Interestingly, Kerner and Hoppel reported an unidentified 53 kDa protein concentrated within the contact sites, and on the inner mitochondrial membrane (92), which could potentially represent FAT/CD36.

### **6.3 The role of mitochondrial FAT/CD36**

FAT/CD36 is an integral membrane protein, containing a hairpin topology with two transmembrane domains, with both C- and N- termini located on the same side of the membrane. There is a large loop that has over 10 potential glycosylation sites, and a hydrophobic segment that is believed to fold back on itself, creating a pocket (70) (Figure 6.1).

QuickTime™ and a  
TIFF (LZW) decompressor  
are needed to see this picture.

**Figure 6.1. Predicted membrane topology of FAT/CD36.** From (70).

Although it has been known for several years that FAT/CD36 can mediate LCFA transport across plasma membranes, it is a recent observation that FAT/CD36 has a role in mitochondrial transport (36). In the current thesis the observation that administration of SSO, a specific inhibitor of FAT/CD36, reduced palmitate oxidation in isolated mitochondria by ~80% is in support of other recent literature (15, 75). While FAT/CD36 appears to be required for LCFA oxidation, in young, healthy lean participants, FAT/CD36 did not correlate with the ability of mitochondria to oxidize LCFAs at rest, as shown elsewhere (15). However, this may reflect the small sample size and uniformity of

participants, as mitochondrial FAT/CD36 was significantly correlated with the ability of mitochondria to oxidize LCFAs in a more diverse population (lean and obese) at rest.

A novel finding in the current thesis is the observation that two hours of continuous exercise at a moderate intensity can induce an increase in the amount of FAT/CD36 on mitochondrial membranes in human skeletal muscle. Concomitantly, exercise induced an increase in the ability of mitochondria to oxidize LCFAs, and this appears to at least be partially regulated by FAT/CD36, as SSO administration alleviated this effect. Given the short duration of the protocol, and the unaltered total muscle FAT/CD36 content, translocation of FAT/CD36 is a likely explanation. Campbell and colleagues, who reported similar findings in mitochondria isolated from rat skeletal muscle following an acute 30-minute electrical stimulation protocol, originally proposed this mechanism (36). To date there are no explanations for how proteins are translocated to mitochondrial membranes, or what the regulatory signals may be. However, given that AMPK, calcium and PKC have all been suggested to induce translocation of FAT/CD36 to the plasma membrane during muscle contraction [reviewed in (111)], these remain likely candidates for targeting FAT/CD36 to mitochondrial membranes. However, although a reallocation of FAT/CD36 to the plasma membrane has been observed in obese humans (26), mitochondrial FAT/CD36 is not different in this population, suggesting that the regulatory signals may be divergent.

Interestingly, in rat skeletal muscle the increase in FAT/CD36 is proportional to the increase in the ability of mitochondria to oxidize LCFAs (36). However, in human skeletal muscle a disproportionate increase in FAT/CD36 protein compared to the capacity to oxidize LCFA was prevalent following exercise. The disparity between the magnitude change in FAT/CD36 and LCFA oxidation remains unexplained. However,

since an association with mitochondrial FAT/CD36 and palmitate oxidation progressively increased with exercise duration, this would suggest a greater regulatory role of FAT/CD36 at higher LCFA oxidative flux rates.

Given that the exact mechanism by which FAT/CD36 mediates LCFA transport is unknown, it is difficult to speculate on its role in mitochondrial transport. However, some insights have been gained in recent years. The observation in the current thesis, and previously (15), that SSO does not inhibit CPTI activity, suggests that FAT/CD36 is located distal to CPTI. This has been further corroborated by the observation that SSO inhibits palmitoyl-carnitine oxidation (15). Interestingly there appears to be a species difference in the location of FAT/CD36, as in rat muscle it was suggested to be located proximal to CPTI, as SSO inhibited CPTI activity (36). In rat skeletal muscle FAT/CD36 may function in a similar fashion to the purported role of porin, as both appear to be located upstream of CPTI (92). Therefore, given the various isoforms of porin, it could be speculated that FAT/CD36 is a porin. However, several differences exist between these two proteins, as porins are typically ~22 kDa and contain 283 amino acids, whereas FAT/CD36 is larger (~88 kDa) and contains 472 amino acids. In addition, amino acid alignment between the rat isoforms of FAT/CD36 and porin show no distinct conserved regions, further strengthening the argument that they are distinct proteins (Figure 6.2).

```

CD36Rat      MGCDRNCGLITGAVIGAVLAVFGGILMPVGDLLIEKTIKREVVLEEGTIAFKNWVKTGTT 60
porin        -----MAVPP-----TYADLGKSARD 16
              : :*                               :: : *:.

CD36Rat      VYRQFWIFDVQNPEEVAKNSSKIKVKQRGPYTYRVRYLAKENITQDPKDSVSVFVQPNGA 120
porin        VFTKGYGFGLIKLDLTKSENGLEFTSSGSANTET---TKVNGSLETKYRWTEYGLTFTE 73
              *: : : *.: : : :*... :... * . . . . : * * : :.* . . : .

CD36Rat      IFEPSSLVGTENDNFVTLNLAVAAAAPHIYTNSFVQGVLSLIKKSKSSMFQTRSLKELLW 180
porin        KWNTDNTLGT---EITVEDQLARGLKLTFDSSFSPTG-----KKNAKIKTGYKREHIN 124
              :.. :*** :** : . . : ** .. .* : ** : * :

CD36Rat      GYKDPFLSLVPYPISTTVGVFYPYNNITVDGVYKVFNGKDNISKVAIIDTYKGRNLSYWE 240
porin        LGCDVDFDIAGPSIRG--ALVLGYEGWLAGYQMNFFETSK--SRVTQSNFVAVG-YKTDEFQ 179
              * :.. .* .. *.: * * : .. ** : : * : . :

CD36Rat      SYCDMINGTDAASFPPFVEKSRTLRFSSDICRSIYAVFGSEVNLKGI PVYRFLVLPANAF 300
porin        LHTNVNDGTEFG-----GSIYQVKNKLETAVNLA-----WTAGNS 215
              : : : ** : . . * : : : : ** .*.

CD36Rat      ASPLQPNPDNHCFCFTEKVISNNCTS YGVLDIGKCKEGKPVYISLPHFLHASPDVSEPIEGL 360
porin        NTRFGIAAKYQVDPDACFSAKVNSSLI GLYTQTLKPG-----IKLTL SALLDGG 266
              : : . : . : : * : .. : : * : * . : * : **

CD36Rat      NPNEDEHRTYLDVEPITGFTLQFAKRLQVNI LVKPARKIEALKNLKRPYIVPILWLN ETG 420
porin        NVNAGGHKLGLEFQA----- 283
              * * . * : * . * :

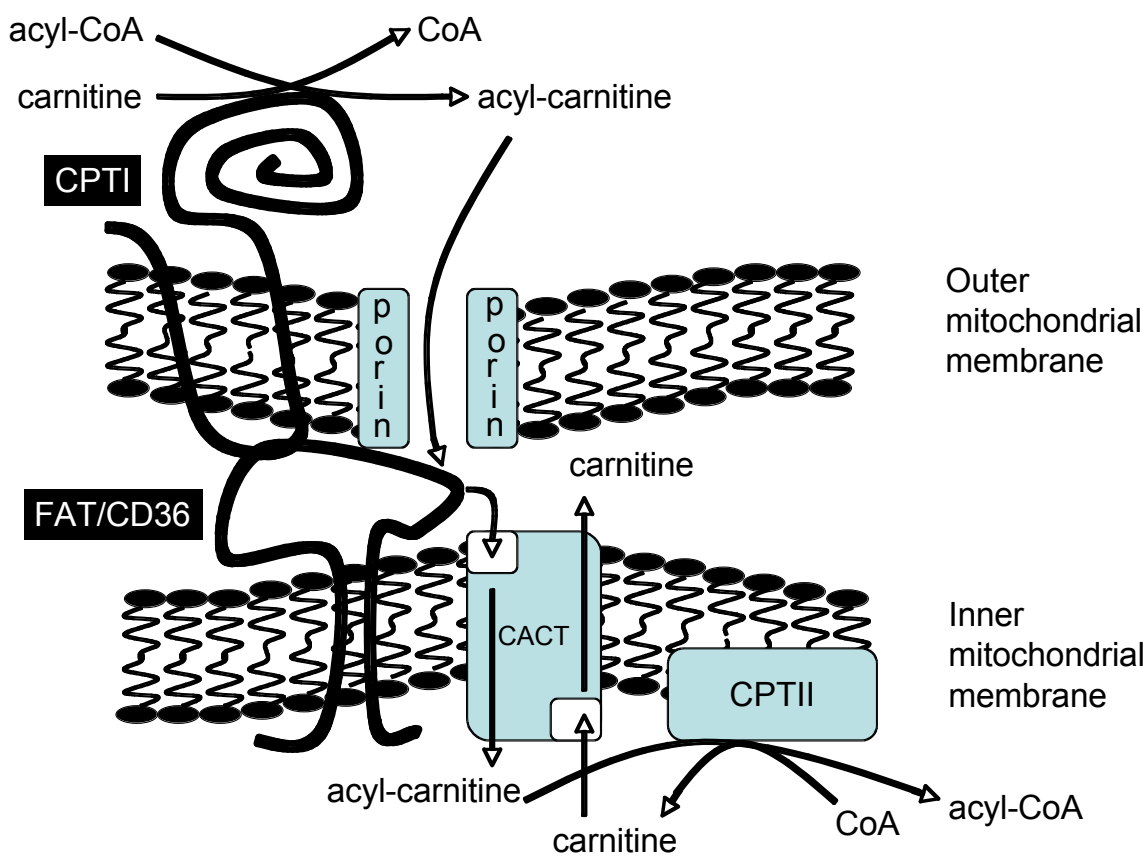
CD36Rat      TIGDEKAEMFRNQVTGKIKLLGLVEMVLLGVGVVMFVAFMISYCACRSKNGK 472
porin        -----

```

**Figure 6.2. Sequence alignment between porin and FAT/CD36.** Alignment was conducted in Clustal W (1.83) software using amino acid sequence NP113749 and Q9Z2L0 for FAT/CD36 and porin, respectively. where, \* indicates an identical amino acid, : indicates a highly conserved amino acid, and . indicates a weakly conserved amino acid.

Given that Kerner and Hoppel have found a previously unidentified protein with the same molecular weight as FAT/CD36 in contact sites and on inner mitochondrial membranes, it is speculated that FAT/CD36 is located down-stream of CPTI on inner mitochondrial membranes. In this location it may facilitate the transfer of acyl-carnitine, either directly or indirectly, from CPTI to the CACT. Interestingly, CACT has been shown to work in a ping-pong like fashion, transferring acyl-carnitine into the mitochondrial matrix, while transporting carnitine from the matrix into the intermembrane space. This enzyme

appears to be regulated by the presence of acyl-carnitine, as carnitine export is negligible without the presence of acyl-carnitine moieties (60). With FAT/CD36 located between CPTI and CACT, it could assist in rapidly moving acyl-carnitines to the binding site of CACT, drastically increasing the transport into the matrix, and accounting for the previously observed absence of acyl-carnitines in the intermembrane space (Figure 6.3).



**Figure 6.3. Proposed model for LCFA transport into mitochondria.** Acronyms are defined in the text.

#### **6.4 CPTI and FAT/CD36 interaction**

Interestingly, FAT/CD36 follows an inverse expression hierarchy to the M-CoA sensitivity of CPTI (RG contains the most FAT/CD36, and the lowest sensitivity of M-CoA CPTI inhibition) (25, 96). In addition, in the current study CPTI M-CoA sensitivity was attenuated most drastically when FAT/CD36 was the most abundant on mitochondrial membranes. It is easy to then speculate that FAT/CD36 somehow alters the CPTI M-CoA sensitivity. With FAT/CD36 located on the inner mitochondrial membrane it can potentially interact with the loop located between the two transmembrane domains, and through an unknown mechanism, induce a conformational change that alters the chemical link between the N- and C-termini of CPTI. Interestingly, FAT/CD36 contains three potential phosphorylation sites, which would have a large negative charge capable of inducing conformational changes in CPTI.

#### **6.5 The Role of FABPpm in Mitochondrial LCFA Oxidation**

Given the interesting and novel regulatory role of FAT/CD36 in mediating mitochondrial LCFA oxidation, it was hypothesized that additional, presently unidentified proteins, may also participate in transport across mitochondrial membranes. FABPpm is a well-recognized plasma membrane LCFA transport protein, and insulin (72), AMPK activation (39) and muscle contraction (72) have been shown to induce a relocation of FABPpm to the plasma membrane in a similar fashion to FAT/CD36. Given the similarities between FAT/CD36 and FABPpm regulation at the plasma membrane in response to various stimuli, the well known presence of FABPpm on mitochondrial membranes (168), and the previous suggestion that FAT/CD36 and FABPpm participate in a concerted manner to transport LCFAs (118), the role of FABPpm in mitochondrial LCFA oxidation was also examined in the current thesis. The observations in current data

that suggests FABPpm does not participate in mitochondrial LCFA transport includes: 1) FABPpm did not translocate to the mitochondria during exercise in rat or human skeletal muscle; 2) and therefore FABPpm did not correlate with the capacity of mitochondria to oxidize fatty acids; 3) electrotransfection drastically increased mitochondrial FABPpm protein content without altering the ability of mitochondria to oxidize fatty acids; and 4) FABPpm protein content significantly correlated with mAspAT activity. Although these data are highly suggestive that FABPpm does not mediate mitochondrial LCFA transport, it is possible that FABPpm is necessary but not limiting. In this regard, increases in FABPpm would not be expected to alter LCFA oxidation. Future studies should include anti-FABPpm antiserum, knock down and knock out techniques to further establish whether FABPpm participates at any level in mitochondrial LCFA oxidation.

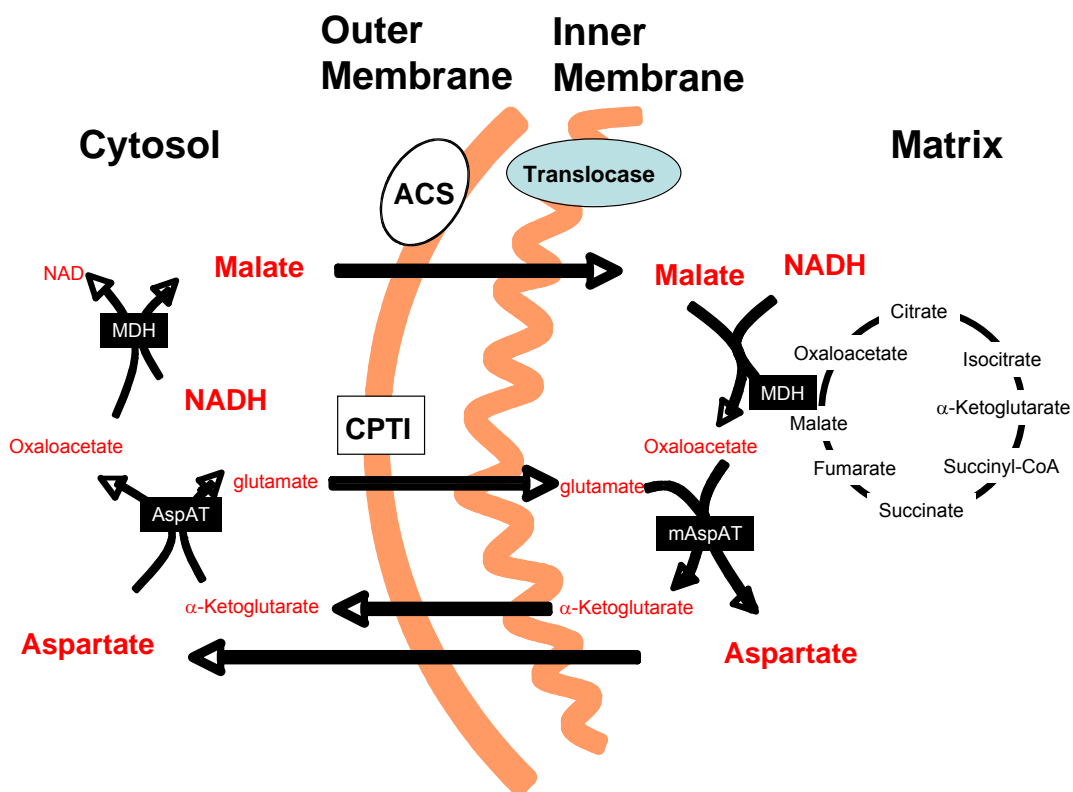


Figure 6.4. Proposed model for NADH transport into mitochondria.

## 6.6 Fatty Acid Oxidation in Lean and Obese Individuals Skeletal Muscle

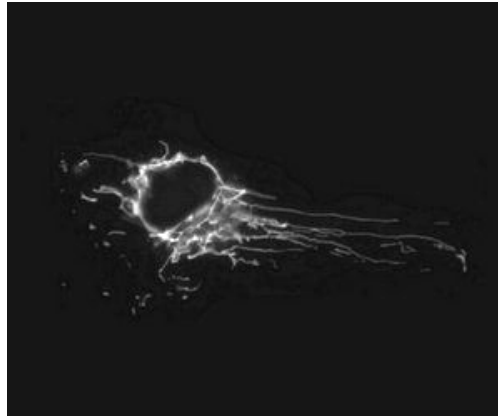
Recent literature has suggested that obesity is associated with impairments in mitochondrial LCFA oxidation. Two plausible explanations exist for alterations in mitochondrial fatty acid oxidation, either mitochondrial content is decreased and/or there is a dysfunction in fatty acid oxidation within mitochondria. Recently the ability of mitochondria to oxidize fatty acids was directly measured in skeletal muscle from type 2 diabetics, and controversially increased (8). It should be noted, however, that mitochondria were isolated from frozen samples, and presumably not metabolically responsive. Data in this thesis showed that mitochondria isolated from lean and obese individuals did not differ in the ability to oxidize LCFAs. In addition, the content of FAT/CD36 was not different in mitochondria obtained from skeletal muscle of lean and obese individuals when expressed per mg mitochondrial protein. However, obesity was associated with reductions in whole muscle CS and  $\beta$ -HAD activities, two common markers of mitochondrial content. Therefore, it was concluded that the whole muscle capacity to oxidize fatty acids is compromised with obesity as a result of reductions in mitochondrial content, and not as a result of some dysfunction within mitochondria.

Since the publication of the present data, methods measuring the oxygen consumption of mitochondria isolated from lean and obese individuals have also suggested that a dysfunction does not exist in mitochondria. Boushel and collaborators have shown whole muscle fatty acid oxidation is impaired with obesity. However, when normalized to CS activity or mitochondrial DNA this result was lost, indicating that obese individuals simply have less mitochondria in their skeletal muscle (28). Importantly, Boushel and colleagues measured mitochondrial oxygen consumption instead of

radioactively labeled substrate consumption, which enables for respiration to be measured in state 4 (quiescent) and state 3 (ADP stimulated) conditions. This provides an indication of the metabolic responsiveness of the mitochondria. Further evidence in support of mitochondria not containing an intrinsic dysfunction comes from the RCR (state 3/state 4) reported by Boushel and colleagues, as mitochondrial responsiveness to metabolic signals in diabetics appeared to be similar to lean controls (28). Interestingly, Mogensen and colleagues isolated mitochondria and measured oxygen consumption in the presence of palmitoyl-carnitine, which is the product of CPTI (128). It was reported that mitochondria isolated from type 2 diabetic individuals compared and obese controls did not differ in the ability to consume oxygen. Since palmitoyl-carnitine was used as a substrate, any differences in CPTI were removed from compromising the interpretation; mitochondria isolated from diabetics did not contain a dysfunction, which was also supported by similar RCR values. However, when pyruvate and malate were used as substrates, a reduction in oxygen consumption was reported, and was associated with a reduction in the RCR value. Therefore, it is possible that mitochondria in diabetic individuals differ in the ability to oxidize substrates, but the impairment might be related to CHO oxidation, as there is now strong evidence that an inherent dysfunction in LCFA oxidation does not exist. However, all of these studies utilized either homogenization or isolation techniques that potentially alter the morphology of mitochondria. In addition, only ~20% of the total mitochondria are recovered, and therefore, it is possible that a mitochondrial dysfunction may be present *in vivo*, and current methodologies limit our understanding.

Recently, in addition to the density of mitochondria, research has shown that the morphology of the mitochondrial membrane network influences the capacity to oxidize

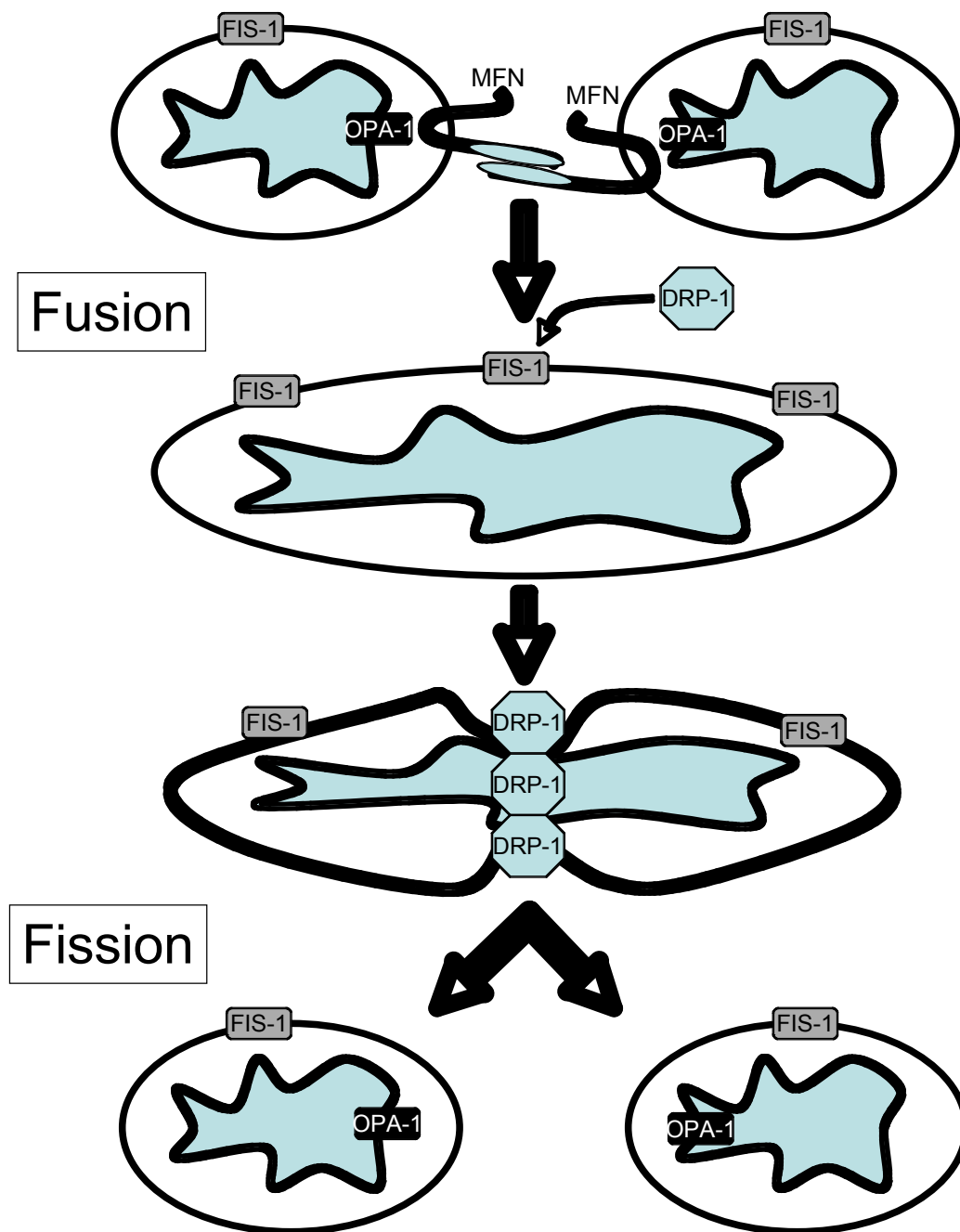
substrates (7). Mitochondria appear to be a complex tubular network, as opposed to individual organelles, that continually undergo fission and fusion reactions (Figure 6.4).



**Figure 6.5. Light microscope image of mitochondrial reticulum shown by Mitotracker green fluorescent staining.** Picture from [www.biocrawler.com](http://www.biocrawler.com)

Although the exact mechanisms responsible for these fission/fusion events remains elusive, gain of function and loss of function studies have revealed several regulatory proteins that appear to be involved. Disruption of dynamin-related protein 1 (DRP-1) and/or fission-1 (FIS-1), generates elongated mitochondrial tubule networks, suggesting roles in fission. In contrast, over-expression of MFN-2, has been shown to increase the mitochondrial tubular network, suggesting a role in fusion (7). DRP-1 is located in the cytosol, contains a GTPase on its N-terminal domain (201), and has been suggested DRP-1 interacts with FIS-1 to couple GTP hydrolysis with mitochondrial membrane constriction and fission (157). For fusion to occur, coordination between the inner and outer mitochondrial membranes must be observed. MFN-1 and 2 are located on

the outer mitochondrial membrane and appear to coordinate fusion events with autosomal dominant optical atrophy 1 (OPA-1), which is located on the inner mitochondrial membrane (Figure 6.5).



**Figure 6.6. Mitochondrial fission and fusion events.**

As a result of the current obesity and diabetic epidemic, attempts have been made to characterize fission/fusion proteins within these populations. Reductions in MFN-2 mRNA and protein (Bach et al., 2006) have been associated with obesity, and BMI appears to be negatively correlated with MFN-2 gene expression (6, 126). These data suggest that mitochondria may be more fragmented within this population, which has been associated with an impaired ability to oxidize CHO in L6E9 and 10T1/2 cells (7). The previous studies suggesting that mitochondria are not dysfunctional utilized isolation techniques, which could alter the reticulum formation, and may not reflect true function. Therefore, future work should also be done utilizing skinned fiber, or permeabilized muscle preparation, techniques that render the mitochondrial reticulum uncompromised.

## **6.7 Conclusions**

In summary, this thesis provides two novel mechanisms that regulate (or contribute to) LCFA oxidation. Specifically, alleviations in CPTI M-CoA inhibition, and increases in the protein content of mitochondrial FAT/CD36, increase the ability of mitochondria to oxidize LCFA during continuous exercise. In contrast, FABPpm, another plasma membrane transport protein, does not appear to regulate mitochondrial LCFA oxidation, as pronounced mitochondrial overexpression did not affect palmitate oxidation. In addition, it appears that FAT/CD36 is distal to CPTI, as SSO administration inhibited palmitate oxidation, without altering CPTI activity. Lastly, while obesity has been associated with reductions in whole muscle LCFA oxidation, this was not attributable to a dysfunction within mitochondria or the content of FAT/CD36, but instead results from a decreased mitochondrial content.

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## **METHODS APPENDIX**