ADAPTATIONS OF SKELETAL MUSCLE PYRUVATE DEHYDROGENASE KINASE IN RESPONSE TO FOOD-RESTRICTION IN MITOCHONDRIAL SUBPOPULATIONS

by

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ABSTRACT

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Pyruvate dehydrogenase (PDH) catalyses the decarboxylation of pyruvate, to form acetyl-CoA. PDH activity is down-regulated by intrinsic PDH kinases (predominantly PDK2 and PDK4 isoforms), but the understanding of the PDK isoform distribution and adaptation to nutritional stresses has been restricted to mixed mitochondrial populations, and not delineated between subsarcolemmal (SS) and intermyofibrillar (IMF) subpopulations. SS and IMF mitochondria exhibit distinct morphological and biochemical properties; however the functional differences are not well understood. This study investigated the effect of fed (FED) versus 48 h total food-restriction (FR) on rat red gastrocnemius muscle PDK2 and 4 isoform content in SS and IMF mitochondria. PDK4 content was ~3-5 fold higher in SS mitochondria compared to IMF ($p=0.001$), and increased with FR ~3-4- fold in both subpopulations ($p<0.001$). PDK2 was ~2.5-4 fold higher in SS mitochondria compared to IMF ($p=0.001$), but PDK2 was unaltered with FR. Citrate synthase activity (μmol/min/mg mitochondrial protein) was not different between either subpopulation. As well there were no significant differences between mitochondrial subpopulations in PDH complex components in both fed and FR states. These results demonstrate that there is a markedly higher content of both PDK isoforms in SS compared to IMF mitochondria. Although PDK2 does not increase in either subpopulation in response to FR, PDK4 increases to a similar extent in both SS and IMF after 48 h food-restriction.

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Finally, this thesis is dedicated to the memory of my cousin and dear friend Lindsay Ellen Hay for her life long friendship and support throughout my schooling. Her outgoing nature and insistence on putting others first are among many of her exceptional traits that made her an exceptional leader and pleasure to be around. As a graduate of Brock University's Child and Youth Studies and a recent graduate of Griffith University's Teachers' College and Master's of Education in Australia, Lindsay had the ability to touch the lives of many students. I am grateful for her love and guidance in all aspects of life and for being there when I needed her the most.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ß-HB	ß (beta)-hydroxybutyrate
BSA	bovine serum albumin
CPT	carnitine palmitoyl transferase
CoA	coenzyme A
CS	citrate syntase
COX	cytochrome c oxidase
EGTA	ethylene glycol tetra acetic acid
E1	pyruvate dehydrogenase
E2	dihydrolipoamide transferase
E3	dihydrolipoamide dehydrogenase
FAT	fatty acid transporter
FFA	free fatty acid
IMF	intermyofibrillar mitochondria
IDH	isocitrate dehydrogenase
Malonyl-CoA	malonyl-coenyze A
MCT	monocarboxylate transporters
N_2	liquid nitrogen
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nictinamide adenine dinucleotide
PCA	perchloric acid
PDH	pyruvate dehydrogenase
PDHa	active pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
RG	red gastrocnemius
SDH	succinate dehydrogenase
SS	subsarcolemmal mitochondria
TCA	tricarboxylic acid cycle
TBST	tris-buffered saline tween
Tris-HCL	tris-hydrochloride
TPP	thiamine pyrophosphate
UCP	uncoupling protein

CHAPTER 1

INTRODUCTION

Fuel utilization of carbohydrate and fat by skeletal muscle has been an important area of research for several decades. The initial investigations made by the Randle laboratory determined the reciprocal relationship between carbohydrate and fat oxidation in mitochondria at rest (Garland et al., 1962). To date there are many aspects of carbohydrate and fat oxidation that currently remain unknown. There is minimal investigation into carbohydrate and fat oxidation in mitochondrial subpopulations specifically and in particular no research has been conducted in the area of carbohydrate oxidation.

The two mitochondrial subpopulations have been named based on their location within the cell and exhibit distinct morphological, biochemical, and functional properties. Subsarcolemmal (SS) mitochondria reside near the sarcolemma or muscle cell membrane and intermyofibrillar (IMF) mitochondria are found between the myofibrils.

In carbohydrate oxidation, pyruvate dehydrogenase (PDH) is an important multienzyme complex that catalyzes the irreversible oxidative decarboxylation of pyruvate producing acetyl CoA for entry into the tricarboxylic acid (TCA) cycle and is the rate limiting step in pyruvate oxidation. When carbohydrate availability is abundant PDH can promote increased oxidation through the TCA cycle. While carbohydrate availability is low or fatty-acid availability is increased, PDH can alter fuel selection in order to preserve carbohydrate stores by decreasing the oxidation of carbohydrate through the TCA cycle. The two intrinsic regulatory enzymes involved in these actions of the complex are PDH kinase (PDK) and PDH phosphatase (PDP). PDK adds

phosphates to the PDH complex and leads to inactivation and decreased carbohydrate oxidation, while the role of PDP is to reactivate the complex through dephosphorylation. Therefore, PDH regulates carbohydrate oxidation, and indirectly the proportion of fat- and carbohydrate-derived acetyl units for the TCA cycle.

PDK exists as four distinct isoforms (PDK1-4), with PDK2 and PDK4 predominating in mammalian skeletal muscle. PDK2 and PDK4 differ in their response to acute allosteric activators and inhibitors. However, all published research pertaining to PDH and PDK was studied in primarily SS mitochondria. To date, there have been no studies directed at investigating the possible differences in distribution of these enzymes between SS and IMF mitochondria.

Therefore, the purpose of this thesis is to determine the protein content of PDK2 and 4. In addition, the PDH complex components will be measured in SS and IMF mitochondria in control conditions and following 48 h of food-restriction to determine whether differences exist in the adaptation response of this key enzyme complex.

CHAPTER 2

LITERATURE REVIEW

In this chapter, the literature reviewed is related to (a) mitochondrial structure and function, as well as subpopulation distribution; (b) conventional regulation of pyruvate dehydrogenase; (c) PDK isoforms 2 and 4; (d) PDH complex components and (e) mitochondrial adaptation will be discussed as these areas of research play an important role in providing background for the present study.

Mitochondria

Mitochondria are highly dynamic organelles and have historically been termed the "powerhouses" of the cell. Without them, cells would be unable to extract significant amounts of energy in the form of adenosine triphosphate (ATP), via the process of oxidative phosphorylation, from nutrients and oxygen and as a result cellular functions would cease (Guyton and Hall, 1997). Mitochondria continuously adjust ATP regeneration to match the changing bioenergetic demands of the cell. They are present in the cytoplasm of the cell, although the volume of mitochondria that are present depends on the specific energy requirements of each distinct cell type. Typically a cell will have hundreds or thousands of mitochondria and they may occupy as much as 25% of the cytoplasm. However, the number of mitochondria per cell, as well as their intracellular locations has been found to vary depending on the cell type and metabolic state (Dyson, 1975). Studies in skeletal muscle mitochondria exploring the response to physiological stimulus (e.g. endurance training) have demonstrated an increase in mitochondrial size,

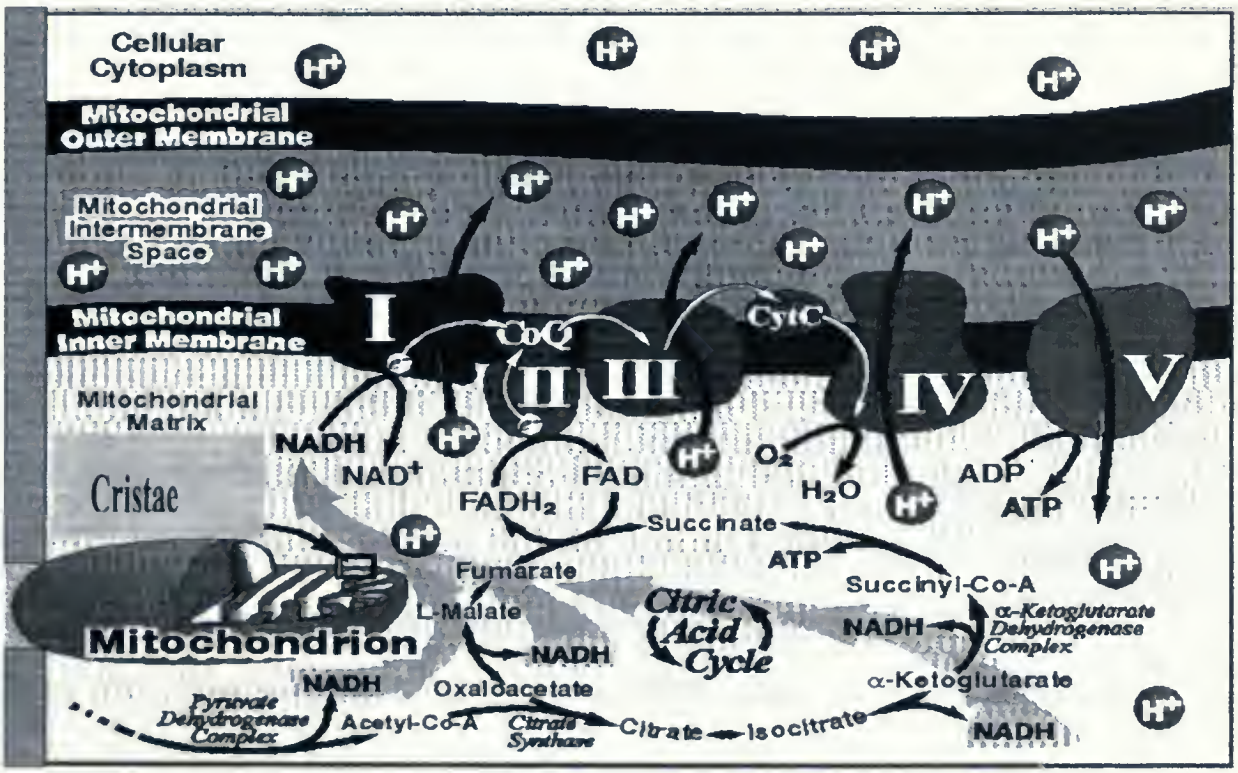
number and/or volume (Hood, 2001). However, there has been minimal research in skeletal muscle mitochondria exploring dietary adaptations.

Mitochondrial Structure

The basic structure of the mitochondrion is illustrated in figure 1. The mitochondrion contains two lipid bilayer-protein membranes: an outer membrane and an inner membrane. The two membranes exhibit distinct functions. The outer membrane functions as a barrier by enclosing the entire organelle. It contains numerous integral proteins known as porins, which contain a relatively large internal channel to allow for the diffusion of small (<5000 daltons) molecules. Larger molecules are only permitted to cross the outer membrane via transporter activity. These highly specific transport mechanisms are used to regulate the influx and efflux of various materials (Brooks et. al, 2005). The inner membrane also allows the passage of specific molecules; however it is more selective, does not contain porins, and is impermeable to protons allowing for the preservation of the chemiosmotic gradient. Passage of specific molecules into and out of the matrix requires protein carriers (Brooks et. al, 2005). There are many infoldings of the inner membrane that form cristae which expand the surface area of the inner membrane. The electron transport chain (ETC) and oxidative phosphorylation enzymes are inner membrane proteins and enhanced inner membrane capacity of oxidative phosphorylation is a result of enhanced inner membrane surface area (Brooks et. al, 2005).

The inside of the mitochondrion contains a compartment called the matrix which contains soluble enzymes, proteins, DNA and ribosomes. It is the site of the oxidation of pyruvate and other small organic molecules (Guyton and Hall, 1997).

Figure 1. **Basic structure of the mitochondria. Displaying structural and functional aspects of the mitochondrial membranes, cristae and matrix.** (Adapted from Illingworth et al., 2006).



Mitochondrial Subpopulations

Mitochondria in skeletal muscle have previously been characterized as discrete, spherically shaped organelles; however current research predicts that mitochondria exist as a continuous reticulum and this notion has been strengthened through the use of electron micrographs of longitudinal sections of skeletal muscle. Although all mitochondria were thought to be similar in function, it has been revealed through the use of electron microscopy that at least two distinct mitochondria subpopulations exist in different subcellular locations and thus may be different in function (Hoppeler, 1986; Kirkwood et al., 1986; Ogata & Yamasaki, 1985; Palmer et al., 1977).

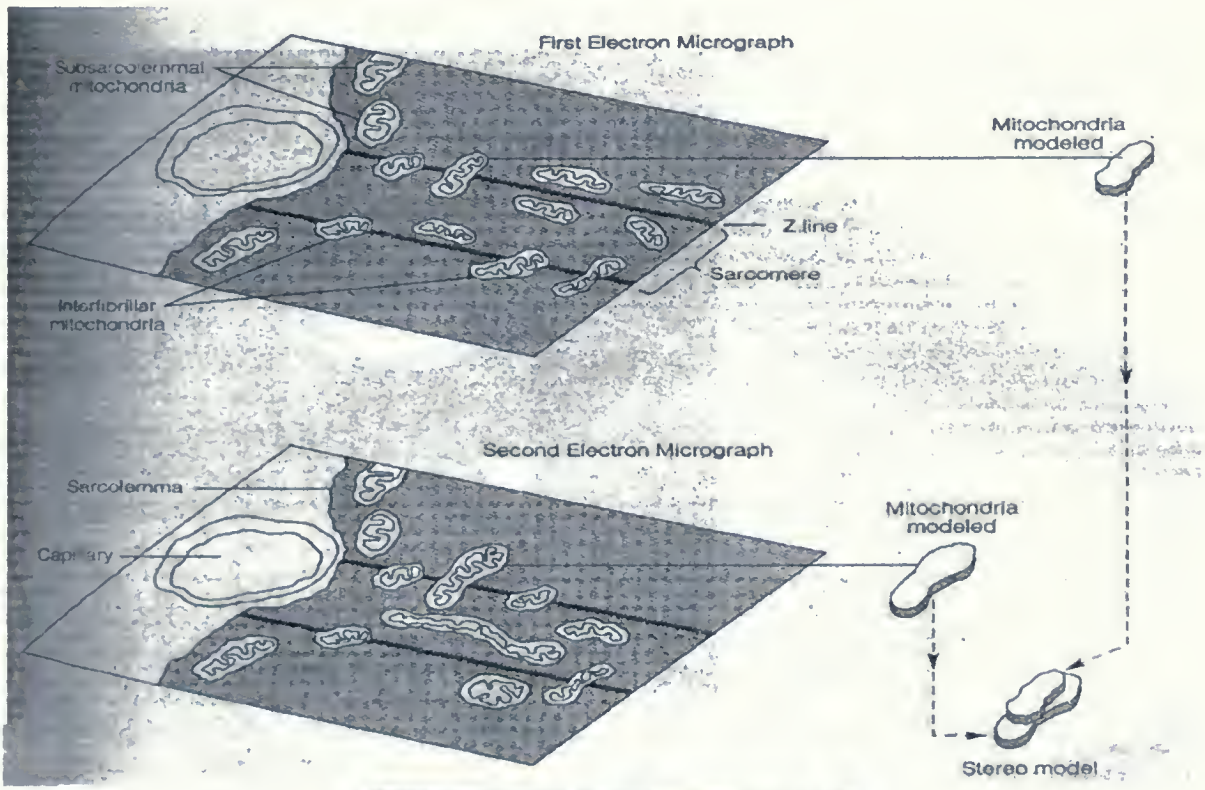
Mitochondrial function in skeletal as well as cardiac muscle, present the added dimension of two distinct mitochondrial subpopulations that might exist as a continuous reticulum, but differ according to their subcellular localization and morphology, as well as biochemical and functional properties (Cogswell et al. 1993; Palmer, et al.1977). In addition, the reticulum has been found to be dynamic and capable of fission and fusion during differentiation, development and energetic changes (Stevens, 1981; Bereiter-Hahn, 1990). The theory of a reticulum is acceptable as it has been demonstrated with the use of ultrastructural analyses of muscle mitochondria that mitochondria exist not as a single organelle but as a reticulum (Bakeeva et al., 1978).

In addition to the evidence for a reticular structure, there is also evidence that distinct mitochondrial structures are found in different locations with different functions occurring. Differences in mitochondrial function signify that physiological benefits are occurring based on the location of the mitochondria. The different susceptibilities or responsiveness of the mitochondria, due to physiological challenges, are occurring at

different rates and/or to differing degrees based on the particular locations of the mitochondria.

For this reason the mitochondrial subpopulations have been named based on their location within the reticular structure. Subsarcolemmal (SS) mitochondria reside near the sarcolemma and appear to be the larger, in size, of the two populations in electron micrographs, while intermyofibular (IMF) mitochondria appear smaller, more compact and are located between the myofibrils (Hoppeler, 1986) (Figure 2). Several studies have shown that the subpopulations exhibit many inherent differences; however some studies have determined conflicting results, which has made it difficult to determine the specific roles of the mitochondria subpopulations. This will be discussed in greater detail.

Figure 2. **Electron micrograph of mitochondria subpopulations and mitochondria modeling.** (Adapted from Brooks et al., 2005).



SS mitochondria have been found to constitute, on average, ~10-15% of the total mitochondrial volume in skeletal muscle. Although SS mitochondria make up a small portion of the total mitochondrial volume they appear to adapt more readily to variations in muscle use and disuse (Krieger et al., 1980). SS mitochondria are the only

mitochondria to contain the monocarboxylate transporters (MCT) 1 and 4 protein according to Benton et al. (2004). There are many conflicting results in the literature in regards to the presence and/or function of many enzymes in the mitochondrial subpopulations. SS mitochondria have been reported to have higher amounts of succinate dehydrogenase (SDH) (Cogwell et al., 1993; Chilibeck et al., 2002), cytochrome c oxidase (COX) (Iossa et al., 2004) and a greater presence of uncoupling protein 3 (UCP3) (Jimenez et al., 2002). As well, the SS mitochondria are thought to be predominately responsible for the regulation of fat oxidation as they have been shown to have higher sensitivity to malonyl-CoA inhibition than the IMF mitochondria (Koves et al., 2005).

IMF mitochondria have been reported to have higher amounts of energy generated by oxidative phosphorylation in support of muscle contraction (Cogswell et al., 1993), higher respiration rates (Kreiger et al., 1980; Cogswell et al., 1993; Bizeau et al., 1998; Roussel et al., 2000), higher protein content (Takahashi et al., 1996) and greater presence of UCP3 (Ljubicic et al., 2004), isocitrate dehydrogenase (IDH) activity (Bizeau et al., 1998), SDH activity (Krieger et al., 1980), succinate oxidase (Menshikova et al., 2004) and COX (Roussel et al., 2000). In addition, IMF mitochondria have higher rates of protein import and protein synthesis and lower levels of cardiolipin (a specialized inner membrane phospholipid) (Hood et al., 2001).

In contrast, although many studies believe functional differences appear to exist between the two subpopulations (Bizeau et al., 1998; Cogswell et al., 1993) there are many studies that believe there are similarities that are shared, including similar maximal activity of carnitine palmitoyl transferase (CPT) I activity, similar citrate synthase (CS)

per mg of mitochondrial protein (Bezaire et al., 2004; Cogswell et al., 1993; Johnson et al., 2006), similar fatty acid transporter (FAT/CD36) protein content (Campbell et al., 2004), COX activity (Jimenez et al., 2002) and F1-ATPase activity (Roussel et al., 2000). Table 1 displays a summary of known differences between mitochondrial subpopulations.

Table 1. **Reported differences in mitochondrial subpopulations in basal state.**

Measurement	**Mitochondrial Subpopulation**	**Study**
Presence of MCT1 and 4 content	SS	Benton et al., 2004
Greater COX activity	SS	Iossa et al., 2004
Higher sensitivity to malonyl-CoA inhibition	SS	Koves et al., 2005
Greater SDH activity	SS	Cogswell et al., 1993; Chilibeck et al., 2002
Greater UCP3 content	SS	Jimenez et al., 2002; Iossa et al., 2004
Greater CS activity/mg protein	SS	Bezaire et al., 2004
Greater SDH activity	IMF	Krieger et al., 1980
Higher state III respiration rates	IMF	Krieger et al., 1980; Cogswell et al., 1993; Bizeau et al., 1998; Roussel et al., 2000
Higher protein content	IMF	Takahashi et al., 1996
Greater UCP3 content	IMF	Ljubicic et al., 2004
Greater IDH activity	IMF	Bizeau et al., 1998
Higher rates of protein import, synthesis	IMF	Hood et al., 2001
Lower levels of cardiolipin	IMF	Hood et al., 2001
Greater COX activity	IMF	Roussel et al., 2000
Greater Succinate oxidase	IMF	Menshikova et al., 2004
CPT I activity	No Difference	Bezaire et al., 2004
CS activity/mg protein	No Difference	Bezaire et al., 2004; Cogswell et al., 1993; Johnson et al., 2006
Fatty acid transporter (FAT/CD36) protein content	No Difference	Campbell et al., 2004
F1-ATPase activity	No Difference	Roussel et al., 2000
COX activity	No Difference	Jimenez et al., 2002

It is not well established how the differences between the subpopulations arise, however it is believed to be linked to the ATP demands in each of the subcellular regions (Hood et al., 2001). It is has been speculated that SS mitochondria contribute to ATP production for active sarcolemmal processes (e.g. Na^{+}-K^{+}-ATPase, Ca^{2+}- ATPase), as well as providing the energy required by nuclei of the cell which are located in the periphery. On the other hand, it would make sense that IMF mitochondria would principally resynthesize ATP for contracting myofibrils (Chilibeck et al., 2002; Hood, 2001).

In summary, biochemical studies have revealed the notion that muscle mitochondria do not exist as discrete subpopulations but rather they exist in a continuous reticulum (Bakeeva et al., 1978; Kirkwood et al, 1986). Although it is widely accepted that there is a presence of a reticular structure, this does not prelude the possibility that regional differences in membrane protein composition may exist, since mitochondria that are located in different regions may adapt to the differing metabolic demands of these regions (Kirkwood et al., 1986). Therefore, the apparent differences in enzyme activities in SS and IMF mitochondria do not discount a mitochondrial reticulum, but provide evidence of mitochondrial heterogeneity within skeletal muscle or within a reticulum. Future research into the mitochondrial subpopulations' biochemical and functional differences are required to fully clarify the role of mitochondrial heterogeneity in skeletal muscle.

Oxidative Enzyme Activities in SS and IMF Mitochondria

Simply comparing the differences in oxidative enzymes between the mitochondrial subpopulations from the literature proves difficult. Results vary from study to study and there are differences in mitochondrial extraction procedures, quality control and the oxidative enzymes measured. Taken together, including all measured ETC, TCA cycle and fat oxidation enzymes and state III respiration, the majority of authors report higher oxidative enzyme content in the IMF preparations (see Table 1 for details). Although a few demonstrate the reverse, and some measurements are not different between the two subpopulations, it is generally believed that the IMF mitochondrial subpopulations have greater oxidative capacity.

CS is measured in most of the studies as a TCA cycle marker, and is one of the few measurements that is consistent from study to study, finding that the two subpopulations contained the same activity when normalized per mg mitochondrial protein. For this reason it was examined in this study as well as a confirmatory measurement.

There are currently no studies that have examined the distribution of PDH activity or its regulators (PDH kinase and phosphatase) in the two mitochondrial subpopulations. It is difficult to summarize the current known research and formulate a hypothesis whether one would expect higher PDH and PDK activity and expression in either SS or IMF preparations. However, given that oxidative capacity is generally greater in IMF mitochondria, it would be reasonable to expect that the PDH complex enzymes would be greater in the IMF subpopulation as well.

The Pyruvate Dehydrogenase Complex

Function of PDH

The pyruvate dehydrogenase (PDH) complex is an intramitochondrial multi-enzyme complex that catalyses the irreversible oxidative decarboxylation of pyruvate with the reduction of NAD^+ to form acetyl-CoA, carbon dioxide (CO_2) and NADH (as reviewed by Sugden et al., 2002). PDH complex links glycolysis to the TCA cycle and contributes to the production of ATP. Therefore, an adequate flux through PDH complex is important in tissues with a high ATP requirement (ie. brain and muscle tissues). The overall reaction of PDH complex is as follows:

$$\text{Pyruvate} + \text{CoA} + NAD^+ \rightarrow \text{Acetyl CoA} + CO_2 + \text{NADH}$$

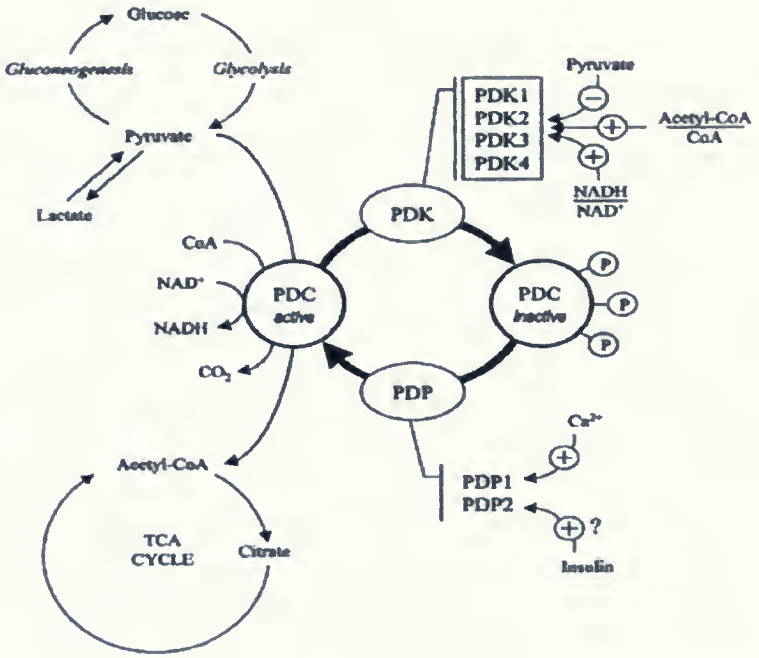
PDH serves as a rate-limiting step in carbohydrate oxidation and it is tightly regulated. The PDH complex reaction is physiologically irreversible and no pathway exists for the conversion of acetyl-CoA to glucose in mammals, and the suppression of the PDH complex is crucial for the conservation of glucose when reserves are scarce. If carbohydrate reserves, including hepatic glycogen, are depleted then pyruvate is required as a precursor for glucose synthesis through gluconeogenesis in the liver. This process allows for the proper regulation of adequate amounts of glucose to sustain proper functioning of important tissues (ie. muscle and brain).

In situations when blood glucose is high, PDH is up-regulated to increase glucose uptake and oxidation by skeletal muscle (Randle et al, 1986). When carbohydrate sources for energy are scarce, PDH is down-regulated and fatty acid metabolism takes over, in order to maximize available glucose for the brain (Randle et al., 1986). It therefore stands to reason that dietary intake has a significant effect on PDH function.

Food-restriction and high-fat diets have been found to decrease PDH activity (Hutson et al., 1978, Kerbey et al., 1982, Orfali et al., 1993). In addition, patients with type II diabetes have diminished skeletal muscle PDH activity. People with Type II diabetes suffer from a decreased muscle glucose uptake and oxidative glucose disposal by skeletal muscle and therefore the down-regulation of PDH and the subsequent suppression of carbohydrate oxidation, which further amplifies this condition (Majer et al., 1998). Therefore, PDH plays such a central role in the balance between fat and carbohydrate oxidation, and is a significant area of research interest.

In mammalian tissues, the PDH complex is regulated by reversible phosphorylation, with the complex being essentially inactive in the phosphorylated state and active in the dephosphorylated state (PDHa) (Santalucia et al., 1992). Activation of the PDH complex via a pair of pyruvate dehydrogenase phosphatases (PDP 1 & 2) (Huang et al., 1998) promotes glucose oxidation, whereas inactivation of the PDH complex via a family of pyruvate dehydrogenase kinases (PDK 1-4) conserves carbohydrate (Johnson & Denton, 2002). Therefore, the PDKs and PDPs regulate glucose oxidation through inhibitory phosphorylation and dephosphorylation of the PDH complex (Sugden, et al., 2003) (Figure 3). The coordinated balance of these two intrinsic enzyme populations will determine what fraction of the complex will be in an active or inactive form at any given time point and will be discussed in more detail later.

Figure 3. **Regulation of the PDH complex.** PDK- Pyruvate dehydrogenase kinase; PDP - Pyruvate dehydrogenase phosphatase. (Adapted from Sugden & Holness, 2003



Structure of PDH

The PDH complex has a molecular mass of approximately 9.5 million Daltons and is composed of 222-226 individual protein molecules (Harris et al., 2002). The subunit composition of the mammalian pyruvate dehydrogenase is summarized in Table 2. The PDH complex is comprised of three major enzymes, PDH, dihydrolipoamide transacetylase, and dihydrolipoamide dehydrogenase. These enzymes are referred to as E1, E2, and E3 respectively (Behal et al., 1993). E1 catalyzes the acetyl-unit formation from pyruvate with the release of carbon dioxide. The acetyl-unit intermediate remains bound to the complex, and is passed from the E1 thiamine pyrophosphate to the E2 lipoyl

domain before being released as acetyl-CoA. E3 participates by regenerating the oxidized lipoyl domain, producing NADH (Behal et al., 1993).

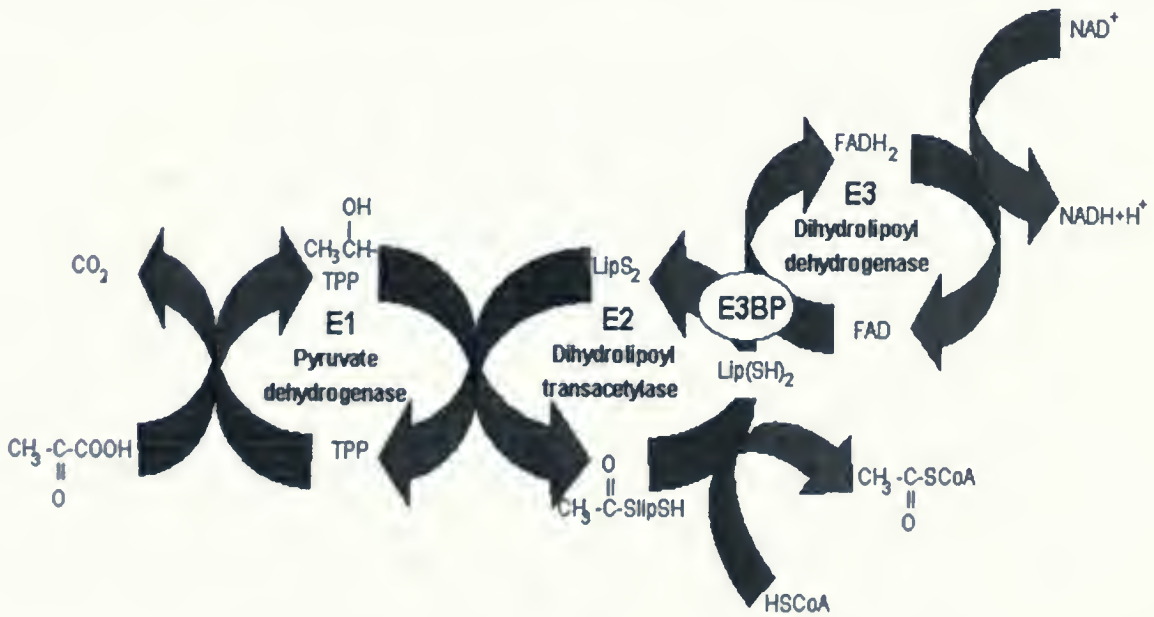
The complex is structured around a large core. This core is proposed to consist of 60 dihydrolipol transacetylase (E2) subunits associated to form a pentagonal dodecahedron (Rahmatulla et, al., 1989). Each E2 complex is comprised of two domains: an inner and outer domain. The inner domain consists of a large oligomeric core and catalyzes the transacetylation reaction (Rahmatulla et, al., 1989). There is also a lipoyl-bearing domain and it is presumably mobile. The complex also contains another lipoyl bearing subunit originally designated as protein X and renamed as the E3 binding protein (Harris et al., 1997). The E1 is less tightly bound and is composed of 30 tetramers, each is composed of two E1-α and two E1-ß subunits (Reed, 1974) (Figure 4). The highly specific regulatory enzymes (PDKs and PDPs) are also associated with the PDH complex.

Table 2. **Subunit composition of mammalian pyruvate dehydrogenase complex.**

Subunit	Size (kDa)	Number of subunits	Size of subunit (kDa)	Subunits per molecule of complex
Complex	~9500			
E1 tetramer E1 α E1 ß	154	 2 2	 41 36	30
E2 core	3100		52	60
E3 dimer & E3 binding protein	110	2	55	12
PDK		4 isoforms PDK1 PDK2 PDK3 PDK4	 48 46 47 46	1-2
PDP		2 isoforms PDP1 PDP2	 61 60	2-3

References: Harris et al., 2002; Gudi et al., 1995; Behal et al., 1993; Linn et al., 1969.

Figure 4. **Overall reaction sequence of the PDH complex.** (Adapted from Behal et al.1993).



Acute Regulation of PDH

The PDKs and PDPs combined catalyze a phosphorylation-dephosphorylation cycle which involves the specific serine residues on the E1-α subunit (Sugden et al., 2003). The phosphorylation of the E1-α subunit renders the PDH complex completely inactive (Linn et al., 1969) and occurs at three specific serine residues (Yeaman et al., 1978). The three specific serine residues are serine-264 (designated at phosphorylation site 1), serine-271 (designated as phosphorylation site 2) and serine-203 (designated as phosphorylation site 3) (Teague et al., 1979). Initial rates of phosphorylation at E1-α were examined in rat heart and it was determined that phosphorylation at site 1 is the most rapid and phosphorylation at site 3 is the least rapid (Sale and Randle, 1981). In addition, a study by Yeamen et al., 1978, determined that site 1 is the major site of inactivation in bovine kidney and is responsible for 60-70% of the inactivation that occurs and site 2 and 3 add to the inactivation. The dephosphorylation has been found to be kinetically ordered, with the dephosphorylation occurring in reverse order and occurring at site 1 last (Sugden et al., 1978). Therefore, increased phosphorylation of sites 2 and 3 impede dephosphorylation of site 1 and activation of the PDH complex. The E1 subunit catalyzes the rate-limiting non-equilibrium reaction, however the E2 and E3 subunits consist of reversible reactions, and therefore are regulated by the concentrations of substrate (pyruvate, NAD^+) and product (acetyl-CoA, NADH).

Acute regulation of PDK is determined by levels of substrate as well as end products. PDK activity is regulated positively by the abundance of acetyl-CoA, NADH and ATP (Denton et al., 1975; Fuller & Randle, 1984), and therefore as stated PDH activity is decreased and carbohydrate oxidation is limited. As well the PDK enzymes

are regulated negatively by the availability of pyruvate, NAD^+, CoASH, ADP, and Ca^{2+} (Fuller and Randle, 1984), all of which downregulate PDK activity, increasing PDH activity and carbohydrate oxidation. PDP1 is activated by the abundance of Ca^{2+} (Severson et al., 1974) and PDP2 is activated by insulin (Behal et al., 1993) which leads to the dephosphorylation and reactivation of PDH and carbohydrate oxidation.

Isoforms of PDK

To date there are four known isoforms of PDK (PDK1, PDK2, PDK3 and PDK 4) that have been identified in mammalian tissues (Gudi et al., 1995, Rowles et al., 1996, Bowker-Kinley et al., 1998). These kinases are expressed in a tissue specific manner and differ substantially in kinetics and allosteric modulation (Bowker-Kinley et al., 1998). PDK1 is found almost exclusively in heart, while PDK2 is expressed in high amounts in all rat tissues except for spleen and lung (Bowker-Kinley et al., 1998). PDK3 is found in very low amounts in rat tissues, except rat testes, which are the only tissues with notable amounts of PDK3 in rodents (Bowker-Kinley et al., 1998). Finally, the PDK4 isoform is most predominantly found in heart and skeletal muscle, with small amounts in lung and liver. The predominant muscle isoforms differ in their sensitivities to mitochondrial effectors (Bowker-Kinley et al., 1998), with PDK2 being sensitive to changes in energy charge and pyruvate, while PDK4 is insensitive to pyruvate. Because of their "specialist properties", PDK2 content increases with endurance training (LeBlanc et al., 2004), while PDK4 content increases with nutritional challenges such as high fat diet, food-restriction and chemically induced diabetes (Peters et al., 2001; Sugden et al., 2000; Wu et al., 1998; Wu et al., 1999).

Isoforms of PDP

It was first suspected that there may be more than one isoform of PDP when it was discovered that PDP activity in different tissues responded differently to various concentrations of Mg^{2+} or Ca^{2+} (Pettit et al., 1972; Severson et al., 1974; Rutter et al., 1989). Through cloning experiments, it was established that the two isoforms (PDP1 and 2) existed (Huang et al., 1998).

Both isoforms are highly specific for the PDH complex, but have differing enzymatic regulators and tissue distribution. These enzymes are both Mg^{2+}-dependent, however the sensitivity of PDP1 to Mg^{2+} ions is almost 10-fold higher than that of PDP2 (Huang et al., 1998). Therefore, PDP2 requires much higher concentrations of intramitochondrial Mg^{2+} for activation (Huang et al., 1998).

Ca^{2+} ions also play a significant role in PDP activation, (Huang et al., 1998; Sugden et al., 2003). PDP1 is more abundant in muscle mitochondria and is stimulated by Ca^{2+} (Huang et al., 1998), while PDP2 is more abundant in liver mitochondria and is Ca^{2+}-insensitive (Huang et al., 1998). An increased concentration of Ca^{2+} stimulates PDP and this binding increases the affinity of PDP for phosphorylated PDH by lowering the apparent K_m for phosphorylated PDH and Mg^{2+} ions, which enhances the dephosphorylation of PDH to occur (Pettit, et al., 1975; Denton, et al, 1975; Behal et al, 1993).

Through PDP, PDH activity is also acutely affected by hormones. Insulin is believed to have a stimulatory effect on PDP, but this mechanism is not well understood (Behal, et al, 1993; Sugden, et al, 2000). Insulin has been observed to mediate PDP stimulation in rat liver and hindlimb preparations (Macaulay et al., 1985). In addition, there have been

more recent discoveries by Huang et al. (1998), that show that since Ca^{2+} ions do not regulate PDP2 activity in rat liver, PDP2 is the likely target of insulin for increasing PDH activity (Huang et al., 1998). Adipose tissue is one of the few tissues where insulin regulation of PDP is dominant, due to the importance of PDH in providing acetyl-units for fatty acid synthesis in this tissue. PDP2 is believed to be the more abundant isoform in rat adipose tissue (Huang et al., 1998). Taken together, these two results strongly suggest that PDP2 is the target isoform for insulin and that PDP2 is indeed insulin-sensitive. Huang et al., (1998) have demonstrated a decreased abundance of PDP2 isoform content in both rat heart and kidney following food-restriction and drug-induced diabetes which can be completely reversed by re-feeding carbohydrates and insulin treatment (Huang et al., 2003).

Finally, recent studies of PDP in rat skeletal muscle have brought forward the findings that PDP activity is specific to fibre type distribution; the predominant PDP isoform in all skeletal muscle fibre types is PDP1, while PDP2 is only detectable in red gastrocnemius (RG) (LeBlanc, et. al. 2006).

Adaptive Regulation of PDH

PDH

The mechanisms of acute regulation of the PDH complex have been well-established through previous research and this form of regulation can be transient and occur within moments. In contrast, the mechanisms involved in adaptive regulation are not as well understood and occur as a result of stable changes in enzyme activity and/or enzyme protein content. The most studied conditions surrounding adaptive regulation of

PDH are those that involve a decrease in carbohydrate utilization and a reciprocal increased reliance on free fatty acid (FFA) metabolism and increased beta-oxidation. These conditions include food-restriction, high fat diets, chemically induced diabetes, and endurance training, where increased PDK activity causes a functional shift in PDH from the active form to the inactive form by means of phosphorylation (Marchington et al., 1990; Sugden et al., 2000; Peters et al., 2001; Wu et al., 1999; LeBlanc et al., 2004). In the short term, an increased reliance on FFA utilization and beta oxidation elicits an increase in both the acetyl CoA/CoA and NADH/NAD^+ ratios, which are responsible for executing the appropriate acute effects as discussed previously. With longer exposure, adaptive increases in PDK activity occur which are independent of the changes in intra-mitochondrial effectors.

There has been limited research into the individual PDH complex components (E1α, E1ß, E2 and E3) in skeletal muscle in response to adaptations such as food-restriction, high fat diets, diabetes, and training. PDH complex components were found to be unaltered after food-restriction (Randle et al., 1986) and have since been used to normalize western blots by other research groups (Peters et al., 2001; Wu et al., 1998,1999). However, LeBlanc et al. (2004) were the first to observe changes in the PDH complex components in primarily SS mitochondria. They determined that 8 weeks of aerobic training increased PDH E1α mitochondrial protein content. In addition, although E1ß was not measured it would be expected that it would increase as well because E1 exists as a functional unit as a tetramer (α_2 $ß_2$). However there was no difference in PDH E2 or PDH E3 binding protein mitochondrial protein content after the training adaptation. Furthermore, the mitochondrial subpopulation distribution of the

PDH complex components is not known. This study will be the first to examine the content of the individual PDH complex components in each of the mitochondrial subpopulations in fed as well as in response to food-restriction, to observe if adaptation occurs differently in each of the mitochondria subpopulations. It is suggested that the content of the individual PDH complex components might be greater in the IMF subpopulation due to the generally higher oxidative capacity of the IMF mitochondria.

PDK

The increased phosphorylation of the PDH complex is a result of an adaptive increase in total PDK activity, which is itself primarily a product of increased PDK4 expression in humans (Peters et al., 2001) and in rats (Sugden and Holness, 2003). PDK4 is up-regulated when there is a sustained change in tissue lipid delivery and/or oxidation in both rats and humans and is considered the "nutritionally sensitive" isoform.

In rat studies PDK4 protein expression increases with food-restriction in heart muscle (Wu et al., 1998), skeletal muscle (Wu et al., 1999), and liver (Sugden et al., 1998; Wu et al., 1998 and 1999). In skeletal muscle, these changes have also been observed as a result of high fat feeding and insulin resistance (Holness et al., 2000; Sugden et al., 2000) and chemically induced diabetes (Wu, et al., 1998). In addition to increased PDK4, food-restriction has also been shown to increase PDK2 expression in rat skeletal muscle (Holness et al., 2000), liver (Sugden, et al, 1998) and kidney (Sugden et al, 1999).

In human studies, similar results were also observed, with an increase in skeletal muscle PDK4 mRNA expression as a result of food-restriction (Spriet et al., 2004) and

both PDK4 mRNA and protein following 6 d of high-fat feeding (Peters et al, 2001). However, PDK2 expression was unaltered in these studies.

In rats, high-fat feeding results are similar to the adaptations of food-restriction. However, 28 d of high fat feeding were necessary to achieve the same changes in PDK activity as was seen with only 48 hr of food-restriction (Orfali et al, 1993). With 28 d high-fat diet, both PDK2 and PDK4 proteins were increased in rat muscle tissue, with the greatest extent in PDK4 (Holness et al., 2000). However, the relative changes in these two isoforms were fibre type specific (Holness et al., 2000). As well, the results of the suppression of PDHa activity after 28 d of high fat-feeding was considerably less than that of the 48 h of food-restriction (Orfali et al., 1993). Therefore, PDK and PDHa activity can be altered faster and to a greater extent with 48 hr food-restriction, rather than a lenghly 28 d high-fat feeding protocol.

There has been focus on fibre-type specific modification of PDK activity and PDK isoform expression in response to high-fat feeding and food-restriction effects. A study examined the effect of high-fat feeding on PDK activity, protein expression and PDHa activity in slow-twitch and fast-twitch skeletal muscle (Holness et al., 2000). It was determined that PDK activity increased similarly in both fibre types, as well changes in PDK2 protein expression increased greater in fast-twitch fibre type and PDK4 protein expression increased greater in slow-twitch fibres. In addition, PDHa activity was greatly decreased in slow-twitch and only slightly decreased in fast-twitch muscle fibres as a result of high-fat feeding (Holness et al., 2000). The observed increase in PDK activity plays a role in the decrease of carbohydrate oxidation and enhanced fat oxidation.

In another study, it was determined that during the basal state PDK activity and PDK4 expression were greater in slow-twitch oxidative (soleus) and fast-twitch oxidative muscle (red gastrocnemius) compared to fast-twitch glycolytic muscle (white gastrocnemius) (Peters et al., 2001). However, food-restriction increased PDK activity in both slow-twitch oxidative (soleus) and mixed fast-twitch (anterior tibialis) muscle (Sugden, et al., 2000), and more specifically in fast-twitch oxidative and fast-twitch glycolytic fibre types (Peters et al., 2001). These results indicated that food-restriction significantly increased PDK4 protein expression in all muscle fibre types, with a greater response in the fast-twitch oxidative fibres (Sugden et al., 2000; Peters et al., 2001). These two studies differed in the length of food-restriction. Peters et al. (2001) examined the effects of 24 h of food-restriction, while Sugden et al. 2000 doubled that time course with 48 h of food-restriction. PDK2 protein was increased after 48 h food-restriction in fast twitch muscle (Sugden et al., 2000) but not after 24 h (Peters et al., 2001). However, although PDK2 protein was unaltered after a 24 h fast, mRNA expression had increased in the fast oxidative fibres, suggesting that the PDK2 isoform adapted more slowly than the PDK4 isoform (Peters et al., 2001). Refeeding for 4 h reversed the effect of 48 h food-restriction on PDK activity and PDK4 expression in both fibre types, however it returned to normal more quickly in slow-twitch fibres (Sugden et al., 2000).

It is clear that PDK activity and isoform content increases as a result of many adaptations including food-restriction, high fat diets, chemical-induced diabetes, and endurance training. Although these adaptations have been studied in great detail, no research has investigated the possible difference that might exist in PDK activity and content in the mitochondrial subpopulations. All research pertaining to adaptations of

PDK activity and content has been studied in mixed mitochondrial preparations which yield primarily SS mitochondria. This study will be the first to examine if food-restriction will alter PDK activity and PDK2 and 4 isoform content in IMF mitochondria to the same extent as SS mitochondria.

PDP

As previously discussed, PDP is the activator of the PDH complex and does so by cleaving a phosphate from PDH to form PDHa. Although there are three sites on the E1 subunit of the complex for phosphate occupancy inactivation of the complex occurs with occupancy of only one site (Sugden et al., 1978). However, further additions of phosphate to the other two sites make it increasingly difficult for reactivation of the complex. This increased site occupancy has been demonstrated with food-restriction and diabetes and is proportional to depleting muscle glycogen stores (Rahmatulla et al., 1989; Sugden et al., 1978).

The increased phosphorylation of the PDH complex in these situations can not be entirely attributed to alterations in PDK activity but also a concurrent down-regulation in PDP activity. In rat heart and kidney, PDP2 isoform has been observed to respond to food-restriction and drug induced diabetes (Huang et al., 1998). While early research in skeletal muscle demonstrated that PDP activity was unaltered with nutritional adaptation (Fuller and Randle, 1984), more recent research in rat red gastrocnemius determined that 48 hr food-restriction did decrease PDP activity (LeBlanc et al., 2006). More specifically this decrease was observed in PDP2, while PDP1 remained unaltered. Therefore, in these tissues, the increased phosphorylation of the complex may be attributed to the combined

action of both the PDH kinase and phosphatase to conserve carbohydrate during periods of food-restriction (LeBlanc et al., 2006).

As well, there is a resistance to reactivation of the PDH complex by PDP with prolonged activation of PDK activity. Some have suggested that this resistance is due to the prolonged increase in PDK activity and multi-site phosphorylation of the complex (Rahmatulla et al., 1989). These findings have been recorded in isolated rat muscle during diet manipulation studies. Holness et al. (1989) saw a proportional relationship between the total time of food-restriction and the reactivation of the complex. It was demonstrated that the longer the food-restriction period the longer the time before reactivation of the complex was observed.

There is limited research pertaining to PDP activity and isoform content in comparison to the abundant research in PDK. Furthermore, there is no research that has investigated the possible difference that might exist in PDP activity and content in the mitochondrial subpopulations. With the recent novel research findings by LeBlanc et al. (2006), the mitochondrial subpopulations will need to be investigated to add to the ongoing understanding of the PDH complex.

Adaptive Oxidative Enzyme Activities in SS and IMF Mitochondria

Training Adaptations in Mitochondrial Subpopulations

Although there is minimal research into dietary adaptations, training adaptations have been an area of intense investigation in skeletal muscle mixed mitochondria. Each training adaptation study has had a different training protocol and investigated different enzyme activities, which has made for a difficult direct interpretation of the collective findings, although trends can be observed among the collective findings. For a summary of the training studies, refer to Table 3.

Studies in human (Chilibeck et al., 2002; Menshikova et al., 2005) and rat (Bizeau et al., 1998; Roussel et al, 2000; Koves et al., 2005; Krieger et al, 1980) skeletal muscle that examined the changes in enzyme activity and oxidative capacity in SS and IMF mitochondria have given various results. While some studies have observed higher increases in IMF mitochondria compared to SS (Krieger et al. 1980; Bizeau et al., 1998; Roussel et. al, 2000), others have observed equal adaptation in the subpopulations (Chilibeck et al., 2002; Menshikova et al., 2005), and one study observed greater oxidative adaptations in the SS compared to the IMF (Koves et al., 2005). Comparison is difficult because the studies have used different training periods ranging from 2 weeks (Roussel et. al, 2000) to 12 weeks or longer (Chilibeck et al., 2002; Krieger et al.; Menshikova et al., 2005); different endpoint determinations [e.g. different TCA cycle enzymes (Bizeau et al., 1998; Koves et al., 2005; Menshikova et al., 2005; Chilibeck et al., 2002; Krieger et al. 1980), ETC enzymes (Bizeau et al., 1998; Roussel et al, 2000; Menshikova et al., 2005; Koves et al., 2005), oxidative capacity (Bizeau et al., 1998; Chilibeck et al., 2023; Krieger et al., 1980), and fat oxidation (Koves et al., 2004)]. In

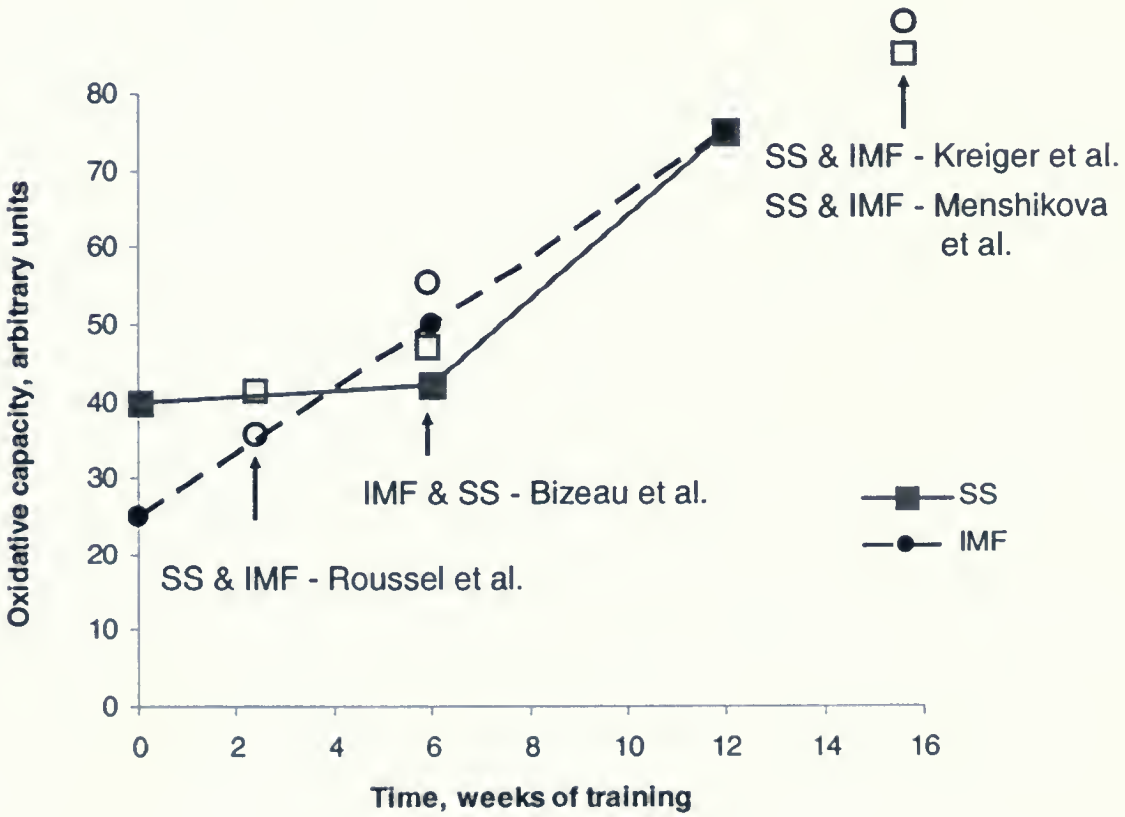
addition, one group highlighted the importance of examining the time course of changes in the subpopulations, since changes in the SS mitochondria were observed later than changes in the IMF (Chilibeck et al., 2002). These reported findings by Chilibeck et al., (2002), are suggested to be the central focus of all of the training findings interms of oxidative capacity of the mitochondrial subpopulations as they appear to fit together across a time course spectrum of adaptability (Figure 5).

Table 3. **Literature review of proposed changes in mitochondrial subpopulations with training.**

Study	Muscle	Measures	Results and Conclusion
Human			
Chilibeck et. al (2002) 12 wk	Human Mixed Muscle (Vastus Lateralis)	SDH activity (through image analysis)	• SDH ↑ in SS during the second half of the training protocol • SDH ↑ in IMF linearly throughout the training protocol ➢ Although SS and IMF adapt at different rates, after a 12 wk training period SDH activity is similar
Menshikova et. al (2004) 16 wk	Human Mixed Muscle (Vastus Lateralis)	SDH	• ↑ in SDH in SS • ↑ in SDH in IMF ➢ ↑ETC activity occurred in both populations equally
Rodent			
Krieger et. al (1980) 16 wk	Female Wistar rats Gastrocnemius	SDH, Rates of Respiration	• ↑↑ in SDH, State II and III respiration in SS • ↑ in SDH, State II and III respiration in IMF and overall + amounts ➢ IMF display a higher oxidative phosphorylation capacity
Bizeau et. al (1998) 6 wk	Female Long Evans rats Soleus, gastrocnemius, quadricep	State II respiration, State III respiration, G3P, SDH, IDH, COX, CS activity	• ↑ in SDH in SS • + State II and III respiration in IMF • ↑↑ in SDH, IDH, in IMF • ↑ in COX equal in both, with + in IMF, • G3P and CS in no significant difference ➢ All enzymes measured (ETC & matrix) were + in IMF, therefore IMF display a higher oxidative phosphorylation capacity and thought to be more important in training adaptation
Roussel et. al (2000) 2 wk	Male Wistar rats Quadriceps	COX F1-ATPase	• +↑ in COX and in F1-ATPase IMF • ↑ in COX and F1-ATPase in SS ➢ IMF display a higher oxidative phosphorylation capacity
Koves et. al (2005) 12 wk	Male Sprawgue Dawley rats Red and white gastrocnemius	CS activity, CPT1, Fatty acid oxidation,	• ↑ CS activity in SS to similar amounts in both • CPT1 similar in both groups and no significant training adaptation • +↑ in Fatty acid oxidation in SS • ↑ in Fatty acid oxidation in IMF ➢ SS has more enhanced fatty acid oxidation after training

NR-Not reported, + - Greater amount after training, ↑ - Increased after training, ↑↑- Greater Increase after training.

Figure 5. **Time course of endurance training adaptations in mitochondria subpopulations.** The diagram below is a time course model of the data from Chilibeck et al., 2002 (closed symbols). Superimposed is the prediction of how studies of different durations might fit into this model (open symbols).



In humans, exercise training over a 12 week period resulted in an early increase in the oxidative capacity of IMF mitochondria, while the SS mitochondria had a latent increase during the final 6 weeks of the training program (Chilibeck et al., 2002). The oxidative capacity in the IMF mitochondria increased linearly throughout the 12 week

training program. This subpopulation is thought to supply energy for cross-bridge cycling (Martin et al., 1987); and they are also thought to be involved in regulating the concentration of sarcoplasmic Ca^{2+} during the contraction-relaxation cycle through sarcoplasmic reticulum active transport (Carafoli et al., 1975). In contrast, SS mitochondria underwent a preferential increase late in the training program and it has been speculated that this could be a result of an adaptive strategy by muscle to increase oxygen delivery from capillaries to muscle fibres due to the proximity of the SS mitochondria to the sarcolemmal membrane and thus the capillaries (Chilibeck et al., 2002). In addition, these authors speculated that the SS mitochondria adapt together with capillarization in response to endurance training programs (Ingjer et al., 1979; Muller et al., 1976). The increase is the SS region is believed to provide energy for active transport of ions across the membrane (Sjodin and Beauge, 1973). However, it has been observed that both subpopulations eventually adapt to a similar extent in SDH activity in humans, with the greatest amount of SDH in the SS mitochondrial subpopulation (Chilibeck et al., 2002). This is in agreement with research conducted by Bourgeois et al. (2004) who determined that with prolonged training SS mitochondria contain greater amounts of SDH activity as well as a greater increase in volume of SS mitochondria are observed. This study was also performed with the use of muscle histology, however the purpose of the study was to investigate a methodological procedure and only contained one subject per condition. Although inconclusive, these findings are interesting and they corroborate the major findings of the Chilibeck et al. (2002).

Although diet is a confounding factor, Menshikova et al., (2005), also found that a 16 wk training intervention led to a highly significant increase in SDH activity in both subfractions. This study is in agreement with Chilibeck et al. (2002) that concluded both

subpopulations adapt to similar extents in ETC activity after prolonged training (12+ wk) in humans. In rats, Krieger et al., (1980), investigated the effects of endurance training in a 16 wk training program and determined that there was a greater increase in SDH activity, state III respiration (oxygen consumption in the presence of ADP) and respiratory control with endurance training in SS mitochondria compared to IMF mitochondria. This appears to be in contrast to the findings of Chilibeck et al., (2002), however due to the extra 4 wks of training, it is possible that the SS mitochondria go on to adapt to a greater extent, possibly due to the latent initial increase observed. As well, in a shorter training study of only 6 wk, results indicated increased oxygen consumption in both mitochondrial populations, with a greater increase in oxygen consumption in IMF mitochondrial populations (Bizeau et al., 1998). Once again, although it appears to contrast, this study may also be in agreement with the previous discussed prolonged training adaptations proposed by Chilibeck et al. (2002) by concluding that IMF mitochondria display a higher oxidative phosphorylation capacity than SS mitochondria early in the training period (6 wk), which would indicate these results are limited to the first portion of the entire prolonged 12 wk training period. A 2 wk training study determined that there was an equal increase in both subpopulations; however a time course for adaptation could not be determined, as only endpoint measurements were obtained (Roussel et al., 2000).

While most training adaptation studies investigated the general oxidative measures, one study examined the actual rate of fat oxidation and how the mitochondrial subpopulations play discrete roles in mediating training-induced increases in skeletal muscle fatty acid oxidation (Koves et al., 2005). They investigated whether the role of malonyl- CoA-resistant carnitine palmitoyltransferase-1 ß (CPT-1ß) would be different in

the mitochondrial subfractions. The addition of malonyl-CoA inhibited oxidation rates in the SS mitochondria by 89% but only 60% in the IMF mitochondria. Endurance training for 10 wk increased palmitate oxidation rates by 100% in the SS mitochondria and only 46% in the IMF mitochondria.

The Koves et al., (2005) study therefore demonstrated that distinct mitochondrial subpopulations display metabolic differences that are dependent upon training status. Furthermore, the results support the idea that the SS mitochondria and the IMF mitochondria might perform distinct bioenergetic functions; however this study was the first to investigate the roles that the mitochondrial subpopulations play in fatty acid oxidation and furthermore report that the SS mitochondria are predominately responsible for the regulation of fat oxidation due to the SS mitochondria being more sensitive to malonyl-CoA inhibition of fatty acid oxidation.

Dietary Perturbation in Mitochondrial Subpopulations

There has been limited research as to the influence of dietary perturbations among the mitochondrial subpopulations. One such preliminary study, by Johnson et. al., (2006) examined if differences in mitochondrial proton leak and mitochondrial energy metabolism, in relation to aging, existed after varying amounts of food-restriction in rats. They investigated the influence of different percentages of food-restriction (25%, 50%, 75% and total food-restriction) compared to control rats over a 3 d period. They determined that mitochondrial proton leak was significantly decreased in the 50% food-restriction group in both subpopulations, but was unaffected in any of the other groups. Further, there was a decrease in state III respiration rates (with pyruvate/malate) and proton leak in SS mitochondria with 50% food-restriction, while IMF mitochondrial

respiration rates remained unchanged. This suggests lower maximal rates of ETC activity, but only in SS mitochondria after 50% food-restriction. However, they reported no changes in CS activity as a result of any of the levels of food-restriction. They concluded that 3 d 50% food-restriction was sufficient to lower mitochondria energetic metabolism in both mitochondrial subpopulations. However, it is unclear why 100% food restriction would not further affect the proton leak. Overall, it was speculated that the rapid adaptation of the SS mitochondria may contribute to the benefit of short term food-restriction. As well, due to the location of the SS mitochondria, and their proximity to the cell membrane, they may be more sensitive to adaptations in substrate availability.

Summary of Adaptive Changes

Taken together, all the research in endurance trained and food-restricted human and rat muscle bring forth many different findings based on various experimental protocols.

When interpreting training adaptation in mitochondrial subpopulations, it is important to consider the length of the training protocol, as Chilibeck et al. (2002) stated that the mitochondrial subpopulations adapt differently throughout the prolonged training period, but they adapt to the same extent after the prolonged training period. Most studies utilized training periods of greater than 12 wk; however some studies examined adaptations of less than 10 wk of training. It is also important to note that rat studies typically determined that IMF mitochondria display a higher oxidative phosphorylation capacity; however the vast amount of these studies were examined for less than a 12 wk training period. Therefore, these results are in agreement with the prolonged training results determined by Chilibeck et al. (2002) which surpassed a 12 wk training period and

noted that in the first half of the training period (<6 wk) IMF mitochondria exhibited a greater increase in SDH activity while SS mitochondria were reluctant to change until the second half of the training period (>6 wk). It is believed that both mitochondrial subpopulations adapt to the same extent after prolonged training, however the differential rate in which they adapt is variable. It is clear that there are disparate results for the effect of changes in muscle contractility on changing oxidative enzymes and capacity in the subpopulations. From these collective studies, it is difficult to predict whether adaptations with food-restriction would be different in the two subpopulations for other oxidative enzymes such as PDH and PDK.

In addition, minimal dietary perturbation work has been conducted in the mitochondrial subpopulations, but the results to date suggest that the SS and IMF mitochondria would adapt differently to nutritional challenges. It is believe that the total PDH activity will be greater in the IMF mitochondria subpopulation because they generally have higher oxygen consumption and are considered more important in providing energy needed for muscle contraction. As well, because the SS mitochondria subpopulation are thought to play a more important role at rest and regulation of oxidation during adaptations in substrate availability and therefore it is thought that food-restriction would effect this subpopulation the most. More specifically, it is expected that a greater increase in PDK2 and 4 isoform content will occur in the SS mitochondria.

There are currently no studies that have examined the mitochondrial subpopulation distribution of the PDH complex in terms of activity and protein content of various aspects of the complex during basal state or following food-restricted conditions. This study will be the initial study into this area of research. Therefore, the purpose of this thesis is to determine the total PDH and PDK activity, as well the protein content of

PDK2 and 4, and PDH complex components in the two different mitochondrial subpopulations during basal state conditions and following 48 h of food-restriction to determine whether differences exist in the adaptive responses of the PDH complex.

CHAPTER 3

STATEMENT OF THE PROBLEM

1. To date, there is limited research into the nutritional adaptations studied in the SS and IMF mitochondria subpopulations.

2. All research pertaining to dietary adaptation of PDK activity and content have been studied in preparations which yield primarily subsarcolemmal (SS) mitochondria.

3. It is not known whether food-restriction will increase PDK activity and PDK2 and 4 isoform content to the same extent in IMF mitochondria compared to previous research conducted in SS mitochondria.

4. The mitochondrial subpopulation distribution of total PDH activity and the PDH complex components are currently unknown.

Purpose

The purpose of the current research was to analyze if differences exist in PDK activity and isoform content in SS and IMF mitochondria in response to 48 h food-restriction. The secondary purpose was to analyze mitochondrial subpopulation distribution of the PDH complex components and total PDH activity.

Hypotheses

1. Total PDH activity would be greater in the IMF mitochondria populations because IMF mitochondria generally have higher oxygen consumption and are considered more important in providing energy needed for muscle contraction.

2. With 48 hour food-restriction, a greater increase in PDK2 and 4 isoform content would occur in the SS mitochondria. This mitochondrial subpopulation is thought to play a more important role at rest, and in the regulation of fat oxidation.

CHAPTER 4

METHODS

Subjects

Sixteen male Sprague Dawley rats (*Rattus norvegicus*) (Charles River, St. Constant, PQ), with a weight of 200-300g were used for this experiment. The animals were housed in a controlled environment with a 12:12 h reversed light-dark cycle. They were fed Purina Rat Chow (Ralston Purina Co.) *ad libitum* until food withdrawal (for the 48 h food-restricted group). All experimental interventions were formally approved by the Brock University Animal Care and Utilization Committee and conformed with all of the Canadian Council for Animal Care (CCAC) guidelines.

Experimental Protocol

Half of the rats (n = 8) were fed immediately up until muscle harvest, while the other half (n = 8) were 100% food-restricted for 48 hr prior to skeletal muscle harvest and mitochondrial extraction. Animals were paired on an experimental day and one fed and one food-restricted were extracted together. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg/100 g body wt.) and the red gastrocnemius (RG) muscles were excised immediately. A portion of the muscle was frozen immediately in liquid nitrogen for later analysis of whole muscle citrate synthase activities. The remaining portion was used immediately for the extraction of SS and IMF mitochondria. Mitochondrial fractions were used to measure PDK activity, as well as citrate synthase activities and western blotting of PDK2 and 4 isoforms and PDH complex components.

Blood Analyses

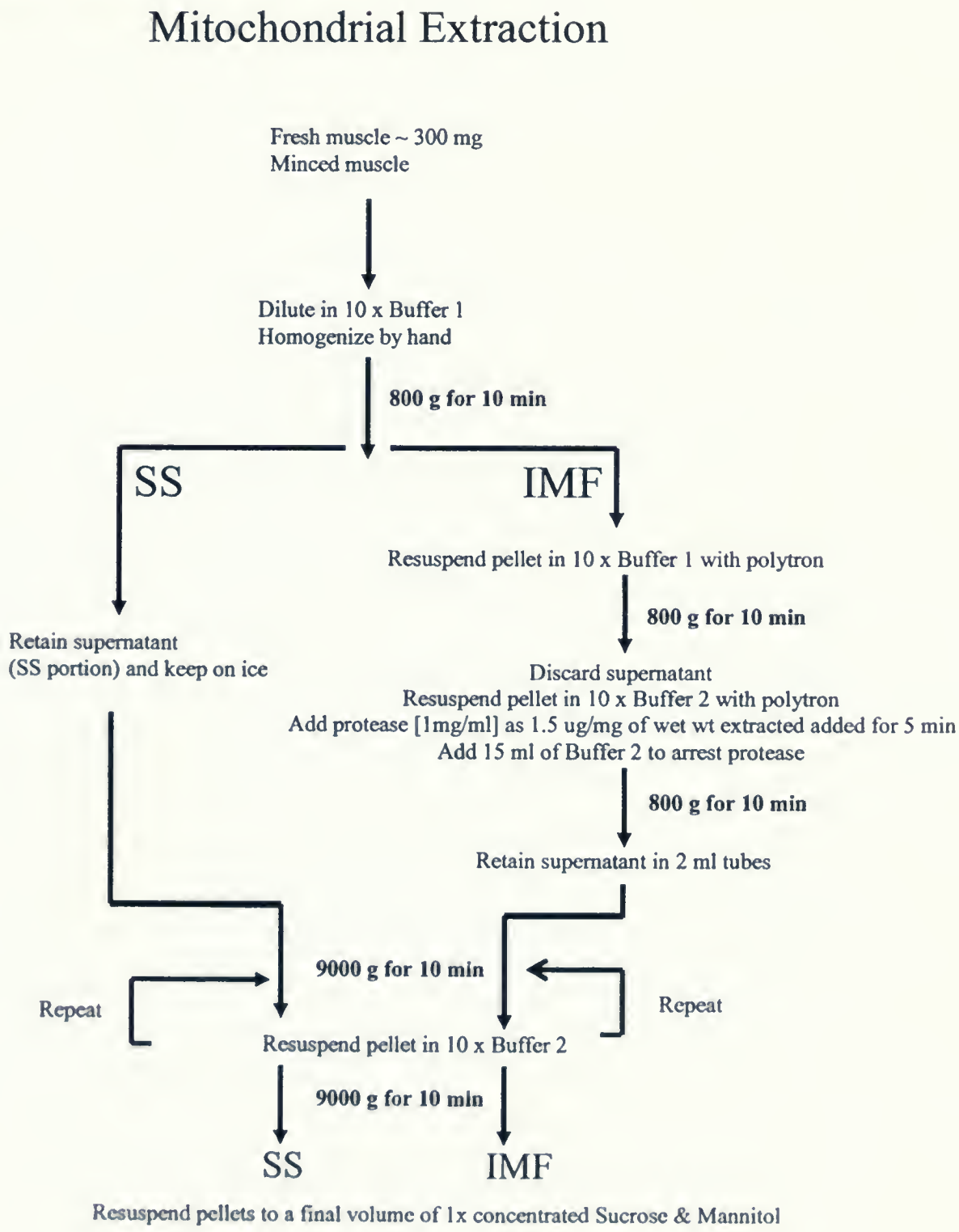
After muscle excision, 2 ml of blood was drawn through an intracardiac puncture with a heparinized syringe. An aliquot (50 µl) of whole blood was deproteinized in a 1:5 ratio with 6% perchloric acid for analysis of ß-hydroxybutyrate, glycerol, and glucose (Bergmeyer et al., 1974). A second portion of whole blood was centrifuged, and an aliquot of plasma (250 µl) was stored at -80° for later analysis of free fatty acids using a Wako nonesterified fatty acid C test kit (Wako Chemicals, Richmond, VA). The remaining plasma was used to determine insulin using a Linco Sensitive Rat Insulin test kit (St.Charles, MO).

Mitochondrial Extractions

Intact mitochondria were extracted by differential centrifugation as originally described by Palmer et al., (1977), with minor modifications made to methods described by Campbell et al., (2004) as displayed in Figure 6. Briefly, minced muscle was diluted 10-fold in Buffer 1 (100 mM KCl, 50 mM Tris-HCl, 5 mM $MgSO_4$, 5 mM EDTA, pH 7.4) and homogenized on ice by hand with the use of a glass potter homogenizer. Samples were homogenized to release the SS mitochondria, leaving the IMF mitochondria between the myofibrils. The SS mitochondria were isolated from the myofibrils by centrifugation at 800 g for 10 min. The SS mitochondria were then pelleted from the supernatant at 9000 g for 10 min. The SS pellets were then washed twice in Buffer 2 (Buffer 1 containing 1 mM ATP) and centrifuged at 9000 g for 10 min and resuspended to a final volume of 1x concentrated sucrose and mannitol solution per mg muscle weight. The myofibrillar pellet containing the IMF mitochondria was rehomogenized using a polytron (Fisher Brand, Power Gen Model 125) set on a low speed for 3 cycles of

5 s on and 55 s off. This was done in order to resuspend the pellet and recentrifuged at 800 g for 10 min, basically a wash to get any residual SS subpopulation away. The supernatant was then discarded analysed to determine that no mitochondrial content was left, then it was discarded. The remaining pellet was diluted in 10-fold Buffer 2 and treated with protease (P8038, Sigma, Canada) (1.5 μl/mg muscle wt.) for exactly 5 min. To dilute the protease 15 ml of Buffer 2 was added and the resulting solution was centrifuged at 800 g for 10 min to remove debris and large particles. The pellet was resuspended in 10-fold Buffer 2 and recentrifuged at 800 g for 10 min. The supernatants were collected and centrifuged at 9000 g for 10 min, while the pellet containing largely myofibrils was discarded. The IMF pellet was washed twice in Buffer 2 and recentrifuged at 9000 g for 10 min and resuspended to a final volume of 1x concentrated sucrose and mannitol solution per mg muscle wt. The resulting SS and IMF mitochondria were then used for further analysis (Figure 6).

Figure 6. **Mitochondrial Extraction.**



Mitochondrial and Total Homogenate CS Activity

Citrate synthase (CS) activity was measured with a spectrophotometer using the enzymatic method to link the release of CoASH to the colourometric agent dithiobis-2-nitrobenzoate (DTNB), as previously described (Srere et al., 1969).

Oxaloacetate + acetyl CoA --------- ***citrate synthase*** --------> CoASH + Citrate

CoASH + DTNB $_{\text{pale yellow}}$ ----------> CoA + DTNB $_{\text{dark yellow}}$

Changes in absorbance were followed at 412 nm. Calculation of units of activity involved the use of the extinction coefficient 13.6. The limited pH range for this assay was 7.4 - 9.0 (Srere et al., 1969). The measurement of CS involved 5 µl of the mitochondrial suspension that was diluted 20-fold with the final sucrose and mannitol buffer and divided into two fractions: extramitochondrial CS (CS_{em}) and total suspension CS (CS_{ts}). The total muscle homogenate CS (CS_{hm}) involved 10-15 mg of muscle sample ground in glass potter homogenizer in 100-fold of homogenizing buffer (0.1 mM KH_2PO_4 + 0.05% BSA).

CS_{em} was measured in the intact mitochondrial preparation as an indicator of the quality of intact mitochondria since any CS activity found outside the mitochondria would have resulted from mitochondrial membrane damage. The preparation of CS_{ts} and CS_{hm} suspensions involved freeze-thawing them twice to break the mitochondrial membranes and release all CS activity. Activity was measured in a 300 µl cuvette with 150 µl of 100 mM Tris buffer, 25 µl of 1mM DTNB, 40 µl of 3 mM acetyl CoA, 10 µl of 10% Triton and 10 µl of mitochondrial suspension or total homogenate. For determination of CS_{em}, 160 µl of 100 mM Tris buffer was used and Triton was not added

to ensure good membrane integrity. To initiate the reaction 15 µl Oxaloacetate (10 mM) was added.

CS activities in the mitochondrial suspensions: extra mitochondrial (CS_{em}) and total suspension (CS_{ts}), as well as total muscle homogenate (CS_{hm}) were used to calculate mitochondrial recovery and quality of the mitochondrial preparations (Peters et al., 1998). The following are the calculations that were used to determine percent intact mitochondria and percent fractional recovery.

% Intact Mitochondria (quality of mitochondria extracted) = $100 \times (CS_{ts} - CS_{em}) / CS_{ts}$

% Fractional Recovery (recovery of intact mitochondria) = $100 \times (CS_{ts} - CS_{em}) / CS_{hm}$

Incubation of Mitochondria for PDK Activity

The final SS and IMF mitochondria suspensions (50 µl/sample) were diluted with 250 µl of buffer containing 10 µM carbonyl cyanide *m*-chlorophenylhydrazone, 20 mM Tris-HCl, 120 mM KCL, 2 mM EGTA, and 5 mM potassium phosphate (monobasic) (pH 7.4) and incubated for 20 min at 30°C, which drove the ATP concentration to zero in the intact mitochondria by forcing the mitochondria to respire due to uncoupling. This also resulted in the complete conversion of PDH to the active form (total PDH activity) as previously described (Fatania, et al., 1986; Peters et. al, 1998). These suspensions were then pelleted at 7,000 g for 10 min and stored in liquid nitrogen for future analysis of PDK activity.

Analysis of PDK Activity

PDK activity was determined as previously described (Peters et al., 1998). The mitochondria pellets were resuspended in ~300 µl of a buffer containing 30 mM KH_2PO_4, 5 mM EGTA, 5 mM dithiothreitol, 25 µg/ml oligomycin B, 1.0 mM tosyl-lysyl-chloro-methyl-ketone (protease inhibitor), 0.1% triton (detergent), and 1% bovine serum albumin (pH 7.0) and freeze-thawed twice to ensure that all mitochondria were ruptured. The suspension was warmed at 30°C, and two aliquots of each of the suspensions were diluted 1:1 in a buffer containing 200 mM sucrose, 50 mM KCl, 5 mM MgCl, 5 mM EGTA, 50 mM Tris-HCl, 50 mM NaF, 5 mM dichloroacetate, and 0.1% Trition (pH 7.8) for further analysis of PDHa activity. Sodium floride (NaF) was used to inhibit the phosphatase (PDP) reaction from occurring and dichloroacetate was used to inhibit the kinase (PDK) reaction from occurring, thus "locking" the PDHa activity. This has been used for more than a decade and has proven effective in sensitive measurement of PDHa activity (Constantin-Teodosia et. al, 1991). This point represents "zero time" or "total PDH'. To initiate the reaction magnesium-ATP (Mg^{+2}-ATP) was added to the remaining suspension to bring the concentration to 0.3 mM, and timed samples were taken every 30 sec for 3 min, as previously described (Fatania et al., 1986; Peters et al., 1998; Vary et al., 1991). The samples were diluted 1:1 in the sodium fluoride-dichloroacetate buffer described above. Samples were stored on ice until further analysis of PDHa activity through radioisotopic measurement of acetyl-CoA production as described previously (Constantin-Teodosiu et al., 1991; Putman et al., 1993). PDK activity has been reported as the apparent first-order rate constant of the inactivation of PDH (min^{-1}), or the slope of ln[%[PDHa activity (with ATP addition)]/total PDH (without ATP addition)] vs. time (Fatania et al., 1986; Vary et al., 1991). The slope was determined using linear

regression analysis. Previous work had determined that there was no appreciable loss of activity to be expected in the absence of ATP over the 2- to 3-min experiment (Peters et al., 1998).

Analysis of PDHa Activity

PDHa activity was determined by adding 15 µl of stopped mitochondrial preparation from PDK assay homogenate to a reagent mixture at 37°C. The reagent mixture contained the necessary coenzymes (3 mM NAD, 1 mM CoA, and 1 mM TPP) to drive the PDHa reaction as formerly outlined (Putman et al., 1993). Pyruvate was added to the reagent mixture to initiate the reaction 15 µl and 70 µl aliquots were removed at precise timed intervals (1, 2, and 3 min) and placed into 40 µl of 0.5 N PCA to stop the reaction. Samples were neutralized with 1 M K_2CO_3 after 5 min following acidification with PCA. The neutralized extracts were then stored at –20°C for subsequent analysis of acetyl-CoA using a radio-isotopic method, as previously described (Cederblad et al., 1990). Linear regression analysis of acetyl-CoA vs. time plots was performed to determine reaction rates for PDHa.

Mitochondrial Protein Concentration

Mitochondrial protein concentration in the prepared sub-populations was determined with the use of Bio-Rad Protein Assay (Bio-Rad Laboratories, International). The Bio-Rad reagent used for this assay was diluted 5 fold: 1 ml BioRad to 4 ml dH_2O. Bovine Serum Albumin (BSA) (775 827, Roche Diagnostics, Indianapolis, IN, USA) was used as the protein standard in 5 differing dilutions (1.0, 0.5, 0.25, 0.125, and 0.05 mM concentrations) plus one blank (0 mM).

Samples were prepared by adding 1µl of mitochondrial sample diluted in 9µl of sucrose and mannitol solution and 200µl of reagent. Standards and samples were read on Ultraspec 2100 pro spectrophotometer at 595 wavelength (Amersham Pharmacia Biotech, Cambridge, United Kingdom).

Western Blotting

Mitochondria were diluted to a final protein concentration of 1.0 µg/µl in 50 mM Tris-HCL, pH 6.8, containing 2% SDS, 0.1 M dithiothreitol, 0.1% bromophenol blue, 10% glycerol, and a protease inhibitor cocktail (1 mM benzamidine, 0.1 mg/ml trypsin inhibitor, 0.1 mM tosyl-lysyl-phenylmethlketone) as previously described (Peters et al., 2001) with minor modifications. Samples were solubilized by boiling for 5 mins and then cooled for 5 mins. Standard SDS-PAGE electrophoresis was performed with 4% stacking and 12% separating gel for PDK 2 and 4 and 10% separating gel for PDH components determination (5 µg of mitochondrial protein per lane for PDK 2 and 4 and purified proteins, and 2.5 µg of mitochondrial protein per lane for PDH components). Electrophoretically separated proteins were transferred onto Protran nitrocellulose membranes (0.45 µm pore size, Schleicher & Schuell, NH, USA) using the tank method of electrophoretic transfer (Bio-Rad, CA, USA) with a transfer buffer containing 34.8 mM Tris base, 31.2 mM glycine, 0.03% (w/v) SDS, and 20% (v/v) absolute ethanol as previously described (LeBlanc et al., 2004). Membranes were incubated in TBST buffer (20mM Tris base, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) with 5% (w/v) non-fat dry milk for 1 h to block all non-specific binding sites. Membranes were then incubated for 1 h in 10 ml 5% milk-TBST containing 50µl polyclonal antiserum against PDK isoforms PDK 2 & 4 or PDH complex components E1α, E1ß, E2 and E3 (gifts from Dr.

R.A. Harris, Indiana University School of Medicine, Indianapolis, Indiana). The membranes were washed with TBST and then incubated for 1 h in 10 ml 5% milk-TBST containing 0.5µl goat anti-rabbit IgG (HRP-conjugated horseradish peroxidase, Santa Cruz Biotechnology, CA, USA) as described by LeBlanc et. al. 2004. Membranes were again washed with TBST and incubated for 5 min with extremely sensitive enhanced chemiluminescent substrate Chemiglow (Alpha Innotech, San Leandro, CA, USA) to develop the immunoblots. Light from the antibody-antigen complexes were visualized with the use of Fluro Chem 5500 (Alpha Innotech, San Leandro, CA, USA). Relative densities were quantified and results are expressed as the intensity of the band in arbitrary units.

Data Analysis and Statistics

All data was presented as means ± SE. For muscle analysis, a two-way ANOVA was employed for treatment (fed and food-restricted) and mitochondrial subpopulation (SS, IMF). Tukey's *post hoc* test was used to identify where significant differences occurred. For blood analysis, an unpaired t-test was employed (fed and food-restricted). Significance was accepted at $p<0.05$.

CHAPTER 5

RESULTS

Animal Characteristics

The rats ranged from 24-30 d old at the time of the experiment. The body weight of the rats did not differ between the fed (263 ± 5g) and food-restricted (236 ± 8g) groups prior to intervention. As well the sample weight of red gastrocnemius muscle samples did not differ between fed and food-restricted groups (Table 4). We had observed that this food-restriction affected mitochondrial yield (unpublished observations).

Table 4. **Rat and muscle sample characteristics.**

Fed	Rat Weight (g)	Sample Weight (mg)	FR	Rat Weight (g)	Sample Weight (mg)
1	257	240.4	1	229	246
2	249	221.6	2	226	226
3	269	223.4	3	255	211
4	272	240.5	4	264	244
5	239	219.1	5	201	235
6	260	234.4	6	212	234
7	280	256.8	7	253	237
8	280	235.2	8	260	259
Mean	263	245.7	Mean	236	248
SE	5	13.2	SE	8	12.5

FR = food-restricted, SE = standard error.

Blood Parameters

Blood glucose concentrations were unaltered between fed and food-restricted rats (7.8 ± 0.7 (FED) vs. 6.4 ± 0.7 (FR)). Plasma insulin concentrations were significantly lower with food-restriction (40.2 ± 7.5 (FED) vs. 14.6 ± 2.9 (FR)) ($p<0.01$). (Table 5).

Plasma FFA were increased 1.5-fold with 48h food-restriction (0.31 ± 0.02 (FED) vs. 0.48 ± 0.06 (FR)) ($p<0.05$). There was a trend towards increased ß-hydroxybutyrate ($p=0.09$) observed with food-restriction (0.38 ± 0.15 (FED) vs. 0.92 ± 0.36 (FR)). No changes were demonstrated in glycerol concentrations between fed and food-restricted groups (12.1 ± 3.5 (FED) vs. 10.8 ± 2.7 (FR)) (Table 5).

Table 5. **Plasma insulin and FFA concentrations, and whole blood β-hydroxybutyrate, glycerol, and glucose concentrations in fed and food-restricted rats.**

	Fed	FR
Insulin (mIU/L)	**40.2 ± 7.5**	**14.6 ± 2.9***
FFA (mM)	**0.31 ± 0.02**	**0.48 ± 0.06***
β-HB (mM)	**0.38 ± 0.15**	**0.92 ± 0.36 (p=0.09)**
Glycerol (µM)	**12.1 ± 3.5**	**10.8 ± 2.7**
Glucose (mM)	**7.8 ± 0.7**	**6.4 ± 0.7**

Values are means ± SE. *significantly different from fed (p<0.05). FR = food-restricted; FFA = free fatty acids; ß-HB = beta hydroxybutyrate, SE = standard error.

Mitochondrial Preparations

The mitochondrial recovery was greater in the fed SS subpopulations (16.4 ± 2.9%) than in the fed IMF subpopulations (5.0 ± 0.9%). As well, the food-restricted SS subpopulations recovery (9.6 ± 1.0%) was greater than the food-restricted IMF subpopulations recovery (4.7 ± 1.0%). The interaction effect of the mitochondrial subpopulations recoveries (SS vs IMF) during the adaptations (fed vs food-restriction) was significant (p=0.01). The mitochondrial qualities of the preparations (percentage of intact mitochondria) were 82 ± 3% in SS subpopulations and 82 ± 2% in IMF subpopulations for fed and were greater than the food-restricted qualities which were 76 ± 4% in SS subpopulations and 75 ± 3% in IMF subpopulations (Table 6).

Table 6. **Mitochondrial recovery and quality of preparations.**

	Fed	FR
SS mitochondria recovery	**16.4 ± 2.9%**	**9.6 ± 1.0%***
IMF mitochondria recovery	**5.0 ± 0.9%†**	**4.7 ± 1.0%†***
SS mitochondria quality	**82 ± 3%**	**76 ± 4%***
IMF mitochondria quality	**82 ± 2%**	**75 ± 3%***

Values are means ± SE. *significantly different from fed (p<0.05). †significantly different from SS (p<0.001). FR = food-restricted, SE = standard error.

Extracted Mitochondrial Protein Yield

There was a higher mitochondrial protein concentration found in the fed group (8.5 ± 1.4 mg/ml) compared to the food-restricted group (7.0 ± 1.0 mg/ml) (p=0.04); and a higher level of extracted mitochondrial protein in the SS preparations (5.09 ± 0.38 mg/ml) compared to the IMF preparations (2.68 ± 0.45 mg/ml) (p<0.001).

CS Activity

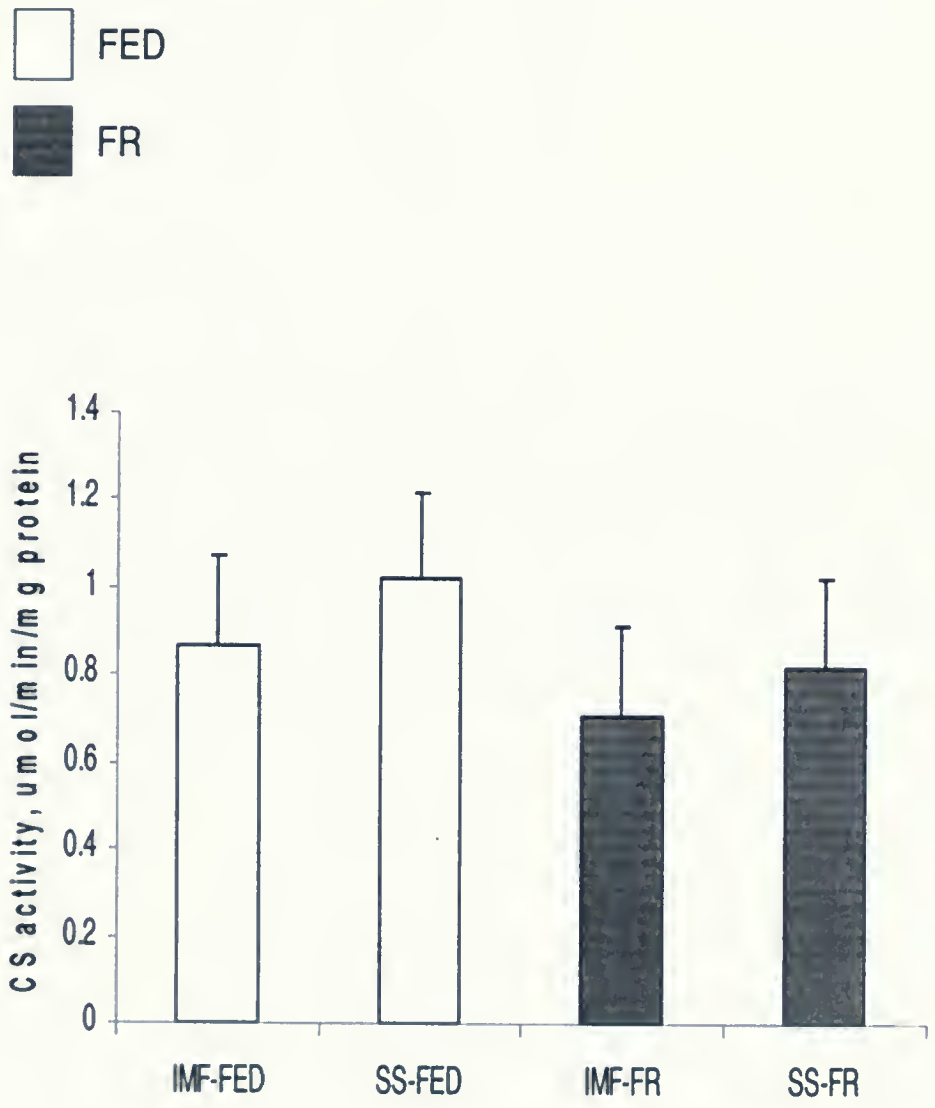
CS was unaltered in total muscle homogenates (Hm) with food-restriction, and was similar to other studies at approximately 30 μmol/min/g wet weight (Table 7). However, when CS activity in the mitochondrial preparations was expressed as μmol/min/ml of mitochondrial extract (representing per g of original muscle sample), SS mitochondria had higher CS activity in the fed state compared with food-restriction (Table 7). Most importantly, there was no significant difference in CS activity between the subpopulations when expressed per mg of mitochondrial protein (Figure 7).

Table 7. **Citrate synthase activities for fed and FR subpopulations (SS and IMF) (μmol/min/g tissue wet weight).** Standard error (SE) given. There was no significance between values for fed vs. food-restricted rat muscles or between subpopulations with fed or food-restriction.

	Fed	FR
Hm	**30.5 ± 3.3**	**31.4 ± 2.3**
SS mitochondria	**5.6 ± 0.8**	**3.6 ± 0.4**
IMF mitochondria	**2.5 ± 0.7**	**1.8 ± 0.4**
Combined mitochondria	**8.17 ± 2.2**	**5.36 ± 1.3***

Values are means (n=9) ± SE. *significantly different from fed (p<0.05). FR = food-restricted, SE = standard error, Hm = total homogenate.

Figure 7. **Citrate Synthase activity µmol/min per mg mitochondrial protein in IMF or SS mitochondria from rats fed (FED) or IMF or SS mitochondria from rats food-restricted for 48 h (FR)**



PDK and Total PDH Activity

Possibly due to methodological problems, there was no measurable PDH activity in the IMF mitochondria subpopulations, making it impossible to compare total PDH activity and PDK activity between the SS and IMF mitochondria. Therefore, these analyses were abandoned.

PDH Complex Proteins

PDK 2 and 4 Protein

PDK2 was ~2.5-4 fold higher in the SS mitochondria compared to the IMF mitochondria ($p=0.001$), however PDK2 was unaltered with food-restriction (Figure 8).

PDK4 content was ~3-5 fold higher in the SS mitochondria compared to IMF mitochondria ($p=0.001$), and increased with food-restriction ~3-4 fold in both sub-populations ($p<0.001$) (Figure 9).

Figure 8. **PDK2 protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 5 ug protein per lane. *, main effect SS mitochondria contained significantly higher PDK2 in both FED and FR rats.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 12% separating gel for PDK 2 determination (5 µg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDK2. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.

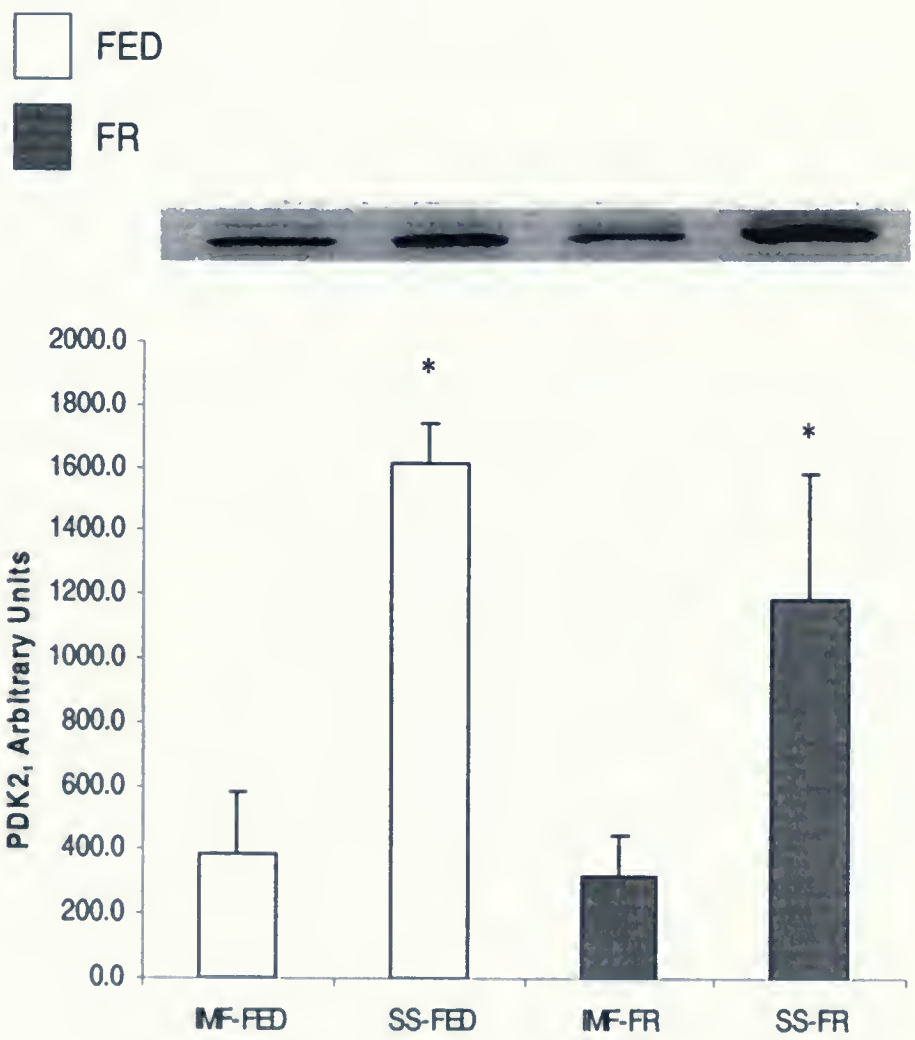
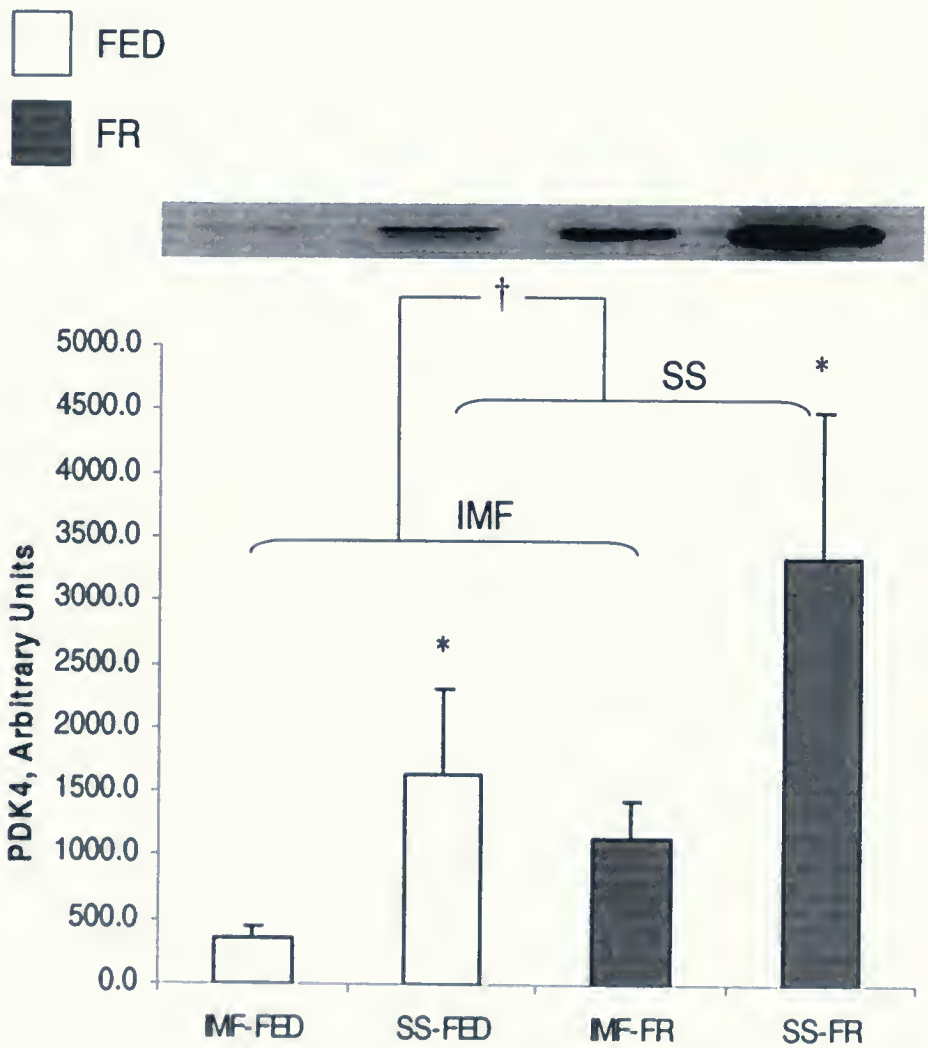


Figure 9. **PDK4 protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 5 ug protein per lane. *, main effect SS mitochondria contained significantly higher PDK4 in both FED and FR rats. †, main effect significantly higher PDK4 following food restriction in both IMF and SS mitochondria.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 12% separating gel for PDK 4 determination (5 μg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDK4. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.



PDC Complex Components

There was no significant difference reported between the SS and IMF subpopulations in the PDC E1α, E2 or E3 components (Figures 10, 12 and 13). However, PDC E1ß displayed a trend towards higher content in SS mitochondria vs. IMF mitochondria ($p=0.09$) (Figure 11). As well there were no significant changes in these PDH complex components with food-restriction.

Figure 10. **PDC E1α protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 2.5 ug protein per lane.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 10% separating gel for PDC E1α determination (2.5 μg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDC E1 alpha. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.

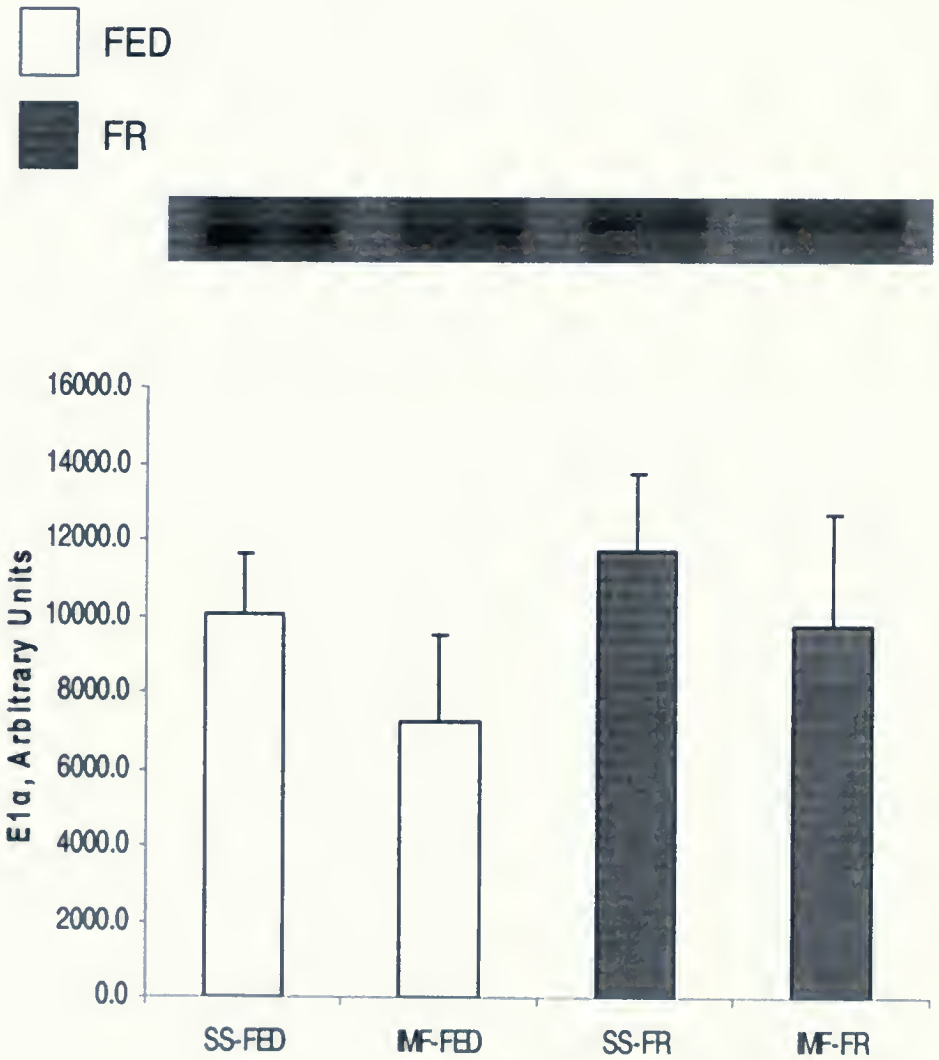


Figure 11. **PDC E1ß protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 2.5 ug protein per lane.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 10% separating gel for PDC E1ß determination (2.5 µg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDC E1 beta. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.

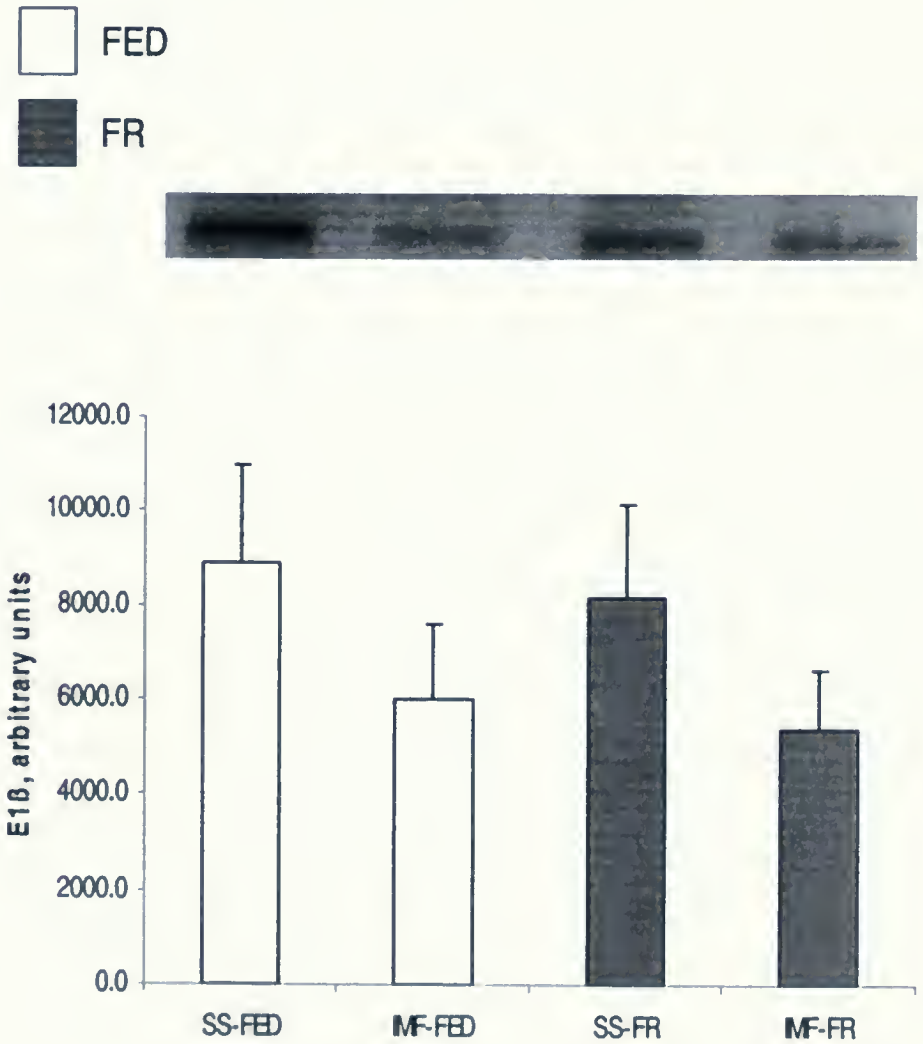


Figure 12. **PDC E2 protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 2.5 ug protein per lane.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 10% separating gel for PDC E2 determination (2.5 µg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDC E2. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.

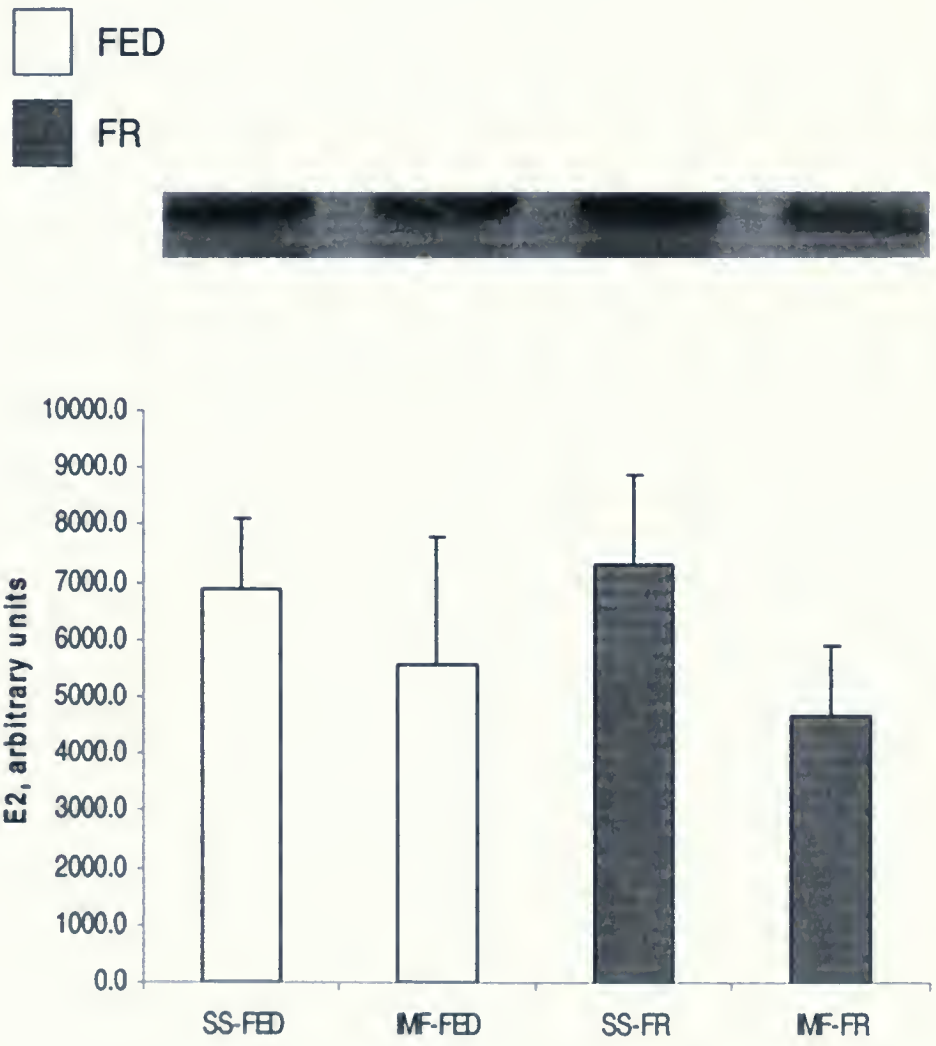
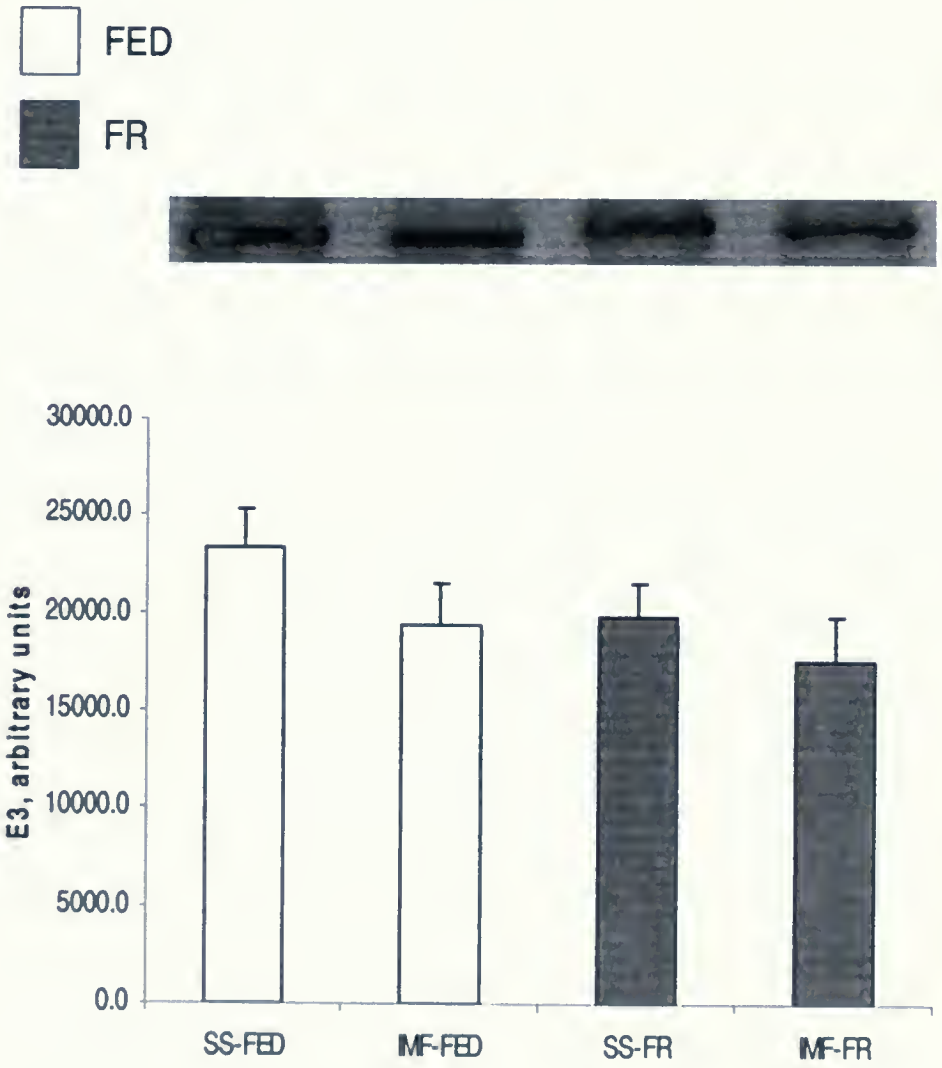


Figure 13. **PDC E3 protein content of red gastrocnemius (RG) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from fed and 48 h food-restricted (FR) rats. Western blotting analysis was performed on 2.5 ug protein per lane.** Standard SDS-PAGE electrophoresis was performed with 4% stacking and 10% separating gel for PDC E3 determination (2.5 µg of mitochondrial protein per lane). Immunodection was using polyclonal antisera directed against PDC E3. Blots were exposed with Chemiglow and visualized with the use of Fluro Chem 5500. Relative density was quantified and results are expressed as the intensity of the band in arbitrary units.



CHAPTER 6
DISCUSSION

This study describes the differences that exist in PDK isoform protein content in SS and IMF mitochondria in response to 48 h food-restriction. As well, the subcellular distribution of the PDH complex components were examined. This study was the first to examine differences in protein content in PDK2 and 4 isoforms and PDC components among the subpopulations in response to dietary perturbation. The major finding was the distribution differences among the mitochondrial subpopulations with greater PDK2 and 4 isoform content observed in the SS subpopulations. The greatest isoform content was found to be in the SS subpopulation in the 48 h food-restricted state of the PDK4 isoform. Furthermore, there was only a significant difference reported in the PDK 4 isoform content after 48 h food-restriction and both subpopulations increased in similar amounts, ~3-4 fold, as a result of the 48 h food-restriction. While there were no differences observed in the PDK2 isoform content after 48 h food-restriction, the SS subpopulation contained the greater amount of PDK2 isoform content, ~2.5-4 fold greater amount in both fed and food-restricted states in comparison to the IMF subpopulation in both fed and food-restricted states. As well, there were no significant differences among all of the PDH complex components in either of the mitochondrial subpopulations during either nutritional state.

Blood Parameters

Blood parameters were examined as confirmatory measures to ensure food-restriction had occurred physiologically and reliance was shifted to fat metabolism. The results were in agreement to previous studies which indicated food-restriction in rodents

(Sugden et al., 2001; Peters et al., 2001; LeBlanc et. al., 2006). Glucose and glycerol were found to be unaltered after the 48 h period of food-restriction; this is most likely due to the large stores of glycogen available for immediate energy usage. As well, this strongly suggests that during this period of food-restriction, hepatic gluconeogenesis was sufficient to maintain output of glucose. Plasma insulin decreased significantly with food-restriction, and there was a significant increase in plasma FFA which is most likely a result of the decline in insulin availability, which facilitated adipose tissue lipolysis during food-restriction. The increase in plasma FFA suggests that the energy requirements are being met by the mobilization of fat, from adipose stores (Dallman et al., 1999). These results were similar to studies by Sugden et. al, (2001), who found that insulin decreased with 48 h food-restriction and FFA levels increased as well. However they observed a decrease in blood glucose with 48 h food-restriction, which we did not observe. As well, in our study there was an increasing trend observed in ß-hydroxybutyrate with food-restriction suggesting increasing the use of fat as a fuel through ketogenesis during the 48 h food-restriction. These results indicate that the physiological changes associated with food-restriction have occurred in the mitochondrial subpopulations after the 48 hr food-restriction period and reliance for energy was shifted to fat metabolism.

Mitochondrial Preparations

Our methodology included the use of a glass potter homogenizer and polytron for homogenization, as well as a protease for mitochondrial isolation, which have been used in combination in many studies (Krieger et al., 1980, Cogswell et al., 1993, Bizeau et al., 1998, and Campbell et al., 2004). While other studies have used a mechanical

homogenization process solely with a Teflon pestle and glass homogenizer in combination with (Bizeau et al., 1998) or without the use of a protease (Jimenez et al. 2002, Bezaire et al. 2004, and Butz et al. 2004). In addition, previous studies differ with the use of a cheese cloth (Bizeau et al., 1998) or gauze filter (Bezaire et al., 2004) or no filter (Campbell et al., 2004) to filter the SS supernatant, as well the thickness of the filter differs as some studies used a single (Bezaire et al., 2004) or double layer (Bizeau et al., 1998) to filter. The purpose of the filter was not explained in the previous work, it may have been used as a quick way to separate large non-mitochondrial portions from the mitochondrial preparations. When the method was tested without the filter in our lab, similar to the procedure described by Campbell et al. (2004), acceptable results were obtained.

Lastly, some studies used a Percoll gradient to prepare the subpopulations for Western blotting analysis (including Campbell et al., 2004; Benton et al., 2004). The principle of the Percoll gradient is to yield mitochondria largely free of contamination by cytosol, peroxisomes, plasmids and other membranes by density separations. However, the vigorous process involves prolonged centrifugation steps and density separation gradients to insure the purified mitochondrial yield (Mickelson, et al., 1980). As well, the Percoll gradient is known to decrease enzyme activity and yield mitochondrial preparations that are no longer metabolically viable (Holloway et al., 2006). The Percoll gradient procedure was not used in studies where enzyme activity was measured (Bezaire et al., 2004; Bizeau et al.1998). For this reason, the Percoll gradient procedure was avoided for the present study because we intended to measure PDK and total PDH activities. As well, the use of the Percoll gradient was deemed unnecessary for the

analysis of PDH complex as it is an intra-mitochondrial enzyme, and is therefore never found in other sub-cellular membrane fractions.

The development of this mitochondrial extraction involved testing several of the previous methods, until we had an acceptable quality and recovery of the preparations, and we were able to extract adequate mitochondrial protein content. The best results were obtained by incorporating minor modifications to the Campbell et al. (2004) method.

Mitochondrial Quality, Recovery and Protein Yield

Few studies report the quality of their mitochondrial preparations. In this study the quality was measured as the proportion of intact mitochondria harvested. In our preparation, the quality was between 75-82% and there was no significant difference between the SS and the IMF mitochondrial subpopulations within each of the fed and food-restricted conditions. However, after food-restriction, mitochondrial qualities were significantly lower (~75%) than in the fed condition (82%). Bezaire et al. (2004) reported similar but slightly higher qualities of ~ 82-85% intact mitochondria for both subpopulations during a fed state in both human and rat skeletal muscle. However, there was one major difference in the mitochondrial extraction procedure between the present study and the Bezaire et al. study, as they did not use a protease treatment for their IMF subpopulation extraction. The protease treatment is known to be hard on the integrity of mitochondria and therefore this might explain their higher IMF quality.

This study is the first to report CS recovery in both mitochondrial subpopulation preparations, but previous work with primarily SS mitochondria has yielded significantly higher recoveries than our SS preparation. We observed an average of ~13% recovery in

our SS preparation (~16% for fed and ~10% for food-restricted) which is slightly lower than recovery for primarily SS mitochondria from rat (21%, Peters et. al, 2000) and human muscle (16%, Turvey et. al, 2005, 25% LeBlanc et. al, 2004). However, there are important differences between the present study and the previous research conducted. In previous work (Peters et. al, 2000; Turvey et. al, 2005; LeBlanc et. al, 2004), the mitochondrial preparations were not separated into the SS and IMF mitochondrial regions and there is a possibility that their mitochondrial preparations may have contained a mixture of the two regions. As well, in the present study the mitochondrial extraction process was lengthier and required the mitochondrial subpopulations to sit on ice for an extended period of time before recovery and quality analysis could take place. The extraction process used in our study was longer because it involved changes in centrifuge speed at different times for each of the mitochondrial subpopulations, which lead to a continual changing of the centrifuge heads back and forth to accommodate each of the mitochondrial subpopulations.

There was a lower recovery observed in the IMF preparation in both fed and food-restricted conditions, which cannot be compared to previous studies. It is likely that our IMF preparation recovery was lower because the IMF extraction had additional steps that involved the use of a polytron for additional homogenization as well as a protease treatment which required more time. Together these additional steps could have contributed to mitochondrial breakage and loss which would contribute to decreased quality and recovery.

The recoveries for both mitochondrial subpopulations decreased significantly with food-restriction with 10% in the SS subfractions and 4.7% in the IMF subfractions after the 48 h food-restriction period. As well, after the 48 h food-restriction period the quality

of both mitochondrial subpopulations was decreased. The reasoning for the decrease in recovery in the mitochondrial subpopulations is unclear but it can be suggested the IMF mitochondria are more fragile than the SS mitochondria based on the lower yield. This is possibly due to the use of the protease during the IMF mitochondrial extraction as well as the lengthier process the IMF mitochondria undergo.

As well, the decrease in quality associated with the 48 h food-restriction period in both mitochondrial subpopulations suggests that both subpopulations are more fragile after food-restriction. There is limited research available to support these speculations but it warrants further research into mitochondrial membrane changes in response to food-restriction.

Many studies report protein yield in the mitochondria preparations rather than the recovery of a mitochondrial enzyme activity. Our study determined that there was a greater amount of mitochondrial protein yield in the SS mitochondrial subpopulations compared to IMF subpopulations, which was different than previous studies that all reported a greater amount of mitochondrial protein yield in their IMF preparations (Krieger et al., 1981, Cogswell et al., 1993, Bizeau et al., 1998, Bezaire et al., 2004, Jimenez et al., 2002). The fact that our results indicate a lower yield in the IMF preparations is directly related to the decreased CS recovery in the IMF preparations. However in the studies that report higher IMF protein yield, mitochondrial recovery was not measured. Therefore, one possibility for the difference observed in IMF preparation protein yield is that other studies might have extra-mitochondrial proteins in their preparations which would have led to contamination. However, the one study that we used in designing our extraction procedure (Campbell et al., 2004) did not report either recovery or protein yield but measured other parameters of purity.

The inconsistency in reporting quality, recovery and protein yield in previous research has made a direct comparison to our preparations difficult. Our SS recovery is similar to what has been previously reported for primarily SS preparations and although our IMF recovery was low, our qualities are comparable to preparations from previous work (Bezaire et al., 2004). In addition, the preparations were comparable in quality within the conditions (Fed vs FR) and differ by less than 10% between conditions.

Citrate Synthase Activity

Citrate synthase is a typical matrix TCA cycle enzyme that is often used to measure oxidative capacity. CS activity was measured in whole muscle homogenate (Hm) for each of the conditions and there was no alteration in activity after the period of food-restriction, as previously demonstrated (Peters et al., 2001). Therefore, the oxidative capacity of the whole muscle did not change with food-restriction.

CS activity was also measured in the subpopulation preparations as μmol/min per ml of mitochondria extraction (as related to per g of original muscle) and differed with a 35% higher activity in fed when compared to food-restricted (combined) preparations. However, there was no significance among SS and IMF in either group which is in good agreement with previously reported research (Bezaire et al., 2004). The decrease in CS activity among the preparations with food-restriction is probably an artefact of the preparation, again indicating decreased recovery of intact mitochondria in the food-restricted condition. Since on a given extraction day, both a fed and food-restricted rat were used, this cannot be due to day-to-day variability in extraction solutions or procedures. Therefore, it suggests that the food-restricted mitochondria may be more vulnerable than those from fed muscle. This increased fragility would explain increased

breakage during extraction and release of the matrix enzyme CS. One could speculate that this is an intriguing result, and could be the result of altered membrane properties in the food-restricted mitochondria which render these mitochondria more susceptible during extraction. This warrants future research to determine if nutritional challenges alter the structure and/or the function of skeletal muscle mitochondrial membranes.

CS Activity/mg protein Concentration

CS activity/mg mitochondrial protein can indicate the differences in oxidative capacity in the mitochondrial subpopulations. In spite of differences in recovery and protein content in our preparations, when CS activity was normalized for mitochondrial protein concentration (μmol/min per mg mitochondrial protein) there was no difference in activity between any of the groups. Therefore, it would appear that the mitochondrial extractions in this study have yielded preparations of the SS and IMF mitochondria that have similar oxidative capacity. These results have also been observed in previous work by Johnson et al. (2006), who determined the effect of various levels of food-restriction (25%, 50%, 75% and 100%) between the SS and IMF subpopulations in rat red gastrocnemius and quadricep muscles found no differences in CS activity/mg mitochondrial protein with food-restriction or between the populations. As well, a study by Cogswell et al. (1993), reported no differences in CS activity/mg mitochondrial protein in rat mixed quadricep muscle samples. In addition, a study by Bezaire et al. (2004), determined that there were no differences in CS activity/mg mitochondrial protein in the mitochondria subpopulations in rat red gastrocnemius. However, in the same study the authors reported differences did exist in rat soleus where CS activity/mg mitochondrial protein was significantly higher in SS mitochondria subpopulations

(Bezaire et al., 2004). The observed difference of CS activity/mg mitochondrial protein in SS mitochondria in rat soleus muscles could be related to the greater presence of oxidative fibres in the soleus. Therefore, it would appear that the mitochondrial extractions of rat red gastrocnemius from our study and previous research yield preparations of SS and IMF mitochondria that have similar oxidative capacity. Taken together this suggests the distribution of oxidative capacity of SS and IMF may be fibre type dependent, with type II fibres more evenly distributing CS activity/mg mitochondrial protein between the mitochondrial subpopulation and type I fibres contain a greater CS activity/mg mitochondrial protein in the SS compared to IMF. However, there have been no studies that directly target the question of differences in mitochondrial subpopulation distribution between the fibre types. Therefore further study is required to answer this question.

PDK and Total PDH Activity

Although previous studies had used CS activity/mg mitochondrial protein as an oxidative marker, other mitochondrial enzymes did not always mirror the distribution of CS activity between the mitochondrial regions (Kreiger et al., 1980; Cogswell et al., 1993; Bizeau et al., 1998). The hypothesis of this study was that total PDH activity would be greater in the IMF mitochondria populations because IMF mitochondria generally have a higher oxidative capacity and have been considered more important in providing energy needed for muscle contraction (Cogswell et al., 1993). As well we predicted that there would be a greater increase in PDK activity in the SS subpopulation after 48 h food-restriction occurred. Both of these hypotheses were unable to be confirmed because we were unable to measure the total PDH and PDK activities in the

IMF subpopulations, most likely due to the use of the protease in the mitochondrial extraction. This was an unforeseen problem, and was not predicted because both of these enzymes are found in the matrix and would not necessarily be vulnerable to protease treatment. A study by Bezaire et al. (2004), did not use a protease because they anticipated a problem would occur in measuring the CPT I activity due to its location on the outer mitochondrial membrane. A more recent study by Koves et al. (2005), reported difficulties measuring malonyl-CoA sensitivity, which is also associated with CPT1 and its relationship to the outer mitochondrial membrane, in the IMF mitochondria due to the use of the a protease. In these studies (Bezaire et al., 2004; Koves et al., 2005) eliminating the use of proteases was necessary because the enzymes are both on or associated with the outer mitochondrial membrane and the use of proteases would have enabled the enzyme activity of interest. For the present study, however, the use of a protease was considered necessary in order to successfully prepare the IMF subpopulations, and previous research examining activities of various intra-mitochondrial enzymes (Krieger et al., 1980; Cogswell et al., 1993; Bizeau et al., 1998; Benton et al., 2004; Takahashi et al., 1996; Rousell et al., 2000; Ljubicic et al., 2004; Menshikova et al., 2004; Campbell et al., 2004; Koves et al., 2005; Adhihetty et al., 2005) did not report problems in the enzyme activity analysis or during the western blotting analysis of the IMF preparations with the use of a protease. For this reason it was an unpredictable problem, and unfortunately the measurement of total PDH and PDK activity was ultimately abandoned.

Although these enzymes are considered to be matrix enzymes, there may be some important interaction with the mitochondrial membrane via a membrane spanning protein

that is disturbed by the protease treatment and further investigation into this area is required.

PDH Complex Proteins

PDH Components

Western blotting to detect the PDH complex components E1α, E1ß, E2 and E3 in the subpopulations were very similar to the CS activity/mg mitochondrial protein results in that there were no significant differences found between the SS mitochondria and IMF mitochondrial subpopulations and no alterations due to food-restriction. This is the first known report of the mitochondrial distribution of the components of PDH. This would suggest that its not just the general oxidative capacity that is similar between the mitochondrial subpopulations but also the maximum capacity for carbohydrate oxidation.

Very little is known about the distinct roles of E1α and E1ß subunit activity of the E1 component in catalyzing the initial reaction. This is because all available information was generated using the isolated E1 as a tetrameric form ($\alpha_2ß_2$) and any attempts to separate E1α and E1ß subunits have been unsuccessful to date (Patel and Roche, et al., 1990). In the present study, although there were no significant differences in either E1 subunit, there was a trend observed in the E1ß that showed a greater amount of the E1ß in the SS subpopulations in comparison to the IMF subpopulations ($p=0.09$). The fact that the E1α content would not change similarly to E1ß is surprising because the E1 component is believed to be a tetramer composed of equal amounts of E1α and E1ß (Behal et al., 1994). Therefore, one would presume both would be present in greater amounts in the SS subpopulation together, if at all.

Like the E1 subunits, E2 and E3 PDH complex components were unaltered with food-restriction and there were no differences in the mitochondrial content in either the SS subpopulation or the IMF subpopulation. The finding that E1, E2 and E3 are unchanged with food-restriction is not unexpected. Previous studies have demonstrated that there is no change in total PDH activity with food-restriction in animals (Peters et al., 2001, Sugden et al., 2001), high fat feeding or refeeding (Holness et al., 2002). Therefore, one would expect no change in PDH complex components with food-restriction, particularly the E1 component, since it is the rate-limiting step in the overall PDH complex reaction. In fact, Peters et al. (2001) and Wu et al. (1998, 1999, 2000, 2003) used the E1α protein to normalize blots, and observed no differences with 24 h or 48 h fasting.

In a different situation, a human study by LeBlanc et al. (2004) found a significant increase in the E1α after 8 weeks of aerobic training in human skeletal muscle, and therefore although it does not appear sensitive to acute nutritional challenges it is sensitive to changes in chronic contractile activity. However, even with 8 weeks of training there were no differences observed in the E2 or E3 binding proteins, which would suggest that these protein concentrations are very stable.

From the comparison of this study and the present research findings it can be assumed that the E1α PDH complex component is altered with the increased energy demand of aerobic training to enhance the PDH activity but unaltered by food-restriction. The increase in E1α would correspond with increased mitochondrial biogenesis. In general, nutritional challenges do not alter total PDH activity and therefore one would not expect alterations in the PDH complex components with food-restriction (Randle et al., 1986; Peters et al., 1998; Holness et al., 1989; Wu et al., 1989 and 1999).

Given that the PDH complex components were not different between SS and IMF mitochondria, had we been able to measure total PDH activity it would presumably be similar in both mitochondrial subpopulations and in both fed and food-restricted conditions.

PDK 2 and 4

Although there were no differences detected in either oxidative capacity (CS activity/mg mitochondrial protein) or maximum potential for carbohydrate oxidation (PDH complex components) between the SS and IMF mitochondria or with food-restriction, there were highly significant differences in PDK isoform content.

There was a 2.5-4 fold greater amount of PDK2 isoform content in the SS subpopulation than the IMF subpopulation regardless of nutritional status. PDK2 protein content remained unaltered with 48 h food-restriction, which is in agreement with previous research in rat skeletal muscle after 24 h food-restriction by Peters, et al. (2001) and rat tibialis anterior and soleus after 48 h food-restriction by Sugden et al. (2000). However, these results disagree with another study by Sugden et al. (2000) who reported small but significant increases in PDK2 isoform content after 48 h food-restriction in fast twitch skeletal muscle. It is not known exactly why there were differences observed in PDK2 content between our study and Sugden et al. 2000, because both studies involved the use of the same PDK2 antibody that was prepared at the same time in the Harris lab. The observed small differences are likely related to variability in results between studies or due to the differences in fast twitch muscle used (tibialis anterior vs. red gastrocnemius).

There was a 3-5 fold greater amount of PDK4 protein content in SS than IMF during the fed state. After 48 h food-restriction there was a 3-4 fold increase in both SS and IMF mitochondria. Although it was previously reported that PDK4 increased with food-restriction (Peters et. al, 2001, Sugden et. al, 2001, and Wu et al., 1998) this is the first study to report that PDK4 protein content increased to a similar extent in both mitochondrial regions. Although we hypothesized that a greater increase would be observed in the SS mitochondria, it would appear that after 48 h, both subfractions have adapted equally to the nutritional challenge.

These results are representative of the endpoint of 48 h of food-restriction and do not allow for an understanding interpretations of how the subpopulations may increase over this period of time, with one or the other population possible increasing at a greater rate early in the food-restriction condition. Chilibeck et al. (2002), demonstrated that the two subpopulations adapted to 12 weeks of endurance training at different rates with a latent increase occurring in the SS subpopulations during the last 6 weeks of training. This observed difference in the rate of adaptation of the subpopulations may be occurring during the adaptation of 48 h food-restriction in the present study. For this reason it must be clarified through a time course analysis of various time points between 12 h and 48 h food-restriction to determine how the subpopulations are adapting to the food-restriction.

Overall, these results demonstrate that there is a greater protein content in the SS mitochondria for both PDK2 and 4, with no difference in PDH complex components regardless of nutritional status, which suggests that the SS subpopulation has a higher capacity for the down-regulation of the PDH complex.

In situations where conditions favour carbohydrate oxidation, SS and IMF mitochondria could have similar PDHa activities. However, when conditions change to regulate PDK

(increased acetyl CoA, NADH and high energy charge) the SS mitochondria would be more sensitive to down-regulation of the PDH complex, decreasing carbohydrate oxidation and switching over to fat oxidation. With respect to fat oxidation in SS mitochondria, Koves et al. (2006) found in resting control rat red gastrocnemius there was no difference in maximum rates of mitochondrial fat oxidation between SS and IMF mitochondria. However, the regulation of fat oxidation was different with SS mitochondria exhibiting higher sensitivity to malonyl-CoA inhibition than IMF mitochondria. Taken together, this suggests that SS mitochondria exhibit more sensitive control of both fat and carbohydrate oxidation than IMF mitochondria in spite of having similar maximal capacities between the mitochondrial subpopulations. It is unclear as to why this would be, but it would suggest a better match between ATP demand and ATP production and a more sensitive switch between the two major fuels.

It is intriguing that both PDK2 and PDK4 have higher protein content in the SS mitochondria because the PDK isoforms are thought to be individually specialized with sensitivity to intra-mitochondrial effectors. PDK2 is linked to training adaptations and is energy sensitive and would normally increase with training, while PDK4 is linked to dietary adaptations and is nutritionally sensitive and pyruvate insensitive (Bowker-Kinley et al., 1998). Surprisingly, regardless of the PDK sensitivity, the same mitochondrial distribution is found in both. It is difficult to speculate why physiologically it would be necessary to have higher content of both of these isoforms, but it allows for a broader range of effector sensitivities and would contribute to the fact that SS mitochondria are more sensitive to down-regulation of carbohydrate and fat oxidation (Koves et al., 2005).

Summary and Conclusions

There are many aspects to fuel regulation that currently remain unknown. There has been minimal research into nutritional alterations in carbohydrate and fat oxidation rates in mitochondrial subpopulations. This study was intended to examine if differences occurred in PDK activity and isoform content in SS and IMF mitochondria in response to 48 h food-restriction. Previous to this study all research pertaining to nutritional adaptation of PDK activity and content have been studied in preparations which yield primarily SS mitochondria. It was not known whether the diet-induced increase in PDK activity and PDK2 and 4 isoform expression would occur to the same extent in IMF mitochondria compared to previous research conducted in the SS mitochondria. The secondary purpose was to analyze mitochondrial subpopulation distribution of the PDH complex components and total PDH activity. The mitochondrial subpopulation distribution of total PDH activity and the PDH complex components currently remained unknown.

The present study determined that CS activity, when normalized for mitochondrial protein, was not different between mitochondrial subpopulations. This was in agreement to previous research and indicates that the mitochondrial populations have similar oxidative capacity. Unfortunately, the PDK and total PDH activities in the IMF mitochondrial subfractions were unable to be analyzed due to methodological difficulties resulting in the inactivation of the PDH complex in the IMF mitochondrial subfractions possible by the use of a protease during the mitochondrial extraction process.

However, in examining the protein contents there were no significant differences between mitochondrial subpopulations in PDH complex components, indicating that both of the mitochondria subpopulations' PDH complex components are unaltered by food-

restriction, which is not surprising and is in agreement with previous research conducted in mixed mitochondrial subpopulations. Taken together, the CS activity when normalized for mitochondrial protein and the PDH complex components, these would suggest that it's not just the general oxidative capacity that is similar between the mitochondrial subpopulations but also the maximum capacity for carbohydrate oxidation.

The major finding of this thesis was in agreement with our hypothesis and is the first study to determine that, PDK4 content was higher in the SS mitochondria compared to the IMF mitochondria. However, contrary to our hypothesis both mitochondrial subpopulations increased with food-restriction. As well, in agreement with our hypothesis, PDK2 content was also higher in the SS mitochondria compared to the IMF mitochondria, however PDK2 content was unaltered with food-restriction. It is intriguing that both PDK2 and PDK4 have higher protein content in the SS mitochondria because the PDK isoforms and these results demonstrate that there is a markedly higher content of both PDK isoforms in SS compared to IMF mitochondria. Potentially this mitochondrial subpopulation may exhibit more sensitive control of both carbohydrate and fat oxidation, in spite of having similar oxidative capacities between the mitochondrial subpopulations.

The present research findings are important and will bring an added dimension into understanding the roles of the mitochondrial subpopulations in carbohydrate and fat oxidation. Investigation into the differences in mitochondrial subpopulations' structure and function is important in order to gain a full understanding of how these subpopulations adapt to various physiological perturbations, including nutritional adaptations, in order to advance the knowledge that is currently available in both carbohydrate and fat oxidation.

Future Studies

Future studies are required to determine if nutritional challenges alter the structure and or/function of skeletal muscle mitochondria membranes. It was observed in this study that the mitochondria membranes were more fragile after the food-restriction period and results indicate that decreased quality and recovery of mitochondria extractions occurred after the 48 h food-restriction period. The proposed increased fragility could be due to increased breakage during the extraction and release of the matrix enzyme CS as a result of altered membrane properties in food-restricted mitochondria. This research has the potential to identify the outcome of nutritional challenges on mitochondrial membrane properties that are not presently known.

As well, future research should be aimed at examining if the SS and IMF mitochondrial subpopulations adapt to dietary changes of food-restriction at different rates through the use of a time course analysis of 12 h, 24 h and 48 h food-restriction. This time course will allow for the observation of each mitochondrial subpopulation to determine if adaptation is occurring at differing rates. It was determined by Chilibeck et al. (2002), that a time course analysis was important in the analysis of training adaptations as the mitochondrial subpopulations adapt to training at different rates throughout the training period analyzed. Therefore, a time course analysis is important in order to gain a full understanding of how dietary adaptations are occurring in the mitochondrial subpopulations.

A limitation to this study is that it is not known for certain that the mitochondrial extraction process did indeed produce pure mitochondrial subpopulations. An additional step that can be made is to determine if the mitochondrial subpopulations contain any extra-mitochondrial enzymes, such as Na^{+}-K^{+}- ATPase, Ca^{2+}- ATPase, through the use of

Western blotting. The absence of any of these extra-mitochondrial enzymes would confirm that the mitochondrial subpopulations are pure and not contaminated. Another approach would be to use a Percoll gradient to prepare highly purified samples of the subpopulations. This step can be added after the mitochondrial extraction if enzymatic activity is no longer being investigating.

In addition, research is required to determine if oxidative capacity, or other mitochondrial functions, differ in the mitochondrial subpopulation distribution between fibre types. This study observed no differences in CS activity/mg mitochondrial protein in mitochondrial subpopulation of type II fibres in comparison to another study that determined a difference in mitochondrial subpopulation of type I fibres. These results suggest that the distribution of oxidative capacity of SS and IMF may be fibre type dependent and investigating this area of research would indicate the preliminary findings of mitochondrial subpopulation distribution between fibre types.

Another area of research that requires further investigation is in the understanding of the interaction of the PDH complex with the mitochondrial membrane. Information in regards to the location of the PDH complex has not been solidified. It is assumed that the complex may be membrane bound however its interaction with the membrane or a membrane spanning protein is not fully understood and requires further investigation. In this study it has been suggested that the protease treatment may be affecting the PDH complex activity and PDK activity as a result of inactivation of an enzyme that may possibly be in association with the mitochondrial membrane, as previous research has addressed that protease treatment has lead to the inactivation of enzymes associated with the mitochondrial membrane.

Lastly, to determine if the protease was the problem, in the inability to measure total PDH activity and PDK activity in the IMF mitochondria, there are two approaches that can be investigated. The first would be to measure if the total PDH activity and PDK activity of the SS mitochondria would be altered in the presence of protease. The second approach would be to develop an extraction procedure that does not use a protease, which would allow for the analysis of total PDH activity and PDK activity in the IMF mitochondria fraction. Together these experiments would allow for the determination into if differences in total PDH activity and PDK activity exist amongst the mitochondria subpopulations. This information would be beneficial to the present study and as well would assist in the general study of the PDH complex. In addition, study into the specific roles of the PDH complex components (primarily E1α and E1ß), as their distinct roles are not known, would greatly assist in the general study of the PDH complex as well would bring clarity into the present study as to why the two components were adapting at differing rates and to differing extents. The next progressive step would be to determine if differences exist in PDP protein content between the mitochondrial subpopulations followed by PDP activity to bring further findings to the understanding of the PDH complex.

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