

Human Skeletal Muscle Pyruvate Dehydrogenase Phosphatase Activity and Expression:  
The Effect of Aerobic Capacity

by

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## **ABSTRACT**

Activation of pyruvate dehydrogenase (PDH), which converts pyruvate into acetyl-CoA, is accomplished by a pair of specific phosphatases (PDP1 & 2). A cross-sectional study investigating the effect of aerobic capacity on PDP activity and expression found that: 1) PDP activity and PDP1 protein expression were positively correlated with most aerobic capacity measures in males (n=18), but not females (n=12); 2) only males showed a positive correlation between PDP activity and PDP1 protein expression ( $r=0.47$ ;  $p=0.05$ ), indicating that the increase in PDP activity in males is largely explained by increased PDP1 protein expression, but that females rely on another level for PDP activity regulation; and 3) PDP1 and E1 $\alpha$  protein expression increase in unison when expressed relative to the E2 core. These data suggest that with increased aerobic capacity there is an increased capacity for carbohydrate oxidation through PDH, via E1 $\alpha$ , and an increased ability to activate PDH, via PDP, when exercising maximally.

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## LIST OF ABBREVIATIONS

<b>ATP</b>	adenosine triphosphate
<b>BMI</b>	body mass index
<b>BSA</b>	bovine serum albumin
<b>CO<sub>2</sub></b>	carbon dioxide
<b>CoA</b>	coenzyme A
<b>CS</b>	citrate synthase
<b>E1</b>	pyruvate dehydrogenase
<b>E2</b>	dihydrolipoyl transacetylase
<b>E3</b>	dihydrolipoyl dehydrogenase
<b>E3BP</b>	E3 binding protein
<b>L2</b>	interior lipoyl-bearing domain
<b>NAD<sup>+</sup></b>	nicotinamide adenine dinucleotide
<b>NADH</b>	reduced nicotinamide adenine dinucleotide
<b>PDH</b>	pyruvate dehydrogenase
<b>PDHa</b>	active pyruvate dehydrogenase
<b>PDK</b>	pyruvate dehydrogenase kinase
<b>PDP</b>	pyruvate dehydrogenase phosphatase
<b>PKC</b>	protein kinase C
<b>PP2C</b>	protein phosphatase 2C
<b>PRP</b>	phosphatase regulatory protein
<b>S &amp; M</b>	sucrose and mannitol
<b>SD</b>	standard deviation
<b>SE</b>	standard error
<b>Ser</b>	serine
<b>T2DM</b>	type II diabetes mellitus
<b>TCA</b>	tricarboxylic acid
<b>TPP</b>	thiamine pyrophosphate

## **CHAPTER 1:**

### **INTRODUCTION**

The human body has many processes in place to react to a variety of stressors in order to maintain a state of homeostasis. This homeostasis is required for blood glucose levels, as they must be kept at a stable level in order for the body to function properly. This homeostasis is accomplished by glucose uptake by the cells of the body whenever blood glucose levels rise or the release of glucose from the cells whenever blood glucose levels become low. Once inside the cell, glucose can be: stored as glycogen; broken down through glycolysis and regenerated through gluconeogenesis; or oxidized. The key enzyme regulating carbohydrate oxidation is pyruvate dehydrogenase (PDH), with this enzyme being of particular importance in skeletal muscle because the large mass of this tissue in the body represents a major site for glucose disposal and whole body glucose homeostasis. In addition, small changes in energy demand through, for example, exercise will cause large overall demand for glucose oxidation and changes in active PDH (PDHa) activity (Fuller & Randle, 1984, Peters et al., 2001b). As PDH itself is regulated by a pair of specific phosphatases (PDP1 & 2; activation) and four kinases (PDK1-4; inactivation), the activity of these regulatory enzymes is of great importance in skeletal muscle in determining overall glucose oxidation, especially during exercise and the adaptations that occur as a result of aerobic exercise training. This thesis describes a cross-sectional study in which the activity of PDP is measured in human skeletal muscle in subjects of varying aerobic capacities. Additionally, the protein expression of the calcium-sensitive, and predominant skeletal muscle PDP isoform, PDP1, will also be measured to determine any correlations with aerobic capacity and total PDP activity.

## **LITERATURE REVIEW**

### ***PDH Function***

PDH acts as the link between glycolysis and oxidative phosphorylation through the irreversible conversion of pyruvate to acetyl-CoA. This step is the first irreversible reaction in carbohydrate oxidative metabolism with the stoichiometry of the reaction being:  $\text{pyruvate} + \text{CoA-SH} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$  (as reviewed by Wieland, 1983). This reaction is made irreversible by the cleaving off of a carbon which is released as carbon dioxide. As PDH exists in both an active and inactive form (which will be discussed later), it acts as a gateway for carbohydrate-derived pyruvate to acetyl-CoA, and can indirectly regulate fat oxidation by determining the proportion of acetyl-CoA from the two major energy sources. In its inactive form, the oxidative disposal of carbohydrate is restricted, which can increase fat oxidation to generate acetyl-CoA units for the TCA cycle, allowing the continuation of oxidative metabolism to meet the energy demand.

### ***PDH Structure***

The PDH complex is located in the matrix of the mitochondria and has been found to be in close proximity to the inner membrane (Matlib & O'Brien, 1975, Stanley & Perham, 1980). Little research has been conducted to date determine if there is a more structured distribution of this complex in the mitochondria. However, a study by Margineantu et al. (2002) investigated the distribution of PDH in the mitochondria, through staining, with the discovery that there were regions within the cristae containing

higher concentrations of PDH (E1 $\alpha$  and E2) interspersed with regions that appeared to be void of PDH. Moreover, these dispersions remained stable for time periods longer than 2hrs, leading the authors to postulate that PDH may be anchored to the inner mitochondrial membrane.

PDH is composed of three different subunits, with each subunit playing a specific role in the conversion of pyruvate to acetyl-CoA. The reaction is initiated at the E1 (pyruvate dehydrogenase) subunit with the cleaving of a carbon off the pyruvate molecule to form CO<sub>2</sub> and an acetyl intermediate. The E1 subunit is a heterotetramer and is composed of both an  $\alpha$  and  $\beta$  subunit (Reed, 1974), with the active site located at the interface of the  $\alpha$  and  $\beta$  subunits (Ciszak et al., 2003). This step also requires the cofactor thiamine pyrophosphate (TPP) which is bound in the cleft between the  $\alpha$  and  $\beta$  subunits (Gutowski & Lienhard, 1976, Robinson & Chun, 1993). The E1 reaction is responsible for the irreversible nature of the PDH reaction because of the CO<sub>2</sub> that is lost at this stage, however, the remaining reactions in the PDH reaction have the capacity to go in either direction (Ravindran et al., 1996). This step is also recognized as being the rate-limiting step for the PDH reaction (Cate et al., 1980). The E1 $\alpha$  subunit is well recognized as being the site of PDH activity regulation because of the presence of three serine residues, which will be discussed in the next section. Following the E1 $\alpha$  reaction, the acetyl intermediate is passed to the E2 (dihydrolipoyl transacetylase) subunit where it is oxidized, combined with CoA, and released as acetyl-CoA. This reaction leaves the E2 subunit in a reduced form, and it must therefore be re-oxidized before accepting another acetyl intermediate. This is accomplished via the E3 (dihydrolipoyl dehydrogenase) subunit, which oxidizes the E2 subunit (becoming reduced itself) and then passing these

electrons on to  $\text{NAD}^+$  to form NADH through an FAD-linked reaction (see Figure 1.1; as reviewed by Behal et al., 1993). Lastly, the complex contains an E3 binding protein (E3BP) which is responsible for binding the E3 subunit to the core (see Figure 1.2; Gopalakrishnan et al., 1989). Additionally, each PDH complex is not composed of a single copy of each subunit, but is in fact composed of multiple copies of each subunit. In a study on purified PDH complex from bovine kidney, it was found that the complex is composed of 30 E1 subunits arranged in a heterotetramer, 6 E3 subunits existing as a homodimer, and 60 E2 subunits which form the core of the complex (Zhou et al., 2001). However, a more recent study using prepared homogenous human components found that the core of the complex may be composed of a different stoichiometry, with 48 E2 subunits and 12 E3BP subunits forming a pentagonal dodecahedral core for the complex (Hiromasa et al., 2004). More recent analysis by Brautigam et al. (2009) on purified recombinant human PDH subunits shows a different stoichiometry, with a core of 40 E2 subunits and 20 E3BP subunits. There is also an E2 'swinging arm' extending from the core that has two lipoyl binding domains for shuttling intermediates between active sites as well as binding and moving the regulatory enzymes between the E1 subunits and an E1 binding domain allowing E1 subunit binding to the E2 core (Liu et al., 1995, Reed & Hackert, 1990). Also extending from the core are linker regions of E3BP which connect to the E3 subunit binding domain as well as a third lipoyl binding domain (Rahmatullah et al 1989a, Rahmatullah et al 1989b). Smolle et al. (2006) found that there may be further interactions occurring in the PDH complex based on the stoichiometries of the complex subunits. Their findings suggested that there are "cross-bridges" formed between two E3BPs by an E3 subunit (as they have a 2:1 stoichiometry), with the strong

possibility that these cross-bridges also occur between two E2 swinging arms by an E1 subunit (also with a 2:1 stoichiometry; Figure 1.3). This model also fits into the theory that the E2 swinging arm shuttles PDK around the complex as the regular spacing of the lipoyl domains of E2 by the cross-bridges of E1 would allow for easier movement of PDK and convenient access to the E1 subunits. However, the findings of Brautigam et al. (2009) contradict the possibility of cross-bridges by showing that E3BP and E3 have a 1:1 stoichiometry (as does E2 and E1), which is in stark contrast to the previously reported ratios of 2:1. The total size of the native complex is approximately 8500 kDa. Aside from the native complex, there are also regulatory proteins that are also associated with the complex (as reviewed by Behal et al., 1993), which will also be discussed in the following section.

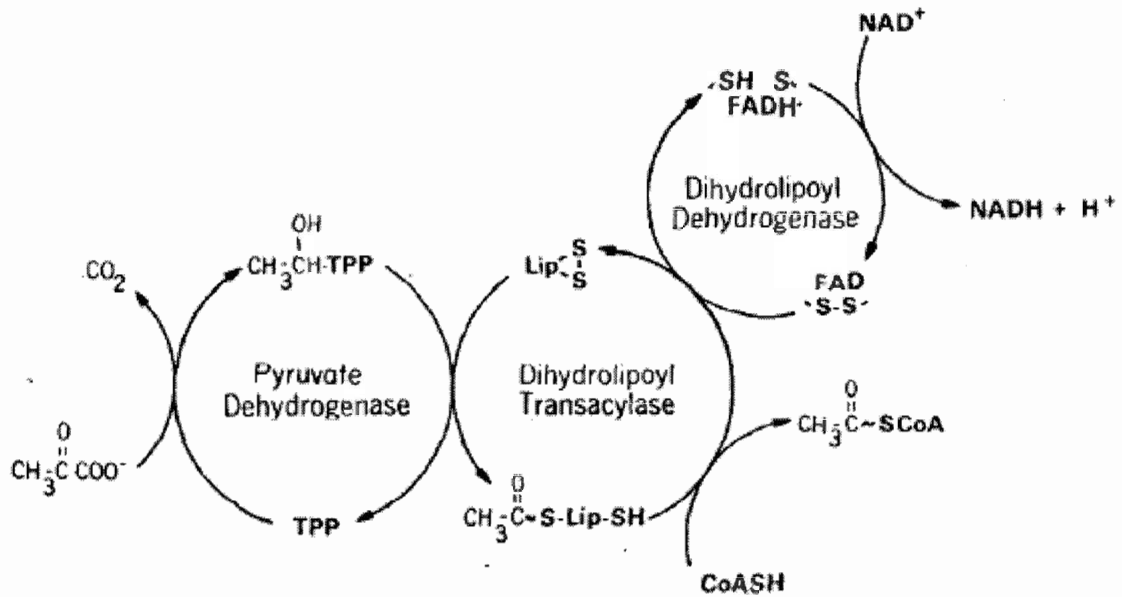


Figure 1.1. Reactions of the three PDH subunits in converting pyruvate to acetyl-CoA. The E1 subunit converts pyruvate to an acetyl intermediate, which is passed to E2 where CoA is added to the acetyl intermediate and it is released as acetyl-CoA. The E3 subunit then re-oxidizes E2, producing  $\text{NADH} + \text{H}^+$  from  $\text{NAD}^+$  in the process. Pyruvate dehydrogenase (E1); dihydrolipoyl transacetylase (E2); dihydrolipoyl dehydrogenase (E3). From Behal, et al. (1993).

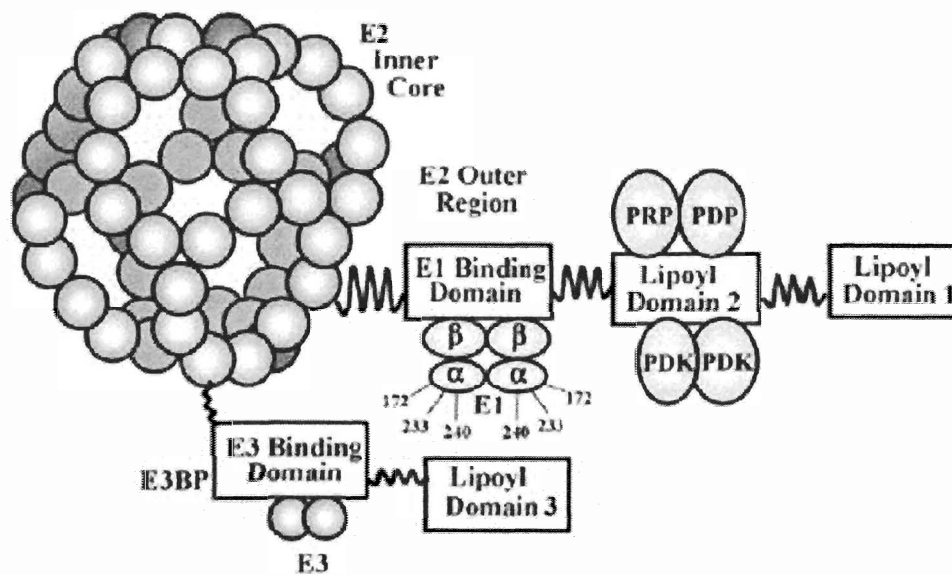


Figure 1.2. Structure of the PDH complex. The inner E2 core with the extending 'super arm' which contains two lipoyl binding domains and the E1 binding domain, where the E1 subunit binds. Also attached to the E2 core is the E3 binding domain, where the E3 subunit binds via the E3 binding protein (E3BP), and a third lipoyl binding domain. PDP, PDH phosphatase; PDK, PDH kinase; PRP, phosphatase regulatory protein; E1, pyruvate dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoyl dehydrogenase. From Harris et al. (2002)

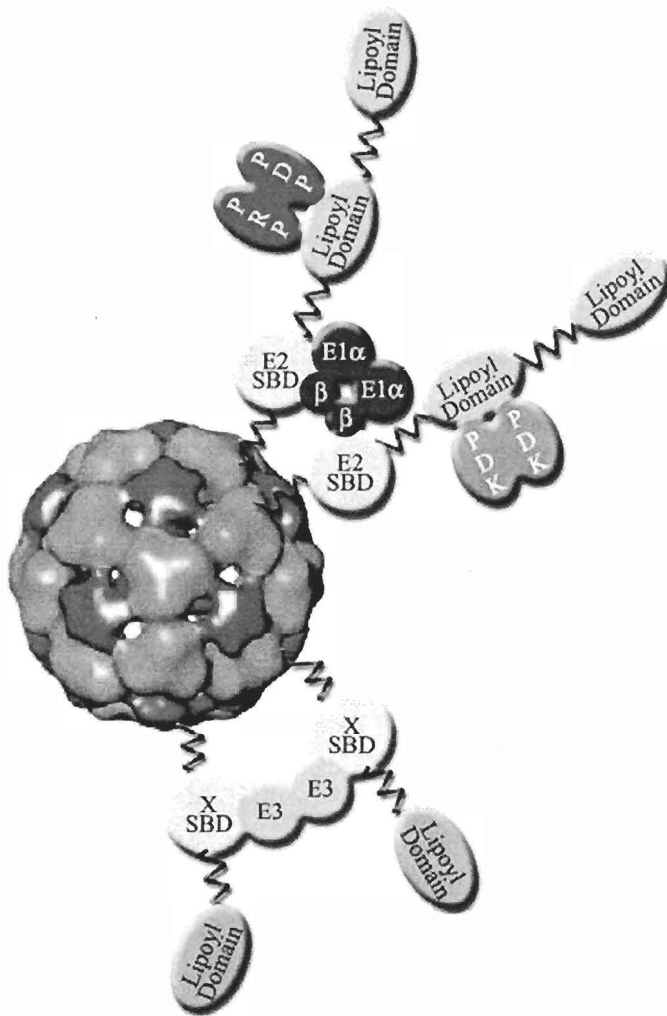


Figure 1.3. Model for cross-bridge formation in the PDH complex. The E3 homodimer binds to the subunit binding domains of two E3BPs to form a cross-bridge, with a similar cross-bridge being formed between two E2 swinging arms at the E1 binding domains by E1. E1, pyruvate dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoamide dehydrogenase; E2SBD, E2 subunit binding domain; XSBD, X subunit binding domain; PDK, PDH kinase; PDP, PDH phosphatase; PRP, phosphatase regulatory protein. From Smolle et al. (2006).

## *Acute Regulation of PDH*

### *Covalent Regulation*

PDH requires tight regulation in order to restrict the utilization of carbohydrates, as this fuel source is critical in certain situations and to certain tissues and has limited storage in the body (as reviewed by Harris et al., 2002). This regulation is accomplished by PDH existing in both an active (PDHa) and inactive form (with the combination of these two forms being total PDH (PDHt)) and this activity being determined covalently by the binding of phosphate to serine residues located on the E1 $\alpha$  subunit of the complex. The enzyme is rendered inactive by phosphorylation and is reactivated by dephosphorylation (see figure 1.4) (Linn et al., 1969). There are three serine residues (Ser<sup>264</sup>, site 1; Ser<sup>271</sup>, site 2; and Ser<sup>203</sup>, site 3) located on each E1 $\alpha$  subunit (Sale & Randle, 1981, Teague et al., 1979, Yeaman et al., 1978). However, only one of the two E1 $\alpha$  subunits needs to be phosphorylated in order to inactivate the complex (Sugden & Randle, 1978). In addition to this, only one of these serine residues needs to be phosphorylated in order for the complex to be inactivated (Korotchkina & Patel, 1995). The binding of additional phosphates is considered to only make the complex more difficult to reactivate because of the additional time required to remove the extra phosphates (Holness et al., 1988, Holness & Sugden, 1989, Sugden & Holness, 1989). The different phosphorylation sites appear to inactivate PDH by different mechanisms. Site 1 (Ser<sup>264</sup>) is located at the substrate channel leading to the active site of E1 $\alpha$  (Ciszak et al., 2003, Seifert et al., 2007) and appears to cause a steric, and to a lesser extent electrostatic, barrier to substrate binding at the active site, as well as preventing E2 lipoyl domain binding, when phosphorylated (Seifert et al., 2007). The mechanism of

inactivation by site 2 and 3 phosphorylation is not known, however, it is postulated that phosphorylation of site 3 (Ser<sup>203</sup>) affects binding of thiamine pyrophosphate (TPP; a required cofactor for the E1 $\alpha$  reaction). However, the complex is only rendered completely inactive when site 1 is phosphorylated, but is still capable of moderate catalytic activity when only sites 2 or 3 are phosphorylated (Korotchkina & Patel, 2001a). Phosphorylation is accomplished by PDH kinases (PDK; Yeaman et al., 1978) and dephosphorylation by PDH phosphatases (PDP; Teague et al., 1979). Therefore, the activity of PDHa is determined by the relative activities of the kinases and phosphatases (as reviewed by Harris et al., 2002 and Sugden & Holness, 1994).

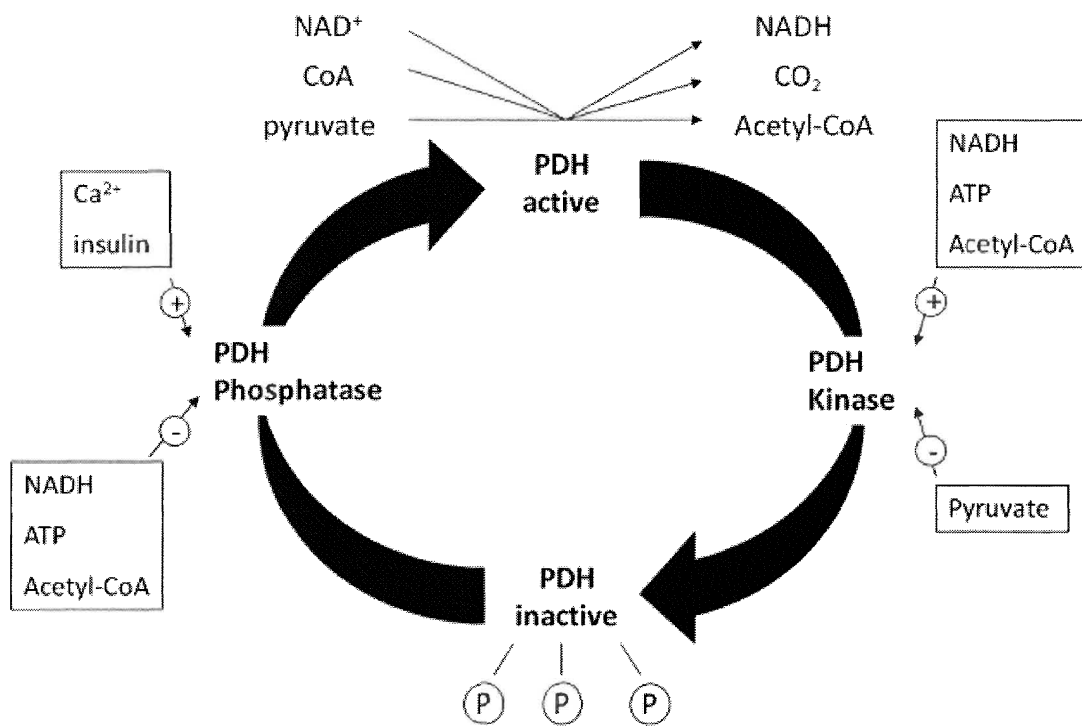


Figure 1.4. Covalent regulation of PDH, with PDK adding phosphate to render the complex inactive, while PDP dephosphorylates PDH to reactivate the complex. There are three sites of phosphorylation on the complex. The activities of PDK and PDP are determined by allosteric regulation. PDH, pyruvate dehydrogenase; PDK, PDH kinase; PDP, PDH phosphatase.

## *PDKs*

There are four PDK isoforms (PDK1-4), which display different activity levels, tissue expression, and allosteric regulation (Bowker-Kinley et al., 1998, Gudi et al., 1995). PDK is largely activated by the products of the PDH reaction (acetyl-CoA and NADH) and increased energy charge (ATP) and is inhibited by the substrates (pyruvate, CoA, and  $\text{NAD}^+$ ) (Kolobova et al., 2001). The products of the PDH reaction also enhance PDK activity by creating a build up of the acetylated form of the E2 component which enhances PDK binding to the lipoyl moiety, thereby increasing PDK activity because of its bound state (Ravindran et al., 1996). However, as mentioned above, each PDK isoform has a differing sensitivity to these regulators. Some important differences in regulation and specific activities are summarized in Figure 1.5. Additionally, each of the PDK isoforms has a different affinity towards the three phosphorylation sites on PDH. Three of the PDK isoforms and their site specificities are shown in Figure 1.6. Of particular note in these specificities is that PDK1 is the only isoform capable of phosphorylating site 3. Taken together, these results yield a phosphorylation rate in which site 1 is 4.6-fold more rapidly phosphorylated than site 2, and 16-fold more rapidly phosphorylated than site 3 (Korotchkina & Patel, 1995). These isoforms are also expressed at different concentrations in different tissues. Analysis of mRNA in rats showed that PDK1 is found predominantly in the heart (Popov et al., 1994, Bowker-Kinley et al., 1998) with smaller concentrations found in skeletal muscle, with the highest concentrations in the more oxidative fibre types (Peters et al., 2001a). PDK2 is expressed in high concentrations in all tissues, while PDK3 is also found in most tissues, but in much lower concentrations. PDK4 is mainly expressed in heart and skeletal muscle, with

the highest concentrations again being in the more oxidative fibre types (Peters et al., 2001a), but is also expressed in low amounts in the brain, liver, and pancreas (Bowker-Kinley et al., 1998). Additionally, PDK exists in both a bound and unbound state in terms of its association to the E2 core of the complex, with the regulatory enzyme being more active in the bound state (Ono et al., 1993). This is important, as a shift in the binding of the isoforms could affect the specific kinase activity without a change in the amount of kinase present (Korotchkina & Patel, 2001b).

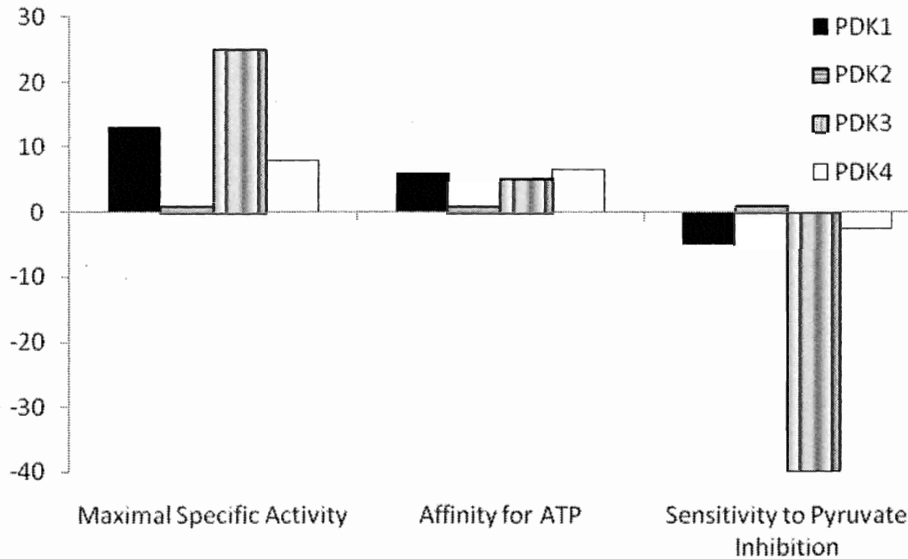


Figure 1.5. Rat PDK isoform activities and sensitivities to various effectors relative to the most abundant PDK isoform, PDK2. Adapted from Bowker-Kinley et al. (1998).

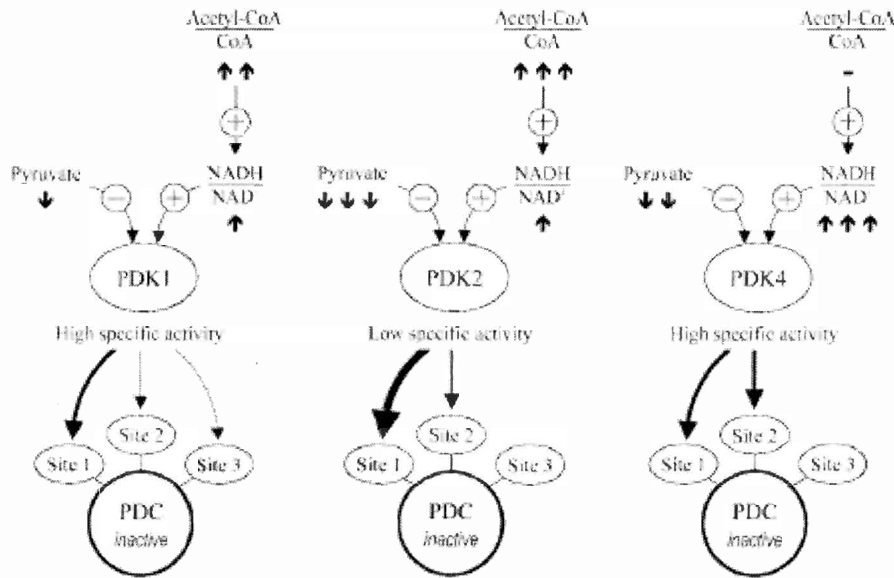


Figure 1.6. The specificity of the major skeletal muscle PDK isoforms toward the three regulatory sites on the PDH complex. Also depicted is the sensitivity of the PDK isoforms to the various allosteric regulators. PDC, PDH complex. From Sugden & Holness (2003).

### *PDPs*

There are two known isoforms of PDP in the body (PDP1-2), with each of these also displaying different activity levels, tissue expression, and allosteric regulation (Huang et al., 1998, Huang et al., 2003, Kolobova et al., 2001). Both isoforms require magnesium for activation (Huang et al., 1998, Lawson et al., 1993, Severson et al., 1974), though PDP2 has approximately a 10-fold lower sensitivity towards  $Mg^{2+}$  than PDP1 (Huang et al., 1998). Although relatively little is known about the catalytic mechanism that PDP follows for dephosphorylation, it is believed that it follows a similar mechanism to that of other protein phosphatase 2C (PP2C) enzymes, which PDP is classified as (Vassilyev & Symersky, 2007). Metal-bound ( $Mn^{2+}$ ) water molecules coordinate the

phosphate group to the enzyme through hydrogen bonds with the oxygens of the phosphate group. It is then believed that a bimolecular nucleophilic substitution occurs with the metal-bound water molecules acting as nucleophiles causing the phosphate group to be expelled (Das et al., 1996). In the case of PDP, however, the two  $Mn^{2+}$  ions are replaced with two  $Mg^{2+}$  ions.

Activation of PDP is accomplished allosterically by calcium and also by insulin stimulation through a mechanism that is not yet fully understood. PDP1 responds solely to calcium (Lawson et al., 1993) while PDP2 is insensitive towards  $Ca^{2+}$  (Huang et al., 1998). Insulin has been shown to activate the PDH complex in skeletal muscle (Nakai, et al., 1999, Denyer et al., 1989) and epididymal adipose tissue (Jungas, 1971, Weiss et al., 1971) of rats, with this activation believed to be mediated through PDP (Thomas & Denton, 1986). Much research has gone into investigating the structure of PDP1 to reveal that it is a heterodimer with a 52.6 kDa catalytic subunit (PDP1c) as well as a 96 kDa regulatory subunit (PDP1r) (Teague et al., 1982, Pratt et al., 1982, Yan et al., 1996). It is the PDP1c component that is stimulated by  $Ca^{2+}$  and requires  $Mg^{2+}$  for activity.  $Ca^{2+}$  stimulates activity by causing binding of PDP1c to the interior lipoyl-bearing (L2) domain of the E2 subunit, however, both of these components (PDP1c and L2) are required as  $Ca^{2+}$  is unable to bind to either component alone (Teague et al., 1982, Yang et al., 1998, Turkan et al., 2004). Without  $Ca^{2+}$  stimulation, PDP1 is very loosely bound to the PDH complex in comparison to the PDK isoforms (Teague et al., 1982). Structural analysis of PDP1c revealed that this is likely due to a more open cavity present on this isoform, compared to the PDK isoforms, which suggests that it must be closed to achieve tight binding to the E2 lipoyl domain (Vassilyev & Symersky, 2007). A similar open

cavity was also found on the PDP2 isoform, which is insensitive to  $\text{Ca}^{2+}$  and has been found to not bind to the E2 lipoyl domain (Huang et al., 1998, Karpova et al., 2004), leading to the theory that  $\text{Ca}^{2+}$  causes a conformational change in this cavity to allow tight binding to the PDH complex (Vassilyev & Symersky, 2007). Once bound to the E2 subunit of the complex, PDP1c activity is greatly increased. However, the minimum subdomains of E2 required for this increase in activity are L2 and the E1 binding domain, with the greatest increase in activity coming when the transacetylase (E2) domain is present with the L2 and E1 binding domain, where activity is indistinguishable from that of the complete E2 complex or the fully assembled PDH complex (Karpova et al, 2003). These findings lend support to the idea that the co-localization of PDP1c and E1 through E2 binding account for the increase in PDP activity. The greater PDP activity seen when the transacetylase (E2) domain is also present could be interpreted to support the hypothesis that this component facilitates delivery of PDP1c to the different E1 subunits, thereby enhancing its function (Chen et al., 1996, Karpova et al., 2003, Pettit et al., 1972).

The other PDP subunit, PDP1r, has an inhibitory effect on PDP activity by decreasing the sensitivity of PDP1c for  $\text{Mg}^{2+}$  by modifying or blocking the PDP1c catalytic site (Yan et al., 1996). It has also been found that PDP1r decreases the sensitivity of PDP1c to  $\text{Ca}^{2+}$ , leading to the belief that PDP1r plays a mediator role between the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  binding sites as an increase in the level of one of these metal ions creates an increase in the sensitivity for the other metal (Turkan et al., 2004). Vassilyev & Symersky (2007) suggest the presence of a low-affinity binding site for  $\text{Mg}^{2+}$  on PDP1r that is unoccupied at low concentrations of  $\text{Mg}^{2+}$ , but binds  $\text{Mg}^{2+}$

whenever higher concentrations of this ion are present. If this  $Mg^{2+}$  binding site is unoccupied, a proposed acidic side-chain of PDP1r binds to the  $Mg^{2+}$  ion of the active site for PDP1c instead, thereby blocking access to the active site. However, if this  $Mg^{2+}$  binding site of PDP1r becomes occupied, the acidic side chain would then switch to this internal  $Mg^{2+}$  ion instead, thereby opening up the active site of PDP1c.

PDP is also further regulated through phosphorylation itself. Caruso et al. (2001) investigated the role of several isoforms in the protein kinase C (PKC) family on the activity of PDP in L6 skeletal muscle cells and immortalized hepatocytes. It was found that upon insulin stimulation all PKC isoforms (PKC $\alpha$ , - $\beta$ , - $\delta$ , - $\epsilon$ , - $\zeta$ ) translocated to the plasma membrane, with PKC $\delta$  also redistributing to the mitochondrial membrane, where it co-localized with the PDP isoforms. Inhibition of PKC $\delta$  was found to nearly eliminate the increase in PDH activity normally seen with insulin stimulation, with inhibition of PKC $\alpha$  and - $\zeta$  having no effect on PDH activity, indicating that PKC $\delta$  is the specific isoform responsible for the phosphorylation of PDP in muscle and liver cells (Caruso et al., 2001). An earlier study investigating the activation of PDH through insulin found that PKC activation is required for this process, in that when they inhibited PKC, insulin was unable to activate PDH (Benelli et al., 1994). They also suggest that there may be a specific pathway for insulin signal transduction with several intermediary steps based on their finding that there was a lag in PDH activation by insulin, but PDH activation occurred much faster whenever 4 $\beta$ -phorbol 12 $\beta$ -myristate 13 $\alpha$ -acetate (a PKC activator) was used directly to activate PKC (Benelli et al., 1994). However, another study found that increased PKC $\delta$  activity prevents recovery of PDH activity during reperfusion, following ischemia, in heart mitochondria, with this prevention in activity inhibited when

PKC $\delta$  translocation to the mitochondria is prevented (Churchill et al., 2005). These contradictory findings for PKC, however, are suspected to be tissue specific (Dekker & Parker, 1994, Caruso et al., 2001), as Caruso et al. (2001) investigated L6 skeletal muscle cells and immortalized hepatocytes, and Benelli et al. (1994) used hepatoma cells, while Churchill et al. (2005) used cardiac mitochondria. These tissue differences are also evident by the different half-lives for PKC activity, as PKC is rapidly degraded in hepatoma cells by 12-O-tetradecanoylphorbol-13-acetate, but degradation is delayed in 3T3 fibroblasts (Lindner et al., 1991), allowing for PDH to be activated longer in these tissues. However, it is not yet known if phosphorylation of PDP (or PDK) plays a physiological role in intact skeletal muscle.

Inhibition of PDP is accomplished by NADH, acetyl-CoA, and ATP (as reviewed by Denton et al., 1975 and Wieland, 1983). PDP also displays tissue specific properties which are essentially based on the activators of the two isoforms. PDP1 (calcium-sensitive) is expressed in heart, brain, skeletal muscle, and testis, while PDP2 (insensitive to calcium) is expressed to a greater degree in the more insulin-sensitive tissues such as liver and kidneys, as well as the heart and brain (Huang et al., 2003), with some expression in skeletal muscle (Bajotto et al., 2004, Huang et al., 1998, LeBlanc et al., 2007). Although both PDP isoforms are present in skeletal muscle, there is a much greater concentration of PDP1 than PDP2 in this tissue (Huang et al., 2003, LeBlanc et al., 2007). LeBlanc et al. (2007) have recently discovered that the expression of PDP within muscle is also fibre-type specific, with PDP1 expression and PDP activity being significantly greater in red gastrocnemius and PDP2 detectable exclusively in red gastrocnemius (LeBlanc et al., 2007). Dephosphorylation of the E1 $\alpha$  subunit by PDP is

thought to occur randomly, demonstrating no site specificity (Korotchkina & Patel, 1995). However, Sale and Randle (1982) documented that in rat heart, the complex was dephosphorylated in the order of site 2 > site 1 = site 3. Using *in vitro* preparations of purified enzymes, Kolobova et al. (2001) also showed that the two isoforms of PDP respond at different rates when the complex is phosphorylated by different isoforms of PDK. It has been shown that both isoforms easily dephosphorylate PDH, at about the same rate, when it has been phosphorylated by PDK2. Both enzymes also display the lowest rates of dephosphorylation when PDK1 has phosphorylated the complex, however, PDP1 dephosphorylates the complex under these conditions at a rate that is approximately 3-fold greater than PDP2. Lastly, there is little difference in reactivating the complex following PDK3 and PDK4 phosphorylation, but again PDP1 is faster than PDP2 (Kolobova et al., 2001). In a similar study, it was demonstrated that both isoforms of PDP dephosphorylated PDH at site 2 faster than site 1 (PDP1: 15-times, PDP2: 10-times) and site 3 two-times faster than site 1 (order: site 2 > site 3 > site 1; Karpova et al., 2003). It was also found that the dephosphorylation of site 1 by PDP1 did not require dephosphorylation of sites 2 or 3 (nor did phosphorylation of site 3 affect the dephosphorylation of site 2). This would suggest that PDP1 follows a random dephosphorylation sequence as phosphorylation of additional sites does not affect the ability to dephosphorylate individual sites (Karpova et al., 2003).

The ability of PDP2 to dephosphorylate site 2 also seems to be unaffected by site 3 phosphorylation, indicating that dephosphorylation of these sites is also random. However, phosphorylation of sites 2 or 3 causes the dephosphorylation rate of site 1 by PDP2 to be approximately two times slower than the dephosphorylation of site 1 alone.

This would indicate that, unlike PDP1, PDP2 does not follow a random dephosphorylation sequence as sites 2 and 3 must be dephosphorylated before site 1 can be dephosphorylated. This finding for PDP2 would translate into an increased difficulty to activate PDH in tissues expressing predominantly this isoform, especially under circumstances causing multi-site phosphorylation of PDH (ex. starvation/diabetes, high-fat diets; Karpova et al., 2003).

### *Adaptive Regulation of PDH*

#### **Changes in Response to Nutritional Perturbations**

##### *PDK*

Of the two regulatory enzymes, PDK has received the greater attention in literature to date. There have been many studies investigating the effect of diet on adaptive changes to PDK activity and expression and PDHa activity. Thus far, research has shown that with a decrease in carbohydrate supply to the cell, there is an increase in PDK activity, causing a decrease in PDHa activity (as reviewed by Peters & LeBlanc, 2004). Fuller & Randle (1984) demonstrated that the skeletal muscle of rats that were starved for 48hrs, or were induced with type I diabetes via pancreatic  $\beta$ -cell damaging drugs, had a significant decrease in the portion of complex that was in the active form compared to controls. Further investigation in this study revealed that PDK activity was increased 2-3-fold with 48hr starvation or drug-induced diabetes. More recent research has investigated the protein content of PDK following other nutritional perturbations. These studies have shown that PDK2 is increased in rat liver, kidney, and mammary

gland (Wu et al., 2000), as well as rat anterior tibialis (Sugden et al., 2000) and liver (Sugden et al., 1998) following starvation. High-fat feeding has also been found to increase PDK2 expression in liver (Sugden et al., 1998), as well as increasing PDK4 expression in the soleus and anterior tibialis of rats (Holness et al., 2000). Research has also shown that the increased PDK4 expression that is induced by starvation is capable of being reversed with refeeding for 4hrs in the skeletal muscle of rats (Sugden et al., 2000).

In human skeletal muscle, Peters et al. (2001b) observed that a high-fat/low-carbohydrate diet caused a continual increase in PDK activity throughout the 3 day period. In looking at the protein content of the isoforms, they found that after just one day on the diet, PDK4 content increased by an average of 70% and remained increased throughout the rest of the diet period, but PDK2 was unaffected by the intervention (Peters et al., 2001b). Because PDK4 is seen as being the most responsive to changes in mitochondrial/lipid state, it has been termed the "'lipid status'-responsive PDK isoform" (Sugden et al., 2001)

There has been the suggestion that this increase in PDK activity and expression and decrease in PDHa activity when a diet is increased in fat is likely due to a decrease in carbohydrates rather than a result of the fat (Turvey et al., 2004). Turvey et al. (2004) conducted a study in which they manipulated the composition of the dietary fat in humans, with the finding that a high-fat diet that is composed of 15% of fat being n-3 fatty acids leads to an attenuated increase in PDK activity in skeletal muscle (Turvey et al., 2004). This finding gives support to the idea that the increased PDK activity and expression following an increase in dietary fat is not solely the result of decreased carbohydrate intake, but is also influenced by the amount and composition of the fat that

is present in the diet. Related to this, Bradley et al. (2008) also found that the short-term consumption of differential dietary fats lead to differences in PDHa activities and fat oxidation during moderate intensity exercise. It was found that fat-loading with n-6 fatty acids over 5 hours attenuated the increase in PDHa activity at the onset of exercise in comparison to saturated fatty acids. This, however, was not accompanied by a change in PDK activity, which is likely due to the short duration (5hrs) of fat consumption, with the attenuated increase in PDHa activity in the n-6 fatty acid consumption likely due to changes in the mitochondrial effectors of PDK. This shows that there is a time course for the adaptive change in PDK activity and expression.

### *PDP*

The explanation for the above decrease in PDHa activity and carbohydrate oxidation with dietary manipulations focussed on the increase PDK activity and content. However, it stands to reason that this same decrease in PDHa activity could be attributed to a decrease in PDP activity or content (Huang et al., 2003). In one of the first studies to look at the effect of nutritional state on PDP activity, Fuller & Randle (1984) found that 48hr starvation or drug-induced diabetes rats had no change in PDP activity in rat hind-limb skeletal muscle. However, more recently a study by LeBlanc et al. (2007), observed a decrease in PDP activity and PDP2 protein content after 48hrs of food deprivation in rat skeletal muscle. These contradictory findings, though, are suspected to be a result of differences in individual fibre type, as Fuller & Randle (1984) studied mixed hind-limb, while LeBlanc et al (2007) studied the individual fibre types of soleus (type I), red

gastrocnemius (type IIa) and white gastrocnemius (type IIb). Similarly, it has also been found that PDP activity decreases in response to starvation in rat mammary gland and kidney (Baxter & Coore, 1979, Cockburn & Coore, 1995). Huang et al. (2003) investigated the effect of 48hr starvation or drug-induced diabetes on PDP activity and isoform expression in rat heart and kidney. They found that starvation and diabetes reduced PDP2 protein in these tissues and that refeeding and insulin treatment returned PDP2 levels to normal. These results also demonstrated that starvation and drug-induced diabetes had no effect on PDP1 content in rat heart, although PDP1 decreased in kidney tissue with starvation and was partially restored with refeeding. Overall, there was a decrease in PDP activity with starvation or drug-induced diabetes in these tissues, which was reversed with refeeding or insulin-treatment respectively (Huang et al., 2003). However, to date there have been no studies examining PDP activity in human skeletal muscle.

## **Changes in Response to Exercise Training**

### *PDK*

In recent years, research has begun to focus on the adaptive changes that occur to PDH as a result of training. Numerous studies have also investigated the effects of prolonged exercise training on total PDH activity (PDHt) and PDHa activities with the results showing no increase in PDHt in rats (Brozinick et al., 1988, Nakai et al., 2002, Nakai et al., 1999). However, there were increases in PDHt following exercise training in diabetic rats (Nakai et al., 2002). PDHa activity has been shown to have mixed results

following exercise training with some finding a decrease in activity in rats at rest (Nakai et al., 1999, Nakai et al., 2002), as well as in humans at rest and after 15min of exercise (LeBlanc et al., 2004a). However, other studies have found an increase in activity in rats (Brozinick et al., 1988), diabetic rats at rest (Nakai et al., 2002) and humans at rest and during exercise (Burgomaster et al., 2006, Ward et al., 1986), while Putman et al. (1998) found no change in PDHa after 7 days of submaximal cycle ergometer training in humans at rest or during exercise. This large discrepancy in results can most likely be attributed to species differences and the various durations of the training (7 days to 5 months). In addition, studies differed in the training methods employed: sprint interval training (Burgomaster et al., 2006), strength training (Ward et al., 1986), or cycling/running (Brozinick et al., 1988, LeBlanc et al., 2004a, Nakai et al., 2002, Nakai et al., 1999, Putman et al., 1998). It has been suggested that these differences in training methods may be of particular importance as the increases in PDHa observed by Burgomaster et al. (2006) and Ward et al. (1986) may be a result of increased sensitivity of PDP to calcium with sprint interval training (Burgomaster et al., 2006) or training that resembles sprint interval training (Ward et al., 1986).

In a more in depth study in human subjects, 8wks of aerobic training lead to a 31% increase in PDHt activity which was accompanied by a 1.3-fold increase in the protein content of only the E1 $\alpha$  subunit, with no significant change in E2 or E3BP. There was also a doubling of PDK activity which was accompanied by a 1.3-fold increase in PDK2 content, while PDK1 and PDK4 were unaltered (LeBlanc et al., 2004b). This increase in PDK2 content is of particular importance because it is the isoform that is most strongly inhibited by pyruvate and ADP (Bowker-Kinley et al., 1998, Gudi et al., 1995),

which both increase during exercise. Therefore, this has led to the suggestion that PDK2 may be the "energy status'-responsive PDK isoform" (LeBlanc et al., 2004b). The increased content of the E1 $\alpha$  subunit is also of importance as this is the subunit that makes the PDH reaction irreversible, as well as being the regulatory subunit that has the three phosphorylation sites (Patel & Korotchkina, 2001b). However, despite the increase in maximal capacity for oxidizing carbohydrates (via increased PDHt activity and E1 $\alpha$  protein content), there is an overall decrease in PDHa activity when exercising at submaximal levels following 7 weeks of aerobic exercise training (LeBlanc et al., 2004a). Seven weeks of aerobic exercise training also leads to a decrease in pyruvate accumulation (LeBlanc et al., 2004a), which would mean a decreased inhibition of PDK2 (the isoform that is most sensitive to pyruvate inhibition), and therefore, increased phosphorylation of PDH during this submaximal exercise. Therefore, the coordinated increases in both PDHt and PDK activities with aerobic training allows for increased capacity to utilize carbohydrates when needed during maximal exercise, but also allows for conservation of carbohydrates during submaximal exercise when they are not needed (LeBlanc et al., 2004b).

### *PDP*

In contrast to the reciprocal changes seen between PDK and PDP with nutritional manipulations, training adaptations do not appear to cause opposing changes in these enzymes. LeBlanc et al. (2008) found that following 8 weeks of endurance training in lean and obese Zucker rats, there was an increase in PDP activity in all skeletal muscles

examined (RG; white gastrocnemius, WG; and soleus) of obese rats, but not lean rats. However, there was a trend toward an increase in PDP activity in the RG and soleus of lean rats suggesting that a greater potential for increase in PDP activity is needed for the observed increase in PDP activity to reach significance. This greater potential for an increase in activity was seen in the obese rats as they had lower PDP activity values before training compared to lean rats, thereby allowing for greater gains to be made in this group with training. Training also caused a significant increase in the expression of PDP1 in the soleus and RG of obese rats and the soleus of lean rats, with no change in the expression of PDP2. This is of particular significance as PDP1 is the calcium-sensitive isoform of PDP, and therefore, when  $\text{Ca}^{2+}$  is released for muscle contraction during exercise, increased levels of PDP1 would allow for enhanced carbohydrate oxidation during maximal exercise by overriding PDK activity and activating PDH.

### ***Summary of Adaptive Changes***

To date, there has been a plethora of research investigating the adaptive changes that PDH undergoes in response to different perturbations. The most commonly investigated of these perturbations is that of a change in nutritional state. Research has shown that an increase in dietary fat for a prolonged time period, as well as starvation, or diabetes leads to an adaptive increase in PDK activity. Specifically this increase in PDK activity is accompanied by an upregulation in the expression of the PDK4 isoform, and in some instances in various rat tissues, the PDK2 isoform. Studies investigating the same nutritional changes on PDP, have found that there is a decrease in PDP activity, with a

specific decrease in the content of the PDP2 isoform. With this increase in PDK activity and decrease in PDP activity there is an overall decrease in PDHa activity, allowing for the conservation of carbohydrates during these situations in which carbohydrates are in limited supply.

During exercise there is an increased need for energy in the working muscles, with this increased demand leading to an increased need for carbohydrate oxidation. This increased demand for carbohydrate also requires an increased regulation so as to conserve carbohydrate when energy demand is not as high, and fat is capable of meeting the body's energy needs. Studies on aerobic training have shown that there is an increased protein expression of the regulatory E1 $\alpha$  subunit of PDH, with no change in the other subunits, allowing for a greater maximal flux through PDH. However, there is also an increase in PDK activity as a result of training, with a specific increase in the protein expression of the PDK2 isoform. In obese rats, and to a certain extent in lean rats, aerobic training has also been seen to cause an increase in maximal PDP activity, which is accompanied by an increase in PDP1 protein expression, with these changes being most evident in obese rats. At present, however, no study has investigated if there is also a change in PDP activity or isoform expression in humans as a result of exercise training.

## **CHAPTER 2:**

### **STATEMENT OF THE PROBLEM**

With the observed coordinate regulation of PDK and PDP in response to dietary perturbations affecting the PDH complex, it would also be expected that such coordinate changes would also exist with endurance training adaptations. To date, the only study that has looked at the effect of training on the PDH regulatory proteins in humans has focused solely on PDK (LeBlanc et al., 2004b), with no investigation of changes in PDP activity in humans. As well, the only studies investigating the adaptive regulation of PDP as a result of dietary (LeBlanc et al., 2007) and training (LeBlanc et al., 2008) manipulations have been done in rats. With the finding that PDP activity and expression are increased with training in rat skeletal muscle, it should be determined if there is a similar increase in PDP activity and expression in humans, which would balance the increase in PDK activity and expression seen with exercise training.

#### ***Purpose***

The purposes of this cross-sectional study are: 1) to determine if it is possible to measure PDP activity in human skeletal muscle; and 2) to investigate if there are differences in PDP activity and expression between subjects with different aerobic capacities.

## **HYPOTHESES**

a) It is expected that PDP activity in skeletal muscle will be positively correlated with aerobic capacity, so as to offset the increase in PDK activity that has been seen with longitudinal aerobic exercise training studies.

b) PDP1 content is expected to increase with aerobic capacity because it is the predominant isoform in skeletal muscle, which is the tissue that is responsible for changes occurring with training (e.g. aerobic capacity). Due to the minimal content of PDP2 in skeletal muscle, and its insensitivity toward calcium (which causes muscle contraction and thereby, training changes), it would be expected that no change in its content would occur with aerobic capacity. However, as no human antibody is commercially available at present for PDP2, it will not be measured in this study.

## **CHAPTER 3:**

### **METHODS**

#### *Subjects*

Male (n=18) and female (n=12) subjects volunteered for this study and were recruited via poster advertisements at McMaster University (Hamilton, ON). All subjects were healthy, non-smokers and non-diabetic, and were free to withdraw at any point during the trial without penalty. For their participation time in this study, subjects were reimbursed with \$100. This research project was given ethics approval from both McMaster University (05-250) and Brock University (05-083).

#### *Experimental Protocol*

Subjects first came in for an interview during which time the study was explained to them, and they filled out a questionnaire regarding current health status and gave a detailed description of their physical activity levels over the past few months. Subjects then signed a consent form to participate in the study and were given an information form regarding the study. A dietary analysis was also done for a typical three day period to record habitual caloric intake of the subject. Once consent was given to participate in the study, a  $VO_{2peak}$  test was conducted on an electromagnetically-braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA, USA) using a metabolic measurement system (Quinton Q-Plex 2, Quinton Instruments, Seattle, WA, USA) following a protocol with incremental workloads. Subjects began the  $VO_{2peak}$  test by

cycling against a resistance of 50W, with the resistance increased to 100W after 1min and increased by 25W every minute after that until the subjects reached exhaustion (unable to maintain 80rpm). At least a week later subjects reported back to have two resting muscle biopsies done in the vastus lateralis with the first immediately placed in buffer at 4°C for mitochondrial extraction and the second immediately frozen in liquid nitrogen for later analysis of citrate synthase activity and Western blotting for specific proteins. Subjects refrained from alcohol, caffeine, and exercise for at least 24hrs before the biopsies and consumed their habitual diet in that time period. Their last habitual meal was consumed at least 3hrs before the biopsies were done.

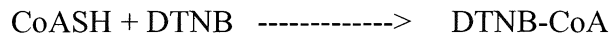
### ***Mitochondrial Extraction***

Mitochondria were isolated from the first muscle biopsy by differential centrifugation, following a method that targets mostly sub-sarcolemmal mitochondria, as originally described by Makinen and Lee (1968) and modified by Jackman and Willis (1996), and has been used previously (LeBlanc et al., 2004b, LeBlanc et al., 2007, LeBlanc et al., 2008, Peters et al., 1998 and 2001b). Briefly, ~75mg muscle was minced and homogenized on ice by hand in 20 volumes of solution 1 (100mM KCl, 40mM Tris HCl, 10mM Tris Base, 5mM MgSO<sub>4</sub>, 5mM Na<sub>2</sub>EDTA, 1mM ATP, pH 7.5). The sample was then centrifuged at 700g for 10min at 4°C to pellet out the contractile proteins, large membranes, and other cellular debris. The supernatant was retained and spun at 14,000g for 10min at 4°C to pellet out the mitochondria after which the supernatant was discarded and the pellet resuspended in 10 volumes of solution 2 (100mM KCl, 40mM Tris HCl,

10mM Tris Base, 1mM MgSO<sub>4</sub>, 0.1mM Na<sub>2</sub>EDTA, 0.25mM ATP, 1% BSA) and centrifuged at 7000g for 10min at 4°C. The supernatant was drawn off and discarded and the pellet was resuspended in solution 3 (same as solution 2, but with BSA omitted) and centrifuged at 7000g for 10min at 4°C. The supernatant was drawn off and discarded and the pellet was resuspended in a 1:1 weight/volume ratio (1mg/1μl) of Sucrose and Mannitol (S&M) buffer (220mM Sucrose, 70mM Mannitol, 10mM Tris HCl, 0.1mM Na<sub>2</sub>EDTA, pH 7.4) for use in PDP activity analysis. A 5ul aliquot of this suspension was added to 100ul of S&M for measurement of mitochondrial total suspension citrate synthase activity (CS<sub>ts</sub>) to determine mitochondrial recovery. Mitochondria for PDP and CS<sub>ts</sub> activity were stored at -80°C.

### ***Citrate Synthase***

A piece (~10mg) of muscle was chipped from the frozen biopsy and homogenized on ice in 100 volumes of homogenizing buffer (0.1M KH<sub>2</sub>PO<sub>4</sub>, 0.05% BSA, pH 7.3) and freeze/thawed twice using liquid nitrogen. Citrate synthase activity for the homogenate (CS<sub>homogenate</sub>) was determined using a spectrophotometer (Biochrom, Cambridge, England) and measuring the formation of DTNB-CoA from dithiobis-2-nitrobenzoate (DTNB) and CoASH at 412nm. This reaction is buffered by 150ul of 100mM Tris buffer (pH 8.3), and with the addition of 25ul of 1mM DTNB, 15ul of 10mM oxaloacetate, 40ul of 3mM acetyl CoA, 10ul of Triton-X, and 10ul of sample (either muscle homogenate or mitochondrial total suspension), the following reactions occur:



This protocol was also followed to analyze the CS activity in mitochondrial TS ( $CS_{ts}$ ) for use in calculating mitochondrial recovery (Srere, 1969).

$$\% \text{ mitochondria recovery} = CS_{ts}/CS_{\text{homogenate}} \times 100$$

### ***Protein Concentration***

Protein concentration of samples was determined by following the standard procedure for the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA) with minor modifications. Briefly, samples were diluted 10-fold with deionized water and 10ul was added to a microtiter plate in duplicate along with several dilutions of a BSA protein standard. A 5min incubation at room temp was then done following the addition of 200ul of diluted Bio-Rad Dye Reagent and absorbance was read at 595 nm using a multi-detection microplate reader (Bio-Tek Instruments, VT, USA).

### ***PDP Activity Assay***

Pyruvate dehydrogenase phosphatase (PDP) activity was determined using the nonradioactive phosphatase assay system from Promega (Madison, WI) and a synthetic peptide substrate from New England Peptide (Gardner, MA) as previously described (LeBlanc et al., 2007, Huang et al., 2003, Caruso et al., 2001) with minor modifications.

Briefly, mitochondria were diluted with S&M to a final protein concentration of 2 $\mu$ g/ $\mu$ l and a total volume of 30 $\mu$ l. Samples were then freeze/thawed three times in liquid nitrogen to disrupt mitochondrial membranes and centrifuged at 21,000g for 1hr at 4°C to pellet out large protein and other insoluble materials, leaving PDP in the supernatant. The supernatant was further purified through Sephadex G-25 in spin columns, which were centrifuged at 600g for 5min at 4°C, to remove excess inorganic phosphate from the supernatant. The spin columns were prepared approximately 1hr before the addition of the sample by equilibration with 10ml Sephadex G-25 storage buffer (10mM Tris Base, 1mM EDTA, 0.02% Sodium Azide, pH 7.5) followed by centrifugation at 600g for 5min at 4°C to remove the remaining buffer from the column. The prepared sample (5 $\mu$ l) was then added to a plate well containing 10 $\mu$ l reaction buffer (250mM Imidazole, 1mM EGTA, 25mM MgCl<sub>2</sub>, 0.1%  $\beta$ -Mercaptoethanol, 0.5mg/ml BSA, pH 7.2), 1 $\mu$ l Phosphatase Inhibitor cocktail (Sigma, St. Louis, MO, USA) to inhibit other phosphatases (alkaline phosphatases, protein phosphatases 1 and 2A), brought to a total volume of 50 $\mu$ l with phosphate-free water and incubated at 37°C for 10min to equilibrate the wells. The reaction was started with the addition of 13 $\mu$ l 1.5mM phosphopeptide and was incubated at 37°C for 30min during which time PDP would remove inorganic phosphate (Pi) from the peptide. The reaction was stopped by adding 50 $\mu$ l of molybdate dye to each well with a 30min incubation at room temperature to allow colour development. Optical density of the plate was read by measuring light absorbance at 600nm. Activity was determined through the use of a standard of 0, 0.1, 0.2, 0.5, 1.0, and 2.0nmols of inorganic phosphate (Pi) that was set up on the same plate. A standard curve regression was used to determine the amount of Pi produced by the mitochondrial

sample. Samples and standards were done in duplicate with the average of the duplicates taken as the accepted activity value. Activity is expressed in nmols Pi/min/mg mitochondrial protein and nmols/min/g wet tissue weight. Activity per gram wet tissue weight was calculated using % mitochondrial recovery in the following formulas:

$$\text{nmols/min/}\mu\text{l mitochondrial solution} = \text{nmols/min} \div 5\mu\text{l (amount added to well)}$$

$$\text{nmols/min/}\mu\text{l extracted mitochondrial solution} = \text{nmols/min/}\mu\text{l mitochondrial solution} \times \text{mitochondrial dilution}$$

$$\text{nmols/min/g wet tissue wt} = \text{nmols/min/}\mu\text{l extracted mitochondrial solution} \div \% \text{ mitochondrial recovery} \times 1000$$

### ***Western Blotting***

Protein content was determined following the methods of LeBlanc et al (2007). A piece (~10mg) of muscle was chipped from the frozen biopsy and homogenized on ice in 100 volumes of homogenizing buffer (250mM Sucrose, 100mM KCl, 2mM EDTA, pH 6.8) which also contained a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Laval, QC). Samples were diluted to a final concentration of 1ug/ul in 1M DTT (10% of total volume), and 5x sample buffer (250mM Tris HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol). Samples were solubilized by boiling for 5min and cooled on ice for 5min. Standard SDS-PAGE electrophoresis was done using 4% stacking and 10% (or 12% for COX-IV blots) separating gels on 5ug of sample (or 2.5ug for PDP1 blots) which was run at 120V for 90min. Proteins were transferred from the gel

to a polyvinylidene fluoride (Immobilon-P, Millipore, Bedford, MA) (0.45µm pore size) using the Mini Trans-Blot System (Bio-Rad Laboratories, CA, USA) and a transfer buffer (25mM Tris Base, 192mM glycine, 1g/L SDS, and 20% methanol) and were run at 100V for 60min. Membranes were blocked for 1hr in TBST (200mM Tris Base, 1.37M NaCl, 38ml 1M HCl/L TBST, 0.1% Tween 20, pH 7.5) with 5% non-fat dry milk to block all non-specific binding sites. Membranes were then incubated overnight at 4°C in 10ml of blocking solution (TBST with 5% skim milk) with a polyclonal antibody against PDP1 (diluted 1:2000; a kind gift from Drs. Brian Robinson and Mary Maj, Genetics and Genome Biology, SickKids Hospital, Toronto, ON), or monoclonal antibodies against PDH E1 $\alpha$  (diluted 1:5000; #A-21323), PDH E2 (diluted 1:5000; #A-21325), COX-IV (diluted 1:1000; #A-21348; Molecular Probes, OR, USA), and actin (diluted 1:5000; #612656; BD Biosciences, Mississauga, ON). Membranes were washed 3-4 times in TBST 5-15min and were incubated at 4°C for 2hrs in 10ml blocking solution with either goat anti-mouse IgG (diluted 1:5000; #A4416; peroxidase conjugated, Sigma, ON) or goat anti-rabbit IgG (diluted 1:10,000; sc-2004; Santa Cruz Biotechnology, CA, USA). Membranes were washed 3 times for 5min in TBST and chemiluminescent substrate (ChemiGlow West, Alpha Innotech, CA, USA) was added for visualization using a Fluorchem 5500 imaging station (Alpha Innotech, CA, USA). Membranes were stored between blotting paper at 4°C, after being soaked in methanol and air dried, for reprobing. Optimization was done on the PDP1 antibody by loading 2.5, 5, and 10µg of protein and diluting the primary antibody 1:1000 and 1:2000. To reprobe for different proteins, membranes were washed for 15min in TBST, washed 5-10min in Restore Plus Western Blot stripping buffer (Pierce Biotechnology, IL, USA) to remove previous

antibodies, followed by a 10min wash in TBST and 1hr block before adding the primary antibody as outlined above. Detection of the membrane revealed that the previous antibody had been stripped off. PDP1 protein was always determined on an original membrane, while E1 $\alpha$ , E2, and COX IV were either on original or reprobed membranes. All protein values were normalized to actin to ensure equal loading (with the exception of E1 $\alpha$ , which could not be reprobed properly for actin due to their near identical molecular weights). Blot density was quantified using the National Institute of Health ImageJ software (downloaded from: <http://rsb.info.nih.gov/ij/>) and is expressed in arbitrary densitometry units. Due to the large number of subjects in this study, several separate membranes were required for protein analysis. To compensate for variations between the membranes, 6-7 subjects were repeated across the membranes which allowed for membranes to be expressed relative to each other by taking the average difference of these subjects between the membranes.

### ***Data Analysis and Statistics***

Differences between males and females were analyzed with independent t-tests and a p-value < 0.05 was accepted as significant. All other comparisons were done through Pearson's correlation coefficients with a p-value < 0.05 for the slope accepted as significant and a p-value < 0.10 accepted as indicating a trend.

## **CHAPTER 4:**

### **RESULTS**

#### ***Subject Characteristics***

Subjects had  $VO_{2peak}$  values ranging from 30.0 to 64.5ml/kg/min (2.10 to 5.35L/min) and citrate synthase (CS) measures ranging from 7.04 to 32.30 $\mu$ mol/min/g wet tissue wt (3.53 to 11.54 $\mu$ mol/min/mg mitochondrial protein; see Figure 4.1 for representative histograms of aerobic capacity). As expected, males were significantly taller and heavier than females, as well as having significantly higher relative and absolute  $VO_{2peak}$  scores (Table 4.1). No other significant differences were found between males and females on any study measures (data not shown).

*Table 4.1.* Subject characteristics and aerobic capacity of males and females.

Variable	Males (n=18)	Females (n=12)
Age (yrs)	24.2 $\pm$ 0.68	22.6 $\pm$ 1.24
Height (m)	1.82 $\pm$ 0.01	1.68 $\pm$ 0.02*
Weight (kg)	81.2 $\pm$ 2.18	66.6 $\pm$ 2.22*
BMI	25 $\pm$ 0.6	23 $\pm$ 0.6
$VO_{2peak}$ (ml/kg/min)	48.0 $\pm$ 1.71	40 $\pm$ 1.46*
$VO_{2peak}$ (L/min)	3.89 $\pm$ 0.15	2.67 $\pm$ 0.11*
CS ( $\mu$ mol/min/g wet tissue)	20.1 $\pm$ 1.14	16.4 $\pm$ 1.54
CS ( $\mu$ mol/min/mg mitochondrial protein)	7.94 $\pm$ 0.45	6.87 $\pm$ 0.49
PDP activity (nmol/min/mg mitochondrial protein)	0.526 $\pm$ 0.073	0.518 $\pm$ 0.097
PDP activity (nmol/min/g wet tissue wt)	7.36 $\pm$ 1.49	5.73 $\pm$ 1.15
PDP1 protein content (arbitrary units)	4206.3 $\pm$ 248.3	4133.4 $\pm$ 517.1

Values are means  $\pm$  SE. BMI, body mass index; CS, citrate synthase. Significantly different from males: \*  $p < .01$ .

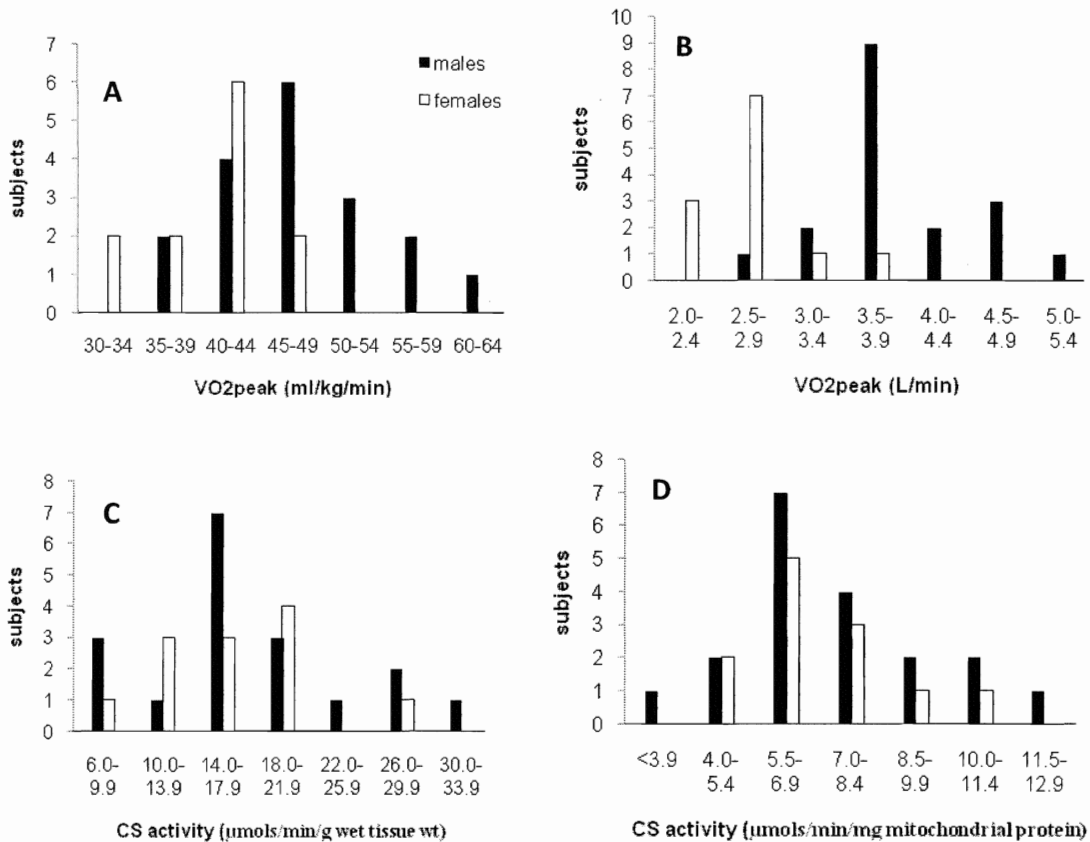


Figure 4.1. Representative histograms of the range of aerobic capacities in male and female subjects for relative (A) and absolute (B)  $VO_{2peak}$ , and CS activity per g wet tissue wt (C) and per mg mitochondrial protein (D).

A significant positive correlation was found between  $VO_{2peak}$  values against CS activity for all subjects together ( $p < 0.05$ ) and for male subjects alone ( $p < 0.01$ ) (data not shown). However, no significant correlations were found within females alone between  $VO_{2peak}$  and CS activity (data not shown).

### ***Dietary Analysis***

Analysis of a self-reported 3-day dietary chart revealed an average consumption of  $2285 \pm 786$  and  $2540 \pm 480$  kcal with a range of 1075 to 3301 kcal and 1754 to 3201 kcal for males ( $n=15$ ) and females ( $n=8$ ) respectively. The composition of these diets was:  $51.0 \pm 7.2\%$  (range: 40.7 to 61.3%) and  $48.3 \pm 7.9\%$  (range: 33.0 to 56.3%) carbohydrate;  $17.5 \pm 5.2\%$  (range: 10.0 to 27.3%) and  $18.8 \pm 5.2\%$  (range: 13.0 to 27.3%) protein; and  $30.6 \pm 4.9\%$  (range: 23.0 to 42.0%) and  $32.3 \pm 6.8\%$  (range: 26.0 to 46.3%) fat for males and females respectively. Values are means  $\pm$  SD.

### ***PDP Activity***

Correlations between PDP activity and aerobic capacity variables are shown in Table 4.2. A strong positive correlation was found in males between PDP activity and absolute  $VO_{2peak}$  ( $r=0.65$ ;  $p<0.01$ ); as well as CS activity (per g wet tissue wt;  $r=0.63$ ;  $p<0.01$  and per mg mitochondrial protein;  $r=0.71$ ;  $p<0.01$ ), with a trend towards a positive correlation for relative  $VO_{2peak}$  ( $r=0.44$ ;  $p=0.08$ ). However, unlike the males, females displayed only a trend towards a positive correlation with absolute  $VO_{2peak}$  ( $r=0.55$ ;  $p=0.10$ ), and no significant correlations found between other aerobic variables and PDP activity. However, despite the differences seen in the way PDP activity in males and females correlates to aerobic capacity (Table 4.2), there was no significant difference in total PDP activity values between males (see Table 4.1).

Table 4.2. Correlations of PDP activity against VO<sub>2peak</sub> and CS measurements.

VARIABLE	PDP activity					
	mg mitochondrial protein			g wet tissue wt		
	r	R <sup>2</sup>	p	r	R <sup>2</sup>	p
<b>MALES (N= 17)</b>						
VO <sub>2peak</sub> (ml/kg/min)	<b>0.44</b>	<b>0.19</b>	<b>0.08</b>	<b><u>0.53</u></b>	<b><u>0.28</u></b>	<b><u>0.03</u></b>
VO <sub>2peak</sub> (L/min)	<b><u>0.65</u></b>	<b><u>0.42</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.65</u></b>	<b><u>0.42</u></b>	<b><u>&lt;0.01</u></b>
CS (μmols/min/g wet tissue wt)	<b><u>0.63</u></b>	<b><u>0.39</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.80</u></b>	<b><u>0.63</u></b>	<b><u>&lt;0.01</u></b>
CS (μmols/min/mg mitochondrial protein)	<b><u>0.71</u></b>	<b><u>0.51</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.77</u></b>	<b><u>0.59</u></b>	<b><u>&lt;0.01</u></b>
<b>FEMALES (N= 10)</b>						
VO <sub>2peak</sub> (ml/kg/min)	0.41	0.17	0.23	0.46	0.21	0.18
VO <sub>2peak</sub> (L/min)	<b>0.55</b>	<b>0.30</b>	<b>0.10</b>	0.52	0.27	0.13
CS (μmols/min/g wet tissue wt)	0.37	0.13	0.30	0.03	<0.01	0.94
CS (μmols/min/mg mitochondrial protein)	0.23	0.05	0.53	0.12	0.01	0.74
<b>ALL (N= 27)</b>						
VO <sub>2peak</sub> (ml/kg/min)	<b><u>0.37</u></b>	<b><u>0.14</u></b>	<b><u>0.05</u></b>	<b><u>0.50</u></b>	<b><u>0.25</u></b>	<b><u>&lt;0.01</u></b>
VO <sub>2peak</sub> (L/min)	<b><u>0.43</u></b>	<b><u>0.19</u></b>	<b><u>0.03</u></b>	<b><u>0.50</u></b>	<b><u>0.25</u></b>	<b><u>&lt;0.01</u></b>
CS (μmols/min/g wet tissue wt)	0.29	0.08	0.15	<b><u>0.63</u></b>	<b><u>0.39</u></b>	<b><u>&lt;0.01</u></b>
CS (μmols/min/mg mitochondrial protein)	<b><u>0.41</u></b>	<b><u>0.17</u></b>	<b><u>0.04</u></b>	<b><u>0.64</u></b>	<b><u>0.41</u></b>	<b><u>&lt;0.01</u></b>

Significant values are underlined in bold italics; trends are shown in bold. CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

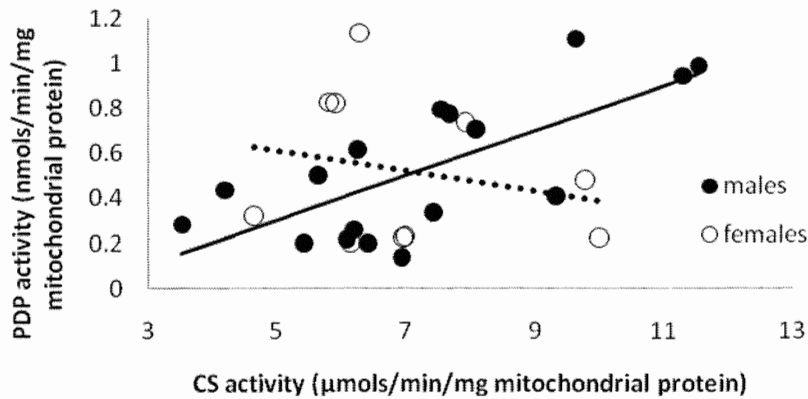


Figure 4.2. Scatterplot of the correlation between CS activity and PDP activity relative to mitochondrial protein in males (solid trendline;  $y = 0.1x - 0.1988$ ,  $R^2 = 0.51$ ,  $p < 0.01$ ) and females (dashed trendline;  $y = 0.0443x + 0.8309$ ,  $R^2 = 0.05$ ,  $p = 0.53$ ). CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

When PDP activity was calculated to the whole muscle (per gram wet tissue weight), there were strong positive correlations found with all aerobic capacity variables (CS and  $VO_{2peak}$ ) for all subjects combined and for males alone. However, no correlations were seen for any aerobic variables against PDP activity in females.

### ***PDP1 Isoform and Mitochondrial Protein Contents***

Significant positive correlations and trends were found between the aerobic variables ( $VO_{2peak}$  and CS) and protein content for all subjects combined and males alone (Tables 4.3 & 4.4). In particular, males demonstrated a significant positive correlation between PDP1 protein content and PDP activity ( $r = 0.47$ ;  $p = 0.05$ ; Figure 4.4). Females showed very few correlations of protein content with aerobic capacity and PDP activity

(Table 4.5). However, significant positive correlations were seen between CS activity (per g wet tissue wt) and both E1 $\alpha$  ( $r= 0.60$ ;  $p= 0.05$ ) and COX IV ( $r= 0.60$ ;  $p= 0.05$ ).

Table 4.3. PDP1, E1 $\alpha$ , E2, and COX IV protein content against VO<sub>2peak</sub>, CS, and PDP activity measurements for all subjects (N= 29).

PROTEIN	PDP1		E1 $\alpha$		E2		COX IV	
	slope	p	slope	p	slope	p	slope	p
VO <sub>2peak</sub> (ml/kg/min)	<b>0.0086</b>	<b>0.08</b>	<b>0.0097</b>	<b>0.10</b>	0.0041	0.26	<b><u>0.0125</u></b>	<b><u>&lt;0.01</u></b>
VO <sub>2peak</sub> (L/min)	0.0567	0.22	0.0407	0.46	0.0215	0.53	<b><u>0.0915</u></b>	<b><u>0.03</u></b>
CS ( $\mu$ mols/min/g wet tissue)	<b><u>0.0179</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0235</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0085</u></b>	<b><u>0.05</u></b>	<b><u>0.0167</u></b>	<b><u>&lt;0.01</u></b>
CS ( $\mu$ mols/min/mg mitochondrial protein)	<b><u>0.0481</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0594</u></b>	<b><u>&lt;0.01</u></b>	<b>0.0224</b>	<b>0.08</b>	<b><u>0.0624</u></b>	<b><u>&lt;0.01</u></b>
PDP activity (nmols Pi/min/mg mito protein)	0.1581	0.24	0.1459	0.39	<b>0.1752</b>	<b>0.09</b>	0.1984	0.11
PDP activity (nmols Pi/min/g wet tissue)	<b><u>13.718</u></b>	<b><u>0.05</u></b>	<b><u>16.85</u></b>	<b><u>0.05</u></b>	<b>9.5368</b>	<b>0.08</b>	<b><u>12.638</u></b>	<b><u>0.05</u></b>

Values are p values and slopes from Pearson's correlation coefficients. Significant values are underlined in bold italics; trends are shown in bold. CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

Table 4.4. PDP1, E1 $\alpha$ , E2, and COX IV protein content against VO<sub>2peak</sub>, CS, and PDP activity measurements for male subjects (N=18).

PROTEIN	PDP1		E1 $\alpha$		E2		COX IV	
	slope	p	slope	p	slope	p	slope	p
VO <sub>2peak</sub> (ml/kg/min)	0.008	0.13	<b><u>0.0205</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0104</u></b>	<b><u>0.01</u></b>	<b><u>0.0157</u></b>	<b><u>&lt;0.01</u></b>
VO <sub>2peak</sub> (L/min)	<b><u>0.1228</u></b>	<b><u>0.04</u></b>	<b><u>0.1829</u></b>	<b><u>0.05</u></b>	<b><u>0.105</u></b>	<b><u>0.04</u></b>	<b><u>0.1894</u></b>	<b><u>&lt;0.01</u></b>
CS ( $\mu$ mols/min/g wet tissue)	<b><u>0.0178</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.026</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0128</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.0172</u></b>	<b><u>&lt;0.01</u></b>
CS ( $\mu$ mols/min/mg mitochondrial protein)	<b><u>0.0377</u></b>	<b><u>0.02</u></b>	<b><u>0.0573</u></b>	<b><u>0.02</u></b>	<b><u>0.0358</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.062</u></b>	<b><u>&lt;0.01</u></b>
PDP activity (nmols Pi/min/mg mito protein)	<b><u>0.2346</u></b>	<b><u>0.05</u></b>	0.1922	0.36	0.1136	0.31	0.2218	0.11
PDP activity (nmols Pi/min/g wet tissue)	<b><u>13.974</u></b>	<b><u>0.02</u></b>	<b><u>18.976</u></b>	<b><u>0.05</u></b>	8.4738	0.11	<b><u>11.831</u></b>	<b><u>0.08</u></b>

Values are p values and slopes from Pearson's correlation coefficients. Significant values are underlined in bold italics; trends are shown in bold. CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

Table 4.5. PDP1, E1 $\alpha$ , E2, and COX IV protein content against VO<sub>2peak</sub>, CS, and PDP activity measurements for female subjects (N=11).

PROTEIN	PDP1		E1 $\alpha$		E2		COX IV	
	slope	p	slope	p	slope	p	slope	p
VO <sub>2peak</sub> (ml/kg/min)	<b>0.0292</b>	<b>0.08</b>	0.0026	0.87	-0.0048	0.68	0.0181	0.14
VO <sub>2peak</sub> (L/min)	0.4089	0.19	0.0739	0.79	-0.0724	0.73	0.2498	0.28
CS ( $\mu$ mols/min/g wet tissue)	0.0189	0.24	0.0192	0.15	-0.0018	0.87	0.0149	0.19
CS ( $\mu$ mols/min/mg mitochondrial protein)	<b>0.0827</b>	<b>0.07</b>	<u><b>0.0759</b></u>	<u><b>0.05</b></u>	-0.013	0.68	<u><b>0.0636</b></u>	<u><b>0.05</b></u>
PDP activity (nmols Pi/min/mg mito protein)	0.0142	0.97	0.0848	0.81	.375	0.12	.1322	0.65
PDP activity (nmols Pi/min/g wet tissue)	19.211	0.50	11.337	0.67	21.057	0.28	19.071	0.39

Values are p values and slopes from Pearson's correlation coefficients. Significant values are underlined in bold italics; trends are shown in bold. CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

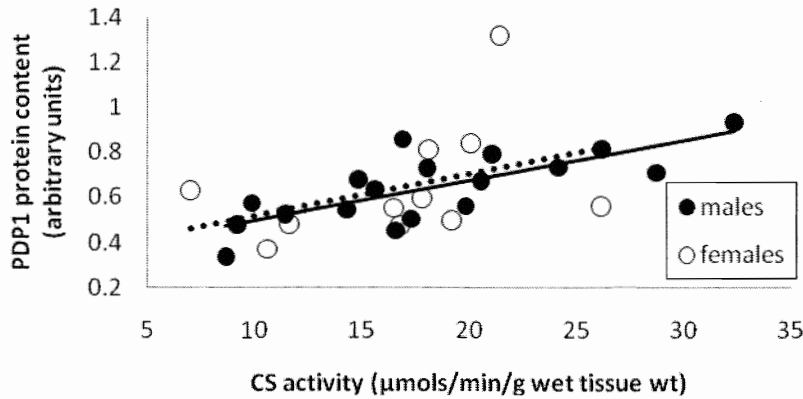


Figure 4.3. Scatterplot of the correlation between CS activity and PDP1 protein content in males (solid trendline;  $y = 0.0178x + 0.3217$ ,  $R^2 = 0.57$ ,  $p < 0.001$ ) and females (dashed trendline;  $y = 0.0189x + 0.3281$ ,  $R^2 = 0.149$ ,  $p = 0.24$ ). CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

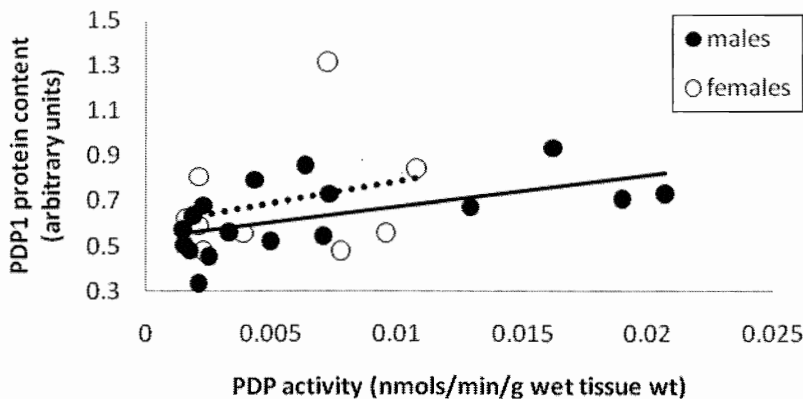


Figure 4.4. Scatterplot of the correlation between PDP activity and PDP1 protein content in males (solid trendline;  $y = 13.974x + 0.538$ ,  $R^2 = 0.33$ ,  $p < 0.05$ ) and females (dashed trendline;  $y = 19.211x + 0.5923$ ,  $R^2 = 0.07$ ,  $p = 0.50$ ). PDP, pyruvate dehydrogenase phosphatase.

Analysis between PDP1, E1 $\alpha$ , E2, and COX IV proteins (Table 4.6) revealed significant positive correlations between PDP1 and E1 $\alpha$  for male subjects ( $r = 0.50$ ;  $p < 0.05$ ) as well as significant positive correlations between PDP1 and COX IV for all

subjects combined ( $r= 0.45$ ;  $p= 0.01$ ) and females alone ( $r= 0.71$ ;  $p= 0.01$ ). E1 $\alpha$  was also found to be positively correlated with COX IV for all subjects combined ( $r= 0.49$ ;  $p< 0.01$ ) and males alone ( $r= 0.46$ ;  $p= 0.05$ ). E2 was also positively correlated with COX IV and E1 $\alpha$  for all subjects and males alone.

*Table 4.6.* Correlations of mitochondrial protein content in males, females, and combined.

Protein		PDP1		E1 $\alpha$		E2	
		slope	p	slope	p	slope	p
E1 $\alpha$	ALL (N= 29)	<b>0.2907</b>	<b>0.07</b>				
	MALES (N= 18)	<b><u>0.3123</u></b>	<b><u>0.04</u></b>				
	FEMALES (N= 11)	0.2539	0.52				
E2	ALL	0.2526	0.34	<b><u>0.7228</u></b>	<b><u>0.02</u></b>		
	MALES	0.4352	0.13	<b><u>0.8837</u></b>	<b><u>0.05</u></b>		
	FEMALES	0.0537	0.92	0.5239	0.23		
COX IV	ALL	<b><u>0.4845</u></b>	<b><u>0.01</u></b>	<b><u>0.623</u></b>	<b><u>&lt;0.01</u></b>	<b><u>0.3473</u></b>	<b><u>0.02</u></b>
	MALES	0.1923	0.36	<b><u>0.6189</u></b>	<b><u>0.05</u></b>	<b><u>0.4728</u></b>	<b><u>&lt;0.01</u></b>
	FEMALES	<b><u>0.9864</u></b>	<b><u>0.01</u></b>	<b>0.6808</b>	<b>0.06</b>	0.1629	0.59

Values are p values and slopes from Pearson's correlation coefficients. Significant values are underlined in bold italics; trends are shown in bold. CS, citrate synthase; PDP, pyruvate dehydrogenase phosphatase.

Interestingly, when both PDP1 and E1 $\alpha$  protein content were normalized to E2 protein content there was a highly significant positive correlation for all subjects combined ( $r= 0.80$ ;  $p< 0.001$ ; Figure 4.5B) and for males ( $r= 0.83$ ;  $p< 0.001$ ) and females ( $r= 0.77$ ;  $p= 0.005$ ) separately.

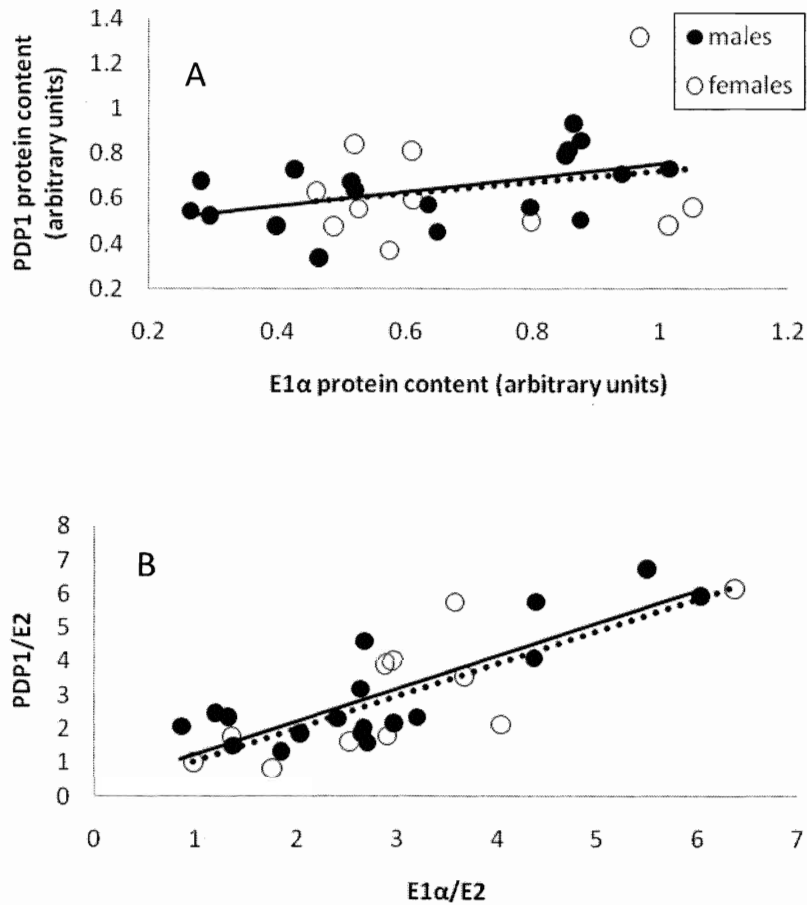


Figure 4.5. Scatterplot of the correlation between PDP1 and E1 $\alpha$  protein content in males (solid trendline; A:  $y = 0.3123x + 0.4436$ ,  $R^2 = 0.25$ ,  $p < 0.05$ ; B:  $y = 0.9672x + 0.2746$ ,  $R^2 = 0.69$ ,  $p < 0.001$ ) and females (dashed trendline; A:  $y = 0.2539x + 0.4706$ ,  $R^2 = 0.05$ ,  $p = 0.52$ ; B:  $y = 0.9594x + 0.0879$ ,  $R^2 = 0.60$ ,  $p < 0.01$ ) before (A) and after (B) both proteins were normalized to E2 protein content. PDP, pyruvate dehydrogenase phosphatase.

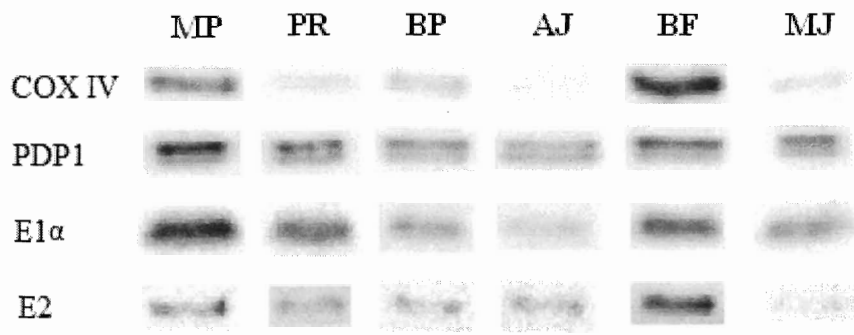


Figure 4.6. Representative Western blots of several subjects for COX IV, PDP1, E1 $\alpha$  and E2 proteins.

## **CHAPTER 5:**

### **DISCUSSION**

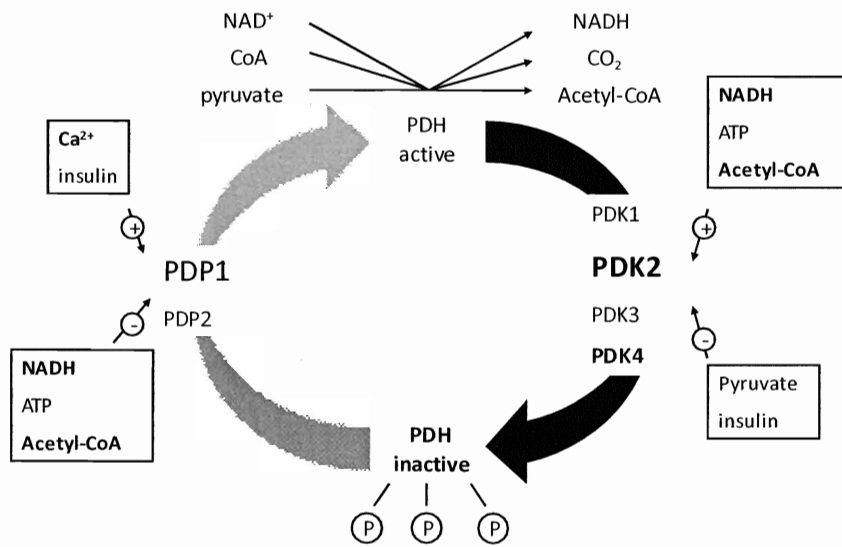
To our knowledge, this is the first study to examine PDP activity in human skeletal muscle, thereby showing that PDP activity can, indeed, be measured in human skeletal muscle. The major findings of the present study were: 1) PDP activity increases with increasing aerobic capacity in males; 2) PDP1 protein increases with increasing aerobic capacity in males, with these two findings resulting in an expected significant correlation between PDP1 protein and PDP activity in males; 3) E2 increased with increasing aerobic capacity, exclusively in males; and 4) when normalized to E2, there was a very strong positive correlation between PDP1 and E1 $\alpha$  in both sexes.

#### ***PDP activity and PDP1 protein content***

One of the most significant findings of this study was that of PDP activity increasing with aerobic capacity, particularly in males. With previous findings that both PDHt activity and PDK activity are increased in human skeletal muscle (in males) following 8 weeks of aerobic exercise training, it would seem necessary that PDP activity would also need to increase to allow for an adequate activation of PDH when it is needed for energy production. Therefore, at the onset of exercise when an increase in ATP production is needed, it can be quickly met through the activation of PDH by Ca<sup>2+</sup> stimulation of PDP1 and then later “fine-tuned” by allosteric regulation of PDK, according to the cellular energy status, to allow for the conservation of carbohydrates (LeBlanc et al., 2004a). This “fine-tuning” by allosteric regulation is accomplished by

the varying concentrations of NADH, ATP, and acetyl-CoA in the cells. With an increase in energy demand by the body (i.e. maximal exercise), the concentrations of these metabolites will decrease as the acetyl-CoA will be used in the TCA cycle to generate more NADH with this NADH in turn being used in the electron transport chain to generate ATP for muscle contraction. During maximal exercise these metabolites will be used very rapidly, meaning that they will not be able to accumulate, thereby releasing their stimulation of PDK to inactivate PDH. At maximal exercise there will also be an increase in glycolysis to help meet the higher energy demands, which will lead to an accumulation of pyruvate, thereby further inhibiting PDK activity (especially PDK2 as it is the most sensitive isoform to pyruvate inhibition; see Figure 5.1B). However, during submaximal exercise there will be an increased reliance on fat oxidation which would cause an increase in NADH and acetyl-CoA, via  $\beta$ -oxidation, and a decreased usage of these metabolites due to the decreased requirement for them at a submaximal workload. As a result, PDK activity (specifically PDK2 & 4, as these are the two isoforms that are most responsive to acetyl-CoA and NADH, respectively) will be stimulated leading to a decrease in PDHa activity. At the same time, these metabolites (NADH, ATP, and acetyl-CoA) also inhibit PDP activity, which means that in situations where these metabolites are increased (i.e. submaximal exercise) PDP activity will be decreased, thereby enhancing the phosphorylated state of PDH and causing inactivation of the complex (see Figure 5.1A). Therefore, after the body's initial responses to exercise (increases in  $[Ca^{2+}]$  and [pyruvate] in the muscle cell, which activate PDH via increased PDP activity and decreased PDK activity, respectively), the concentrations of the products of the PDH reaction (NADH and acetyl-CoA) and ATP provide more specific

### A. Submaximal Exercise



### B. Maximal Exercise

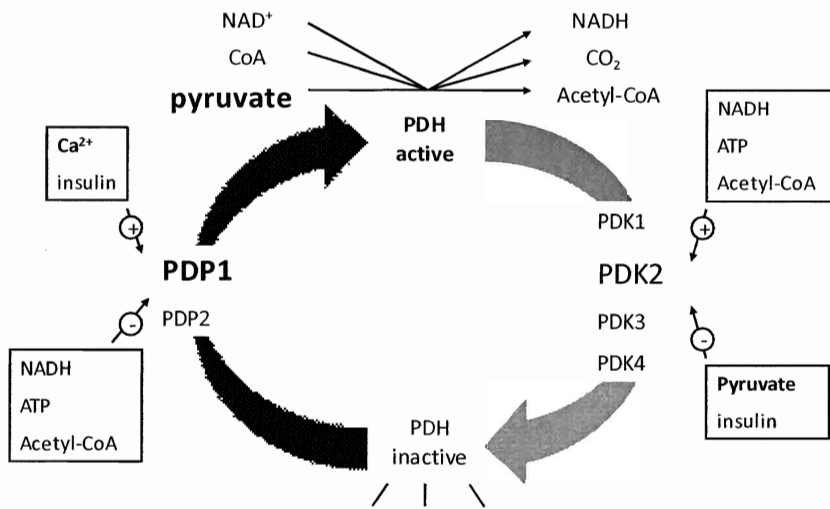


Figure 5.1. Representation of a model of the adaptive changes to PDH complex regulation at submaximal (A) and maximal (B) exercise following aerobic training. Text size indicates an increase in protein content; bold indicates an increase in enzyme activity or metabolite expression. PDH, pyruvate dehydrogenase; PDP, PDH phosphatase; PDK, PDH kinase.

adjustments to the activities of the regulatory proteins of the PDH complex, thereby “fine-tuning” PDHa activity. Therefore, it would seem that with training the body has an increased maximal capacity for carbohydrate oxidation, but is also able to more tightly regulate carbohydrate oxidation (LeBlanc et al., 2008), by allowing it to be used when needed for quick energy production at higher work intensities, but conserving carbohydrates during submaximal exercise with an increased reliance on fat oxidation (as reviewed by Holloszy & Coyle, 1984). This finding of increased PDP activity with increased aerobic capacity is in agreement with LeBlanc et al. (2008) who found that 8 weeks of endurance training lead to an increase in PDP activity in obese rats, with a trend towards increased activity in lean rats. Of note here is that our data also showed that the PDP activity values of human skeletal muscle ( $\sim 0.52$  nmols/min/mg mitochondrial protein) are consistent with those of rat skeletal muscle ( $\sim 0.55$  nmols/min/mg mitochondrial protein; LeBlanc et al., 2007, LeBlanc et al., 2008). Although none of the subjects in the current study could be classified as obese according to BMI ( $< 30$ ; see Table 4.1), it would appear that our cross sectional data is consistent with what has been observed in rats in a longitudinal study. The fact that lean rats were shy of statistical significance may be attributed to training duration, as the rats underwent a training period of 8 weeks, whereas the current study employed a  $VO_{2peak}$  test in which subjects had maintained a consistent activity level for at least 6 months prior to the test. Therefore, a longer duration of training may be required to see significant differences in PDP activity in leaner individuals. However, one cannot dismiss the fact that the current study is cross-sectional in nature, and therefore, any changes observed in PDP activity cannot necessarily be directly attributed to training. It is also possible that these differences in

PDP activity are a result of insulin sensitivity as insulin sensitivity is known to increase with aerobic exercise training (see Zanuso et al., 2009 for review) and since PDP is known to be stimulated by insulin, this may be the cause of the increased PDP activity with aerobic capacity. We cannot rule out the effect of genetics either as this has been shown to be a primary determinant of aerobic capacity (Klissouras, 1971), especially at the whole body level (i.e.  $VO_{2peak}$ ), and may therefore be playing a role in the correlations with enzyme activity. Lastly, recent findings demonstrating the phosphorylation of PDP (Caruso et al., 2001) represent another potential source for these differences as it is possible that the changes in PDP activity may be a result of PDP phosphorylation.

Along with the increase in PDP activity seen with increasing aerobic capacity, there was also a significant increase in PDP1 protein content with muscle aerobic capacity (i.e. citrate synthase activity) in all subjects, with male subjects also showing a significant correlation with whole body aerobic capacity (absolute  $VO_{2max}$ ). This is also similar to what was previously observed in rats (LeBlanc et al., 2008). This increase in protein content would be expected, as PDP1 is the calcium-sensitive isoform of PDP and would, therefore, allow for increased activation of PDH during muscle contraction when cytosolic calcium levels are raised and there is an increased requirement for carbohydrate oxidation to meet energy needs.

In comparing PDP activity with PDP1 protein content, a positive correlation was found in all subjects when PDP activity was expressed relative to wet tissue weight, and in male subjects alone when activity was expressed relative to mitochondrial protein. This suggests that the increase in PDP1 protein content seen with increasing aerobic capacity is largely responsible for the increase in PDP activity seen with increasing

aerobic capacity in males. This is not surprising since PDP1 is the predominant isoform in skeletal muscle (Huang et al., 1998), and therefore, any changes in its content (i.e. an increase) should result in an increase in maximal activity as well.

This finding of the relationship between protein content and activity has also been seen on the other side of PDH regulation, with that of PDK. LeBlanc et al. (2004b) found that with 8 weeks of aerobic training there was an approximate doubling of PDK activity, which was accompanied by a 1.3-fold increase in PDK2 protein content, again showing that increased protein content seems to be a significant cause for increased enzyme activity. Therefore, it would appear that there is a strong correlation between the protein content of the predominant PDH regulatory proteins in skeletal muscle, PDK2 and PDP1, and the maximal activity levels of the kinase and phosphatase in this tissue, respectively.

### ***PDH subunit content***

The present study found higher E1 $\alpha$  content with increasing muscle aerobic capacity in all subjects, which is consistent with previous findings that the protein content of E1 $\alpha$  and PDK2 increase with 8 weeks of aerobic exercise training in humans (LeBlanc et al., 2004b). This increase in E1 $\alpha$  is of particular importance though, as it is the rate-limiting step in the PDH reaction. Therefore, an increase in this subunit with training would allow for a greater maximal flux of carbohydrates through PDH, which would lead to an increased rate of energy production. In addition to this, E1 $\alpha$  and PDP1 protein content were shown to increase together. This is largely to be expected though, as an

increase in E1 $\alpha$  would require an increase in the regulatory subunit to activate it, especially with the previously observed increase in PDK2 with training which would inactivate E1 $\alpha$ . These findings will be discussed in greater detail later.

Previous work looking at the effects of training on changes in the content of the various subunits of PDH found that the subunits do not increase in a uniform manner as E2 and E3BP (unlike E1 $\alpha$ ) demonstrated no significant increases in activity following 8 weeks of endurance training (LeBlanc et al., 2004b). In contrast, however, the present study, in which subjects had maintained a similar training status for at least 6 months, did find a correlation between muscle aerobic capacity and E2 protein content for all subjects, with males in particular showing strong correlations with all aerobic capacity measures. Therefore, taken together, it may be suggested that the protein content of certain subunits (E2 in this case) requires a greater duration of training to observe significant differences. This suggestion is also supported by the finding of a clear trend for an increase in the protein contents of E2 and E3BP in humans following 8 weeks of training, despite a lack of significance (LeBlanc et al., 2004b). Taken together, the trend towards increased E2 after 8 weeks of training and a significant correlation with aerobic capacity in the present study, it would seem that E2 (and very likely E3BP) also increase in protein content with training, albeit at a slower rate than that of E1 $\alpha$ . This may be explained in the context that, as already stated, E1 $\alpha$  is the rate-limiting step in the PDH reaction (Cate et al., 1980), therefore it would be of greater importance to increase the content of this subunit before that of E2. Therefore, with training there is an increase in E1 $\alpha$  content which allows for a greater maximal flux of carbohydrates for oxidation, and therefore, a greater amount of energy to be produced for continued exercise at high

intensities. Following the more rapid increase in E1 $\alpha$  content, there is also an increase in E2 (and very likely other PDH subunits as well, as noted by the trending increase in E3BP) which would again allow for increased carbohydrate oxidation by creating more lipoyl binding domains for E1 $\alpha$  to attach to, allowing for a more rapid transfer of the acetyl intermediate to E2 for further oxidation. In addition, when E1 $\alpha$  is bound to the complex there is a greater ability for PDP and PDK to dephosphorylate and phosphorylate the subunit (Ono et al., 1993, Pettit et al., 1972). Therefore, an increase in E2 could also be seen as enhancing the ability to tightly regulate the activity of the complex by allowing for the more rapid activation or deactivation of the complex through the binding of E1 $\alpha$  to its binding domain.

With the finding that the core of the PDH complex, E2, is more highly expressed with a higher aerobic capacity, we decided to investigate whether E1 $\alpha$  and PDP1 still showed significant increases when increases in the PDH core were compensated for. It was found that the content of both PDP1 and E1 $\alpha$  proteins was greater than the E2 core, but more interestingly, that these proteins co-ordinately increase relative to E2 (Figure 4.5B). Accounting for the E2 core is also important as the binding domains for both E1 $\alpha$  and PDP1 are located on the “swinging arm” of E2. Therefore, it would be inefficient if either E1 $\alpha$  or PDP1 were to increase faster than the other in comparison to E2, as both proteins must be bound to their respective binding domains on E2 in order for dephosphorylation to occur, and each E2 subunit has only one binding domain for both E1 $\alpha$  and PDP1. An increase in either protein over the other would most likely result in the lesser protein being a limiting factor in the PDH reaction, thereby making the increase in that protein futile. Interestingly, females also showed a strong positive correlation

between E1 $\alpha$  and PDP1 when expressed relative to E2, despite showing very few correlations with aerobic capacity when E1 $\alpha$  and PDP1 were expressed on their own. With both females and males showing significance in this correlation, it suggests that the PDH complex varies together as a whole, with E1 $\alpha$  and PDP1 increasing at the same rate relative to the core of the PDH complex regardless of the magnitude of change relative to oxidative capacity.

### *Sex differences*

Despite numerous significant correlations between aerobic capacity and PDP activity and several proteins of the PDH complex (E1 $\alpha$ , E2, and PDP1) in male subjects, there were very few correlations (even considering the trends) between aerobic capacity and PDP activity and proteins of the PDH complex in females alone. However, this is not entirely unexpected, since numerous studies have shown that females differ from males in terms of fuel selection during aerobic activities. In these studies it has been found that recreationally active and endurance trained women utilize less carbohydrate than men, who were matched by  $VO_{2peak}$  to the women, during moderate-intensity exercise (Devries et al., 2006, Tarnopolsky et al., 1990). Therefore, it is entirely possible that the enzymes involved in carbohydrate metabolism and those regulating them would be affected by, if not causing the effect of, a decreased usage of carbohydrates in females. Therefore, the lack of a correlation seen between PDP activity and aerobic capacity in females may be a result of their decreased carbohydrate utilization, in comparison to males, and therefore, they require less activation of PDH by PDP. However, it should

also be noted that there have been differences seen in substrate utilization between the phases of the menstrual cycle, with the common finding that women use more carbohydrates in the follicular phase than the luteal phase (Devries et al., 2006, Campbell et al., 2001, Zderic et al., 2001). In the present study, menstrual cycle phase was not accounted for, making it possible that the reason for differences in sex were a result of the majority of females being tested in the luteal phase, however, this is only speculation and cannot be confirmed. However, there have also been several studies demonstrating no differences in substrate utilization between the menstrual cycle phases (Casazza et al., 2004, Suh et al., 2002, Suh et al., 2003). Therefore, future research in this area should give consideration to the differences between the sexes as well as possible differences in the phases of the female menstrual cycle.

Although there was no significant correlation in females between aerobic capacity and PDP activity, there was a trend for an increase in PDP1 protein content with relative  $VO_{2peak}$  and CS activity relative to mitochondrial protein. Therefore, the protein content does appear to be there if increased PDH activation is required. Additionally, there was no difference seen in total PDP activity between males and females (Table 4.1). This is not what would be expected, as females would be expected to have lower activity values for PDP since they rely less on carbohydrate metabolism for energy production, and therefore, would have less of a need for PDH activation by PDP in comparison to males. However, the activity values in this study are maximal in nature, therefore it is possible that there is a disconnect between maximal activity and that which is observed acutely *in vivo*. Taken together, with a trend toward increased PDP1 protein content with aerobic capacity, and a lack of difference between maximal enzyme activities between males and

females, it is possible that females rely on another level of post-translational PDP regulation.

### ***Possible sources for sex differences***

A possible source of these differences is that females may rely more on the phosphorylation of PDP to regulate PDP activity, as this would lend some explanation to the differences between PDP1 protein content and that of maximal activity when compared to aerobic capacity. However, research investigating the possibility of PDP phosphorylation is still ongoing and has not yet been proven to be physiologically relevant in skeletal muscle (Caruso et al., 2001). Alternatively, the levels of PDP2 protein and its activity cannot be overlooked, as females may rely on it more heavily than males. Numerous studies have found that  $17\beta$ -estradiol ( $E_2$ ; the main form of estrogen in the body) plays a role in insulin sensitivity in the body (Puah & Bailey, 1985, Kumagai et al., 1993, Gonzalez et al., 2000, Ordonez et al., 2008) and with estrogen being the main female sex hormone this could play a large role in substrate utilization by females. Puah & Bailey (1985) found “evidence that  $E_2$  promotes insulin-mediated glucose uptake and utilization in skeletal muscle.” Therefore, given the higher concentrations of  $E_2$  in females and its effects on insulin, it is possible that females do rely more on the insulin sensitive isoform, PDP2, for activation of the PDH complex. If females do indeed rely more heavily on PDP2, this could represent another factor influencing the decreased utilization of carbohydrates by females as PDP2 has been found to have a decreased ability to reactivate the PDH complex than that of PDP1, especially when more than one

site has been phosphorylated. This would lead to a decrease in PDHa activity, and therefore, there would be a decreased utilization of carbohydrates. However, further research would be required to investigate this theory.

The possibility of differences in muscle fibre types between the sexes influencing our results cannot be dismissed either, as no analysis was done to determine the proportion of type I and IIa fibres in the biopsies taken. Several studies have found that females have a smaller proportion of type II fibres (with values ranging from 7-19% less; Simoneau & Bouchard, 1989, Miller et al., 1993, Carter et al., 2001) and a greater proportion of type I fibres (with values ranging from 9-11% more; Simoneau & Bouchard, 1989, Carter et al., 2001) when compared with males (Brooke & Engel, 1969), leading to a strong possibility that this was also the case in the present study. The possibility of fibre type being a cause for the sex differences observed between males and females is given greater weight by the findings of LeBlanc et al. (2007 & 2008) which found that PDP1 protein content is significantly greater in the red gastrocnemius (predominantly type IIa fibres, with some type I) than white gastrocnemius (predominantly type IIb) and soleus (predominantly type I) of rat skeletal muscle. In addition to this, it was found that PDP2 protein is only expressed in red gastrocnemius of rats, with no detectable levels in other fibre types. Therefore, with the decreased proportion of type II fibres seen in females compared to males (Simoneau & Bourchard, 1989, Miller et al, 1993, Carter et al., 2001), it would seem that females would have less of both PDP isoforms since both isoforms seem to be expressed to a greater degree in type IIa muscle fibres. With a decrease in the content of PDP as a result of these fibre type differences, this could further explain the lack of significant correlations seen in

females between PDP1 content and aerobic capacity measures. However, more research is required on this as these studies were done on male rats, therefore it cannot be said for certain that PDP1 and PDP2 proteins would be expressed in female fibre types the same as that of males. Nevertheless, a study by Carter et al. (2001) demonstrates the strong possibility that fibre type differences between the sexes is a large factor for the differences in carbohydrate and fat metabolism, as they found that males and females do not have differences in the maximal activities of several enzymes responsible for  $\beta$ -oxidation, the TCA cycle, and the electron transport chain. Without differences in the maximal enzyme activities of these metabolic pathways, especially  $\beta$ -oxidation, it could be postulated that the differences in substrate metabolism between the sexes are a result of different expressions and locations of these enzymes in the body (particularly muscle).

When comparing whole body aerobic capacity ( $VO_{2peak}$ ) against mitochondrial oxidative capacity (CS activity), our data demonstrated that males had a significant correlation between the two measures, while females did not show any correlation. However, this discrepancy can probably be explained by body composition as it has long been established that females have a higher body fat percentage than males, with males possessing a much greater lean mass (see Wells, 2007 for review). When expressed relative to fat free mass, it has been found that there is no difference in  $VO_{2peak}$  between males and females (Devries et al., 2006). Therefore, it would be expected that CS measures would be far more representative of the true aerobic capacity for females as this would eliminate the confounding factor of their excess adipose tissue. This appears to be the case as significant correlations that were seen in males between aerobic capacity (both

VO<sub>2peak</sub> and CS measurements) and protein content were usually only seen in females when comparing protein content against CS measures.

However, it should be noted that in the present study there were fewer females (n= 12) than males (n= 18), with a smaller range in values for aerobic capacity in the females as well. Therefore, it is possible that due to the smaller sample size of our female volunteers, and the fact that they represented a less heterogeneous mixture of aerobic capacity compared to our males, it was simply more difficult to make solid significant correlations.

### ***Conclusions***

These data strongly suggest that the changes seen in PDP activity and PDP1 protein content are a result of aerobic capacity relative to training status, but cause and effect cannot be conclusively proven due to the cross-sectional design of this study. However, many of our observations are in agreement with previous longitudinal training studies in both rats and humans, lending support to the strong possibility that the novel findings in this study are indeed because of differences in aerobic training status. Therefore, since our results demonstrate that E1 $\alpha$  and PDP1 content increase coordinately relative to the complex, and previous work showed that E1 $\alpha$  subunit content increased with 8 weeks of aerobic training (LeBlanc et al., 2004b), it follows that a similar increase in PDP1 protein content would occur with 8 weeks of aerobic training. Additionally, an increase in PDP activity and PDP1 protein content has already been noted in a rat model, making it highly possible that such differences also exist in other

mammals (e.g. humans). However, further longitudinal training studies are necessary to confirm the findings in the present study, and it will be necessary to study males and females separately.

Some caution should be taken in extrapolating PDP activity values to *in vivo* maximal activity rates, as the present study investigated activity using a synthetic peptide substrate (rather than the natural intact complex substrate) under *in vitro* assay conditions. In addition, our assay measures total PDP activity and cannot differentiate between the PDP1 and PDP2 contributions. As it has been found that PDP1 and PDP2 display different sensitivities toward the different phosphorylation sites on E1 $\alpha$  (Kolobova et al., 2001, Karpova et al., 2003), it is difficult to determine how the increased activity of PDP would specifically relate to E1 $\alpha$  activation *in vivo*. However, as the data in this study would suggest, the increase in PDP activity is largely the result of an increase in the content of PDP1 (at least in male subjects), and as this is the isoform that has been found to have a higher specific activity towards the E1 $\alpha$  phosphorylation sites (Kolobova et al., 2001, Karpova et al., 2003), it would be expected that the increased activity seen here should lead to an increased activation of the E1 $\alpha$  subunit in exercising human skeletal muscle.

Taking the training studies together (LeBlanc et al., 2008, LeBlanc et al., 2004b) with the data from this study, a model of PDH complex regulation during exercise is emerging. As a result of training, there is an increase in the ability of the body to oxidize carbohydrates for immediate energy production due to an increase in the E1 $\alpha$  subunit of PDH. However, there is also an increase in the body's ability to regulate this depending

on the intensity at which the body is working. During submaximal exercise the body is able to decrease carbohydrate oxidation by inactivating PDH through PDK2, which increases in content following training, and is strongly activated by acetyl-CoA which would be produced in great amounts by  $\beta$ -oxidation of fats (see Figure 5.1A). However, at maximal exercise PDH is activated by the elevated content of PDP1, which is itself activated by the increased levels of cytosolic  $\text{Ca}^{2+}$  that occur with muscle contraction. Additionally, the elevated levels of PDK2 are inactivated due to this isoform's high inhibition by pyruvate, which will be produced in large amounts by glycolysis in order to meet the body's high energy demands at maximal exercise, allowing for greater activation of PDH (see Figure 5.1B).

### **Future Directions**

The present study was, to the best of our knowledge, the first to examine PDP activity in human skeletal muscle and found that with increasing aerobic capacity there is an increase in PDP activity and PDP1 protein expression. However, future work should focus on extending the results of this cross-sectional study by examining PDP activity and expression in humans in a longitudinal training study. Also, in determining protein expression, only the PDP1 isoform was investigated (as there is no commercially available antibody for human PDP2), meaning that we cannot rule out the influence that PDP2 may have had on overall PDP activity. Therefore, future analysis should include both PDP1 and PDP2 isoforms when investigating the effects of training.

Additionally, the general lack of correlation for females in many PDP activity and expression measures indicates that more research is needed to investigate the differences in PDH complex regulation between males and females, specifically concerning the female menstrual phase, as well as lean body mass and skeletal muscle fibre type. Previous studies have found that there are differences in substrate utilization between the phases of the menstrual cycle (Devries et al., 2006, Campbell et al., 2001, Zderic et al., 2001), therefore the effects of this on PDP activity and expression, as it relates to carbohydrate oxidation, should be further investigated in both the luteal and follicular phases of the menstrual cycle.

Analysis of the different skeletal muscle fibre types would also be an important consideration for future studies in humans, since previous studies have already determined differences in PDP activity and expression in rodent models (LeBlanc et al., 2007, LeBlanc et al., 2008). These differences between fibre types could also lead to a better understanding of the divergent results observed between males and females in this study, as males and females appear to have different fibre type distributions.

Lastly, the 1hr 21,000g spin used in the methods of the present study removed the mitochondrial membrane from the sample, meaning that PDP activity was analyzed from the mitochondrial matrix alone. However, studies have suggested that PDP may be closely associated with the inner mitochondrial membrane (Simonot et al., 1997), making it possible that there may be some PDP activity (if not greater activity) in the membrane portion if it were to be analyzed. Therefore, future studies should also include analysis of the mitochondrial membrane for PDP activity.

## Implications and Perspectives

Carbohydrates are an exceedingly important fuel source for the body because they can supply ATP at a rapid rate when needed, and certain tissues rely on them solely for energy production (see Fehm et al., 2006 for review). Additionally, the tremendous increase in the prevalence of type II diabetes mellitus (T2DM) over the past decades, largely due to the obesity epidemic, has led to an even greater focus on carbohydrate metabolism and glucose homeostasis in the body. Therefore, understanding the body's uptake, storage, and usage of carbohydrates is of great importance in helping curb the present trends in the health status of society. A main area of focus for this research has been in establishing ways to maintain normal blood glucose concentrations, possibly by reducing the plasma glucose concentration through oxidative skeletal muscle disposal (see Stump et al., 2006 for review). Therefore, PDH may play a large role in maintaining blood glucose levels at normal concentrations in those with T2DM or some level of insulin resistance. Recent findings seem to indicate this very theory, as they found that in the skeletal muscle of PDK4 knockout (PDK4<sup>-/-</sup>) mice that were fed a high-fat diet there was greater PDHa activity in comparison to wild-type mice that were fed the same diet. Along with this, they found that PDK4<sup>-/-</sup> mice had improved glucose tolerance, lower fasting blood glucose levels, and greater insulin sensitivity in comparison to their wild-type counterparts (Jeoung & Harris, 2008). These findings imply that PDHa activity can play a role in enhancing insulin sensitivity and ameliorating high plasma glucose concentrations in those with T2DM. An increase in PDP1 protein with exercise would be of great importance to this, as those with T2DM (or some level of insulin resistance) have a decreased ability to respond to insulin, which means that they will have to rely more on

the calcium-sensitive isoform of PDP1, than the insulin-sensitive PDP2 isoform, in order to activate the PDH complex to decrease glucose levels. Therefore, the findings in the present study that PDP activity and PDP1 protein expression increase with aerobic capacity (which increases with training; see Powers & Criswell, 1996 for review) potentially presents good prospects for those with T2DM to correct insulin sensitivity and hyperglycaemia by allowing for increased PDHa activity.

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## APPENDIX

Table A.1. Raw data for male subjects

Subject	VO <sub>2peak</sub>		CS activity (μmols/min)		PDP activity (nmols/min)		Protein Content, A.U.			
	(ml/kg/min)	(L/min)	(mg mitochondrial protein)	(g wet tissue wt)	(mg mitochondrial protein)	(g wet tissue wt)	PDP1	E1α	E2	COX IV
AJ	36	2.7	6.092	14.78	0.217	2.304	0.681	0.281	0.329	0.160
AT	48	3.8	7.736	19.35	0.437	4.704	0.337	0.464	0.145	0.179
BF	57	4.7	11.106	26.10			0.818	0.855	0.357	0.708
BP	54	3.7	11.287	28.67	0.946	18.954	0.713	0.939	0.353	0.374
CO	49	3.7	6.944	17.21	0.137	1.533	0.507	0.874	0.324	0.381
JY	49	3.2	9.316	16.49	0.413	2.519	0.455	0.648	0.245	0.634
KM	55	4.1	6.412	19.75	0.201	3.323	0.565	0.796	0.432	0.422
MP	65	5.4	11.536	32.30	0.991	16.160	0.938	0.863	0.631	0.692
NJ	41	3.9	8.091	14.24	0.708	7.059	0.548	0.265	0.223	0.402
NP	48	4.6	9.628	24.07	1.112	20.667	0.736	1.013	0.342	0.527
PR	40	3.7	7.679	17.97	0.776	7.287	0.732	0.426	0.160	0.218
RE	41	3.6	6.508	18.36	0.285	3.505	0.481	0.398	0.152	0.281
RM	42	3.2	5.418	15.55	0.202	1.899	0.638	0.520	0.095	0.112
RN	49	3.7	5.648	16.83	0.503	6.355	0.860	0.875	0.145	0.075
RP	47	4.0	7.433	20.96	0.339	4.322	0.796	0.851	0.195	0.382
RW	40	3.6	6.355	18.53	0.261	2.788	0.575	0.634	0.312	0.379
SL	54	4.6	7.554	20.47	0.796	12.888	0.675	0.513	0.117	0.387
SM	51	3.9	8.196	20.39	0.616	8.875	0.525	0.295	0.224	0.263

Table A.2. Raw data for female subjects

Subject	VO <sub>2peak</sub>		CS activity (μmols/min)		PDP activity (nmols/min)		Protein Content			
	(ml/kg/min)	(L/min)	(mg mitochondrial protein)	(g wet tissue wt)	(mg mitochondrial protein)	(g wet tissue wt)	PDP1	E1α	E2	COX IV
AA	43	3.0	7.930	20.05	0.736	10.790	0.839	0.521	0.146	0.278
CB	44	2.7	6.988	17.77	0.222	2.170	0.592	0.613	0.096	0.452
ChO	35	2.6	6.171	18.08	0.199	2.177	0.808	0.610	0.451	0.236
EF	46	3.6	6.307	11.29	1.136	9.748				
HR	39	2.6	5.836	11.64	0.829	7.748	0.477	1.014	0.578	0.382
KP	46	2.8	9.782	21.33	0.476	7.257	1.316	0.967	0.329	0.821
LH	40	2.6	10.000	26.14	0.220	3.953	0.557	1.051	0.260	0.286
MiK	40	2.1	4.654	7.04	0.318	1.663	0.626	0.460	0.160	0.111
MJ	30	2.3	4.749	10.64			0.372	0.573	0.228	0.216
MK	42	2.9	6.997	16.78	0.226	2.295	0.474	0.489	0.133	0.184
SG	33	2.3	7.074	19.16			0.494	0.797	0.275	0.353
SW	42	2.5	5.924	16.47	0.820	9.546	0.556	0.525	0.540	0.314

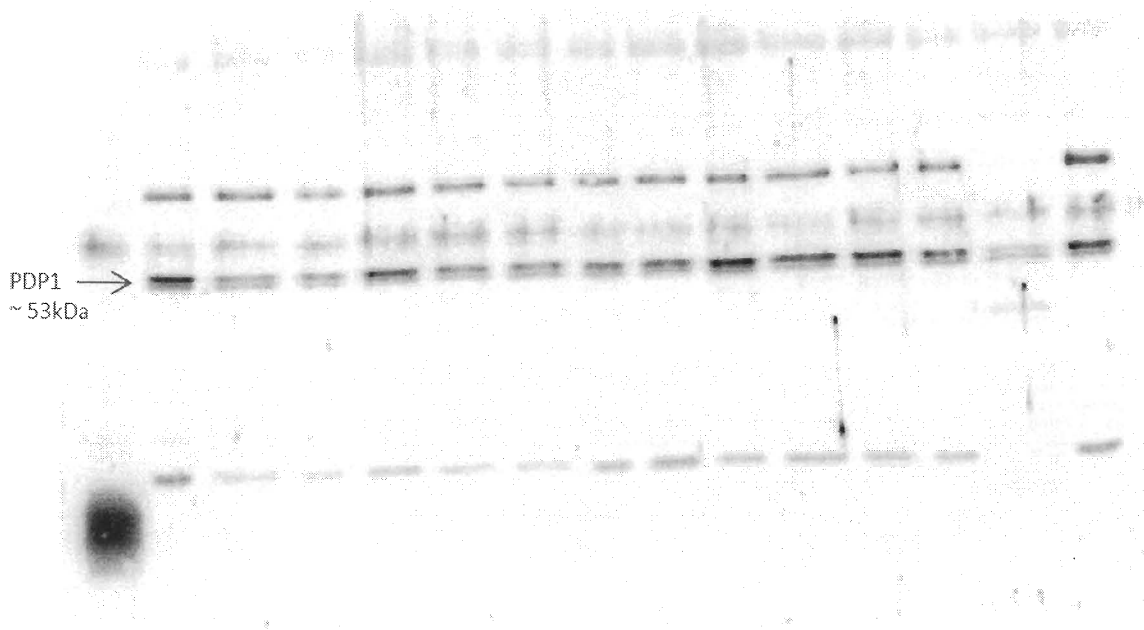


Figure A.1. Representative Western blot for PDP1.

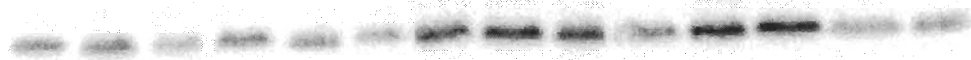


Figure A.2. Representative Western blot for E1 $\alpha$ .



Figure A.3. Representative Western blot for E2.



Figure A.4. Representative Western blot for COX IV.

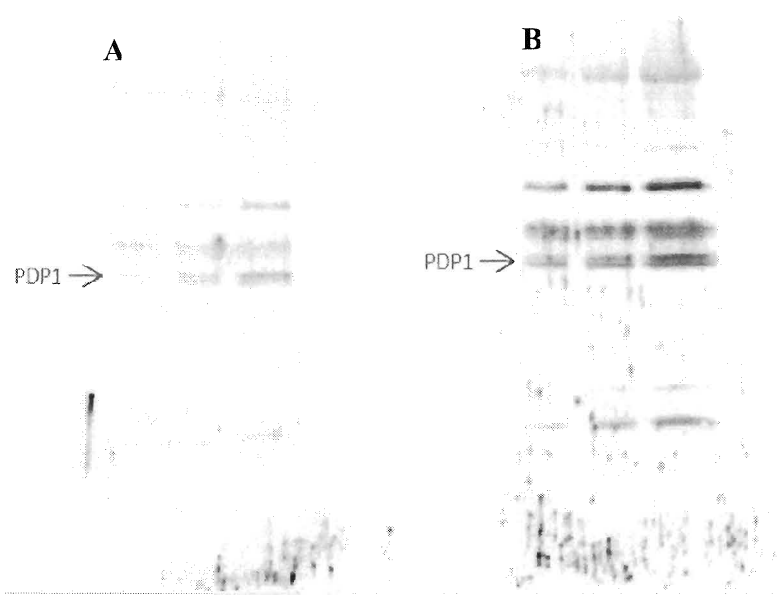


Figure A.5. Optimization blots for PDP1. Lanes were loaded (from left to right) with 2.5, 5, and 10µg of protein with the primary antibody diluted 1:2000 (A) and 1:1000 (B).

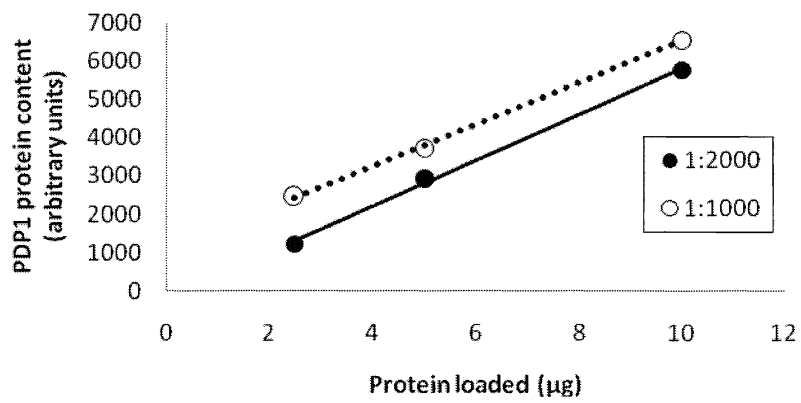


Figure A.6. Scatterplot of optimization values for PDP1 for 1:1000 and 1:2000 primary antibody dilution.