DYNAMIC AND STABLE REGULATION OF PYRUVATE DEHYDROGENASE IN HUMAN SKELETAL MUSCLE.

by

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DYNAMIC AND STABLE REGULATION OF PYRUVATE DEHYDROGENASE IN HUMAN SKELETAL MUSCLE.

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ABSTRACT

The mechanisms regulating the rate-determining enzyme of carbohydrate oxidation, pyruvate dehydrogenase (PDH), were examined in human skeletal muscle at rest and during exercise in response to acute respiratory alkalosis and short- and long-term aerobic training.

Voluntary hyperventilation-induced respiratory alkalosis (R-Alk) delayed PDH activation during the transition from rest to submaximal exercise. A mismatch between pyruvate production and its oxidation in R-Alk resulted in a 35% higher lactate accumulation. These effects were not seen during steady state exercise. The results from this study suggest that respiratory alkalosis may play an important role in lactate accumulation during the transition from rest to exercise in acute hypoxic conditions, but that other factors mediate lactate accumulation during steady state exercise.

Short-term aerobic training did not alter resting skeletal muscle total PDH (PDHt) and PDH kinase (PDK) activities, or their respective protein expressions compared to pre-training. In contrast, long-term aerobic training (Post) compared to pre-training (Pre) resulted in a 31% increased total PDHt activity, partially due to a 1.3 fold increased protein expression of a PDH subunit, $PDHE_1\alpha$. Despite the increased PDHt activity post-training, there was an approximate 37% attenuated activation of PDH Post after 15 min of exercise at the same absolute submaximal workload compared to Pre. PDK demonstrated a 2 fold increased activity Post, partially attributed to a 1.3 fold increased PDK2 isoform protein expression. The training-induced increased PDK2 isoform expression, pyruvate-sensitive PDK isoform, coincided with an attenuated skeletal muscle pyruvate content

Post during submaximal exercise at the same absolute workload. The results of these findings suggest that aerobically trained human skeletal muscle has an increased maximal capacity to utilize carbohydrates, evident by increased PDHt, but increased metabolic control sensitivity to pyruvate during submaximal exercise through increased contribution of PDK2 to total PDK activity.

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Format and Organization of Thesis

The present thesis was prepared in the "sandwich format" as outlined in the School of Graduate Studies' Guide for the Preparation of Theses. This thesis is comprised of three original research papers. One of the papers (Chapter 1) has been published and the two others (Chapters 2 and 3) have been submitted to peer reviewed journals with the candidate as first author.

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIP

CHAPTER 1

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Contribution

The experiments were coordinated and conducted by P.J. LeBlanc with the assistance of the co-authors. The primary supervisor for this study was Dr. G.J.F. Heigenhauser in conjunction with Dr. N.L. Jones. Muscle biopsies were obtained by Dr. G.J.F. Heigenhauser. Respiratory gas measurements and muscle and blood samples were collected, processed, and analyzed by P.J. LeBlanc. Statistical analyses and manuscript preparation were completed by P.J. LeBlanc.

CHAPTER 2

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The experiments were coordinated and conducted by P.J. LeBlanc with the assistance of Drs. S.J. Peters and G.J.F. Heigenhauser. The primary supervisor for this study was Dr. G.J.F. Heigenhauser in conjunction with Dr. S.J. Peters. Muscle biopsies were obtained by Dr. G.J.F. Heigenhauser. The training program was monitored by P.J. LeBlanc with assistance from B. Easterbrook. Muscle samples were collected, processed, and analyzed by P.J. LeBlanc, with the exception of mRNA analysis which was processed and analyzed by P.J. LeBlanc with the assistance of R.J. Tunstall and Drs. D. Cameron-Smith and M. Hargreaves. Statistical analyses and manuscript preparation were completed by P.J. LeBlanc.

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Contribution

The experiments were coordinated and conducted by P.J. LeBlanc with the assistance of the co-authors. The primary supervisor for this study was Dr. G.J.F. Heigenhauser in conjunction with Dr. M.J. Gibala. The training program was monitored by K.R. Howarth. Muscle biopsies were obtained by Dr. G.J.F. Heigenhauser. Respiratory gas

measurements and muscle samples were collected, processed, and analyzed by P.J. LeBlanc with assistance from K.R. Howarth. Statistical analyses and manuscript preparation were completed by P.J. LeBlanc.

General Introduction

The rate of ATP demand during exercise in human skeletal muscle can increase by up to 100-fold over rest (Hochachka & Matheson, 1992). The intramuscular content of ATP is limited however and would become quickly depleted if not for the precise metabolic control exhibited by the muscle. As a result, the rates of ATP production and ATP utilization are closely matched. This is accomplished primarily through enzyme regulation, utilizing metabolic pathways that release the chemical energy from their respective available fuels. At rest and during exercise, the primary fuels utilized by skeletal muscle are carbohydrates, in the form of plasma glucose and muscle glycogen, and fats, in the form of plasma free fatty acids (FFA) and intramuscular triglycerides (TG; see Henriksson, 1995;Holloszy *et al.*, 1998;Hultman, 1995 for review). Amino acids are also metabolized, but account for less than 3-6% of the energy requirements during exercise (see Hargreaves & Snow, 2001 for review). Skeletal muscle metabolism is a complex yet integrated series of processes that lead to a common goal of generating ATP. Once produced, ATP is the only available form of energy that is utilized by the contractile apparatus within the muscle. Depending on the source of fuel, ATP regeneration can occur either through substrate-level or oxidative phosphorylation. Substrate-level phosphorylation occurs mainly in the cytosolic compartment of the cell via phosphocreatine (PCr) breakdown and glycolysis. On the other hand, oxidative phosphorylation occurs in the mitochondria and is dependent on the availability of NADH, O_2, ADP and inorganic phosphate (P_i).

At rest, skeletal muscle utilizes approximately equal amounts of carbohydrates, in the form of plasma glucose, and fat, in the form of plasma FFA (Van Loon *et al.*, 2001). During low to moderate levels of exercise (up to approximately 55% maximal workload), total fat and carbohydrate oxidation rates increase from rest, with contributions from muscle glycogen and intramuscular triglycerides accounting for over 65% of energy expenditure (Romijn *et al.*, 1993;Van Loon *et al.*, 2001). However, the relative contribution of fat and carbohydrate to total energy expenditure remains similar to rest (Van Loon *et al.*, 2001). At higher workloads (>70% maximal workload), carbohydrates, in the form of muscle glycogen, become the dominant fuel source (Romijn *et al.*, 1993;Van Loon *et al.*, 2001).

Pyruvate dehydrogenase complex

The pyruvate dehydrogenase enzyme complex (PDH), found in the mitochondrial inner membrane-matrix space, is an important regulatory site between carbohydrate-derived substrate-level and oxidative phosphorylation in resting and exercising skeletal muscle (Fig 1). Carbohydrate-derived oxidative phosphorylation is dependent on the activity level of PDH, as it is the rate-determining enzyme that regulates the entry of glycolytically produced pyruvate into the tricarboxylic acid (TCA) cycle. The PDH enzyme reaction is catalyzed sequentially by three components of the complex. Pyruvate dehydrogenase (E_1) is the first component of the enzyme complex that irreversibly decarboxylates pyruvate. This results in a cascade of enzyme reactions through the other two enzymatic subunits (dihydrolipoamide transacetylase, E_2;

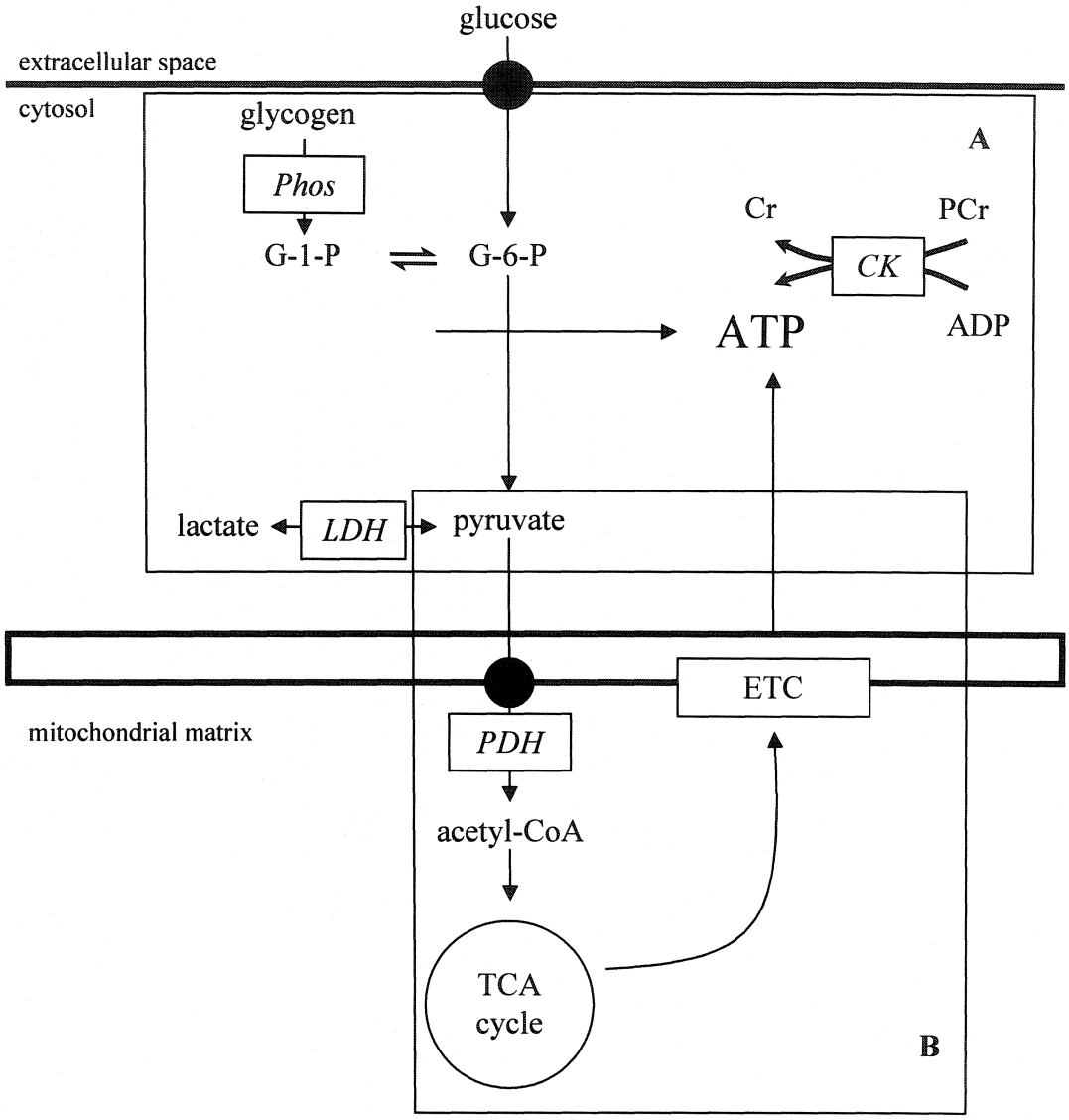

Figure 1. Simple schematic of skeletal muscle carbohydrate metabolism. Pyruvate dehydrogenase (PDH) is situated in an important regulatory step between A) substrate-level phosphorylation and B) oxidative phosphorylation. Phos, glycogen phosphorylase; CK, creatine kinase; LDH, lactate dehydrogenase; ETC, electron transport chain; PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

dihydrolipoamide dehydrogenase, E3) resulting in a net production of acetyl-CoA (Voet & Voet, 1995). In addition, PDH contains a tightly-associated protein (E3bp), anchoring E3 subunits to the E2 core (Harris *et al.*, 1997), and two regulatory enzymes, PDH kinase (PDK) and PDH phosphatase (PDP; Denton *et al.*, 1975;Wieland, 1983). Structurally, the PDH enzyme complex is built around an inner core of 60 E_2 subunits (see Harris *et al.*, 2002 for review). To this core, 30 E_1 heterotetrameric ($\alpha_2\beta_2$) and 12 E_3bp subunits are bound noncovalently, with 12 E_3 homodimers bound to the complex by way of the E_3bp. Including 1-2 homodimers of PDK and 2-3 heterodimers of PDP, the PDH multienzyme complex is composed of 222-226 subunits (Harris *et al.*, 2002).

PDH exists in two interconvertable forms; a phosphorylated inactive form and a dephosphorylated active form (Fig 2). Interconversion of PDH is accomplished by the two covalent modulators PDK and PDP, which catalyze the phosphorylation and dephosphorylation of PDH, respectively (Denton *et al.*, 1975;Stansbie, 1976). The PDH-$E_1\alpha$ subunit possesses 3 seryl residues that, when phosphorylated, determine the activation state of PDH. Phosphorylation of the first site (Ser-264; Korotchkina & Patel, 1995;Yeaman *et al.*, 1978) is sufficient for more than 98% inactivation of the enzyme complex. In addition, the heterotetrameric E_1 which processes 2 α subunits, exhibits half-of-the-site reactivity, as phosphorylation of one of the two Ser-264 is needed for inactivation of the PDH complex. The other two sites are considered barrier sites and phosphorylation of these sites (Ser-271, site 2; Ser-203, site 3) result in a negligible decrease in PDH activity (Korotchkina & Patel, 1995;Yeaman *et al.*, 1978), however,

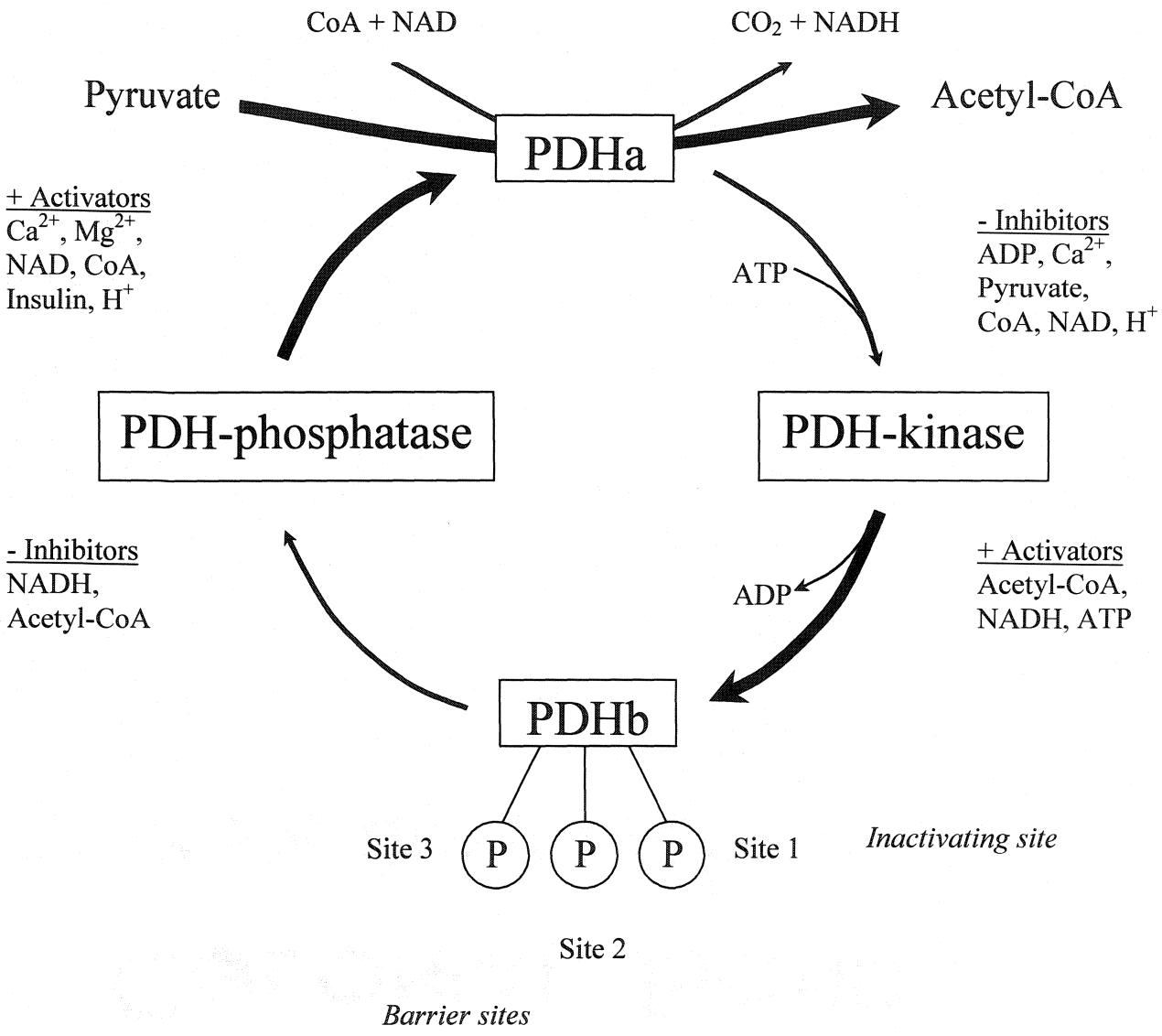

Figure 2. Pyruvate dehydrogenase enzyme complex control by a phosphorylation and dephosphorylation cycle.

represents a hyperphosphorylated enzyme complex that requires a greater degree of dephosphorylation by PDP before activation.

The complexity of PDH control by the two covalent modulators in mammals is enhanced by the presence of four PDK isoforms (PDK1-4; Bowker-Kinley *et al.*, 1998) and two PDP isoforms (PDP1 and 2; Huang *et al.*, 1998). Inter comparison of both PDK and PDP isoforms reveals differences in specific activities and kinetic properties, tissue specific expression and concentration, species-specific tissue distribution, and their responsiveness to different allosteric regulators, resulting in unique responses to certain metabolic demands. The isoform-specific response to allosteric modulators will be discussed in the *Dynamic Regulation* section below.

PDK1 has a limited tissue distribution, being only found in cardiac tissue of rats (Bowker-Kinley *et al.*, 1998) and humans (Gudi *et al.*, 1995). However, in humans, small amounts are found also in skeletal muscle, liver, pancreas, brain, placenta, lung, and kidney (Gudi *et al.*, 1995). PDK1 demonstrates a high specific activity compared to the other isoforms (Gudi *et al.*, 1995). The site specific phosphorylation of the three sites on $E_1\alpha$ by PDK1 compared to the other isoforms is unique in that it has mid (site 1) to low (site 2) phosphorylation activity, however, it is the only isoform that phosphorylates site 3 (Korotchkina & Patel, 2001).

PDK2 has the lowest specific activity of all the isoforms and is the most ubiquitous in terms of tissue distribution, being found in heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis of rat (Bowker-Kinley *et al.*, 1998). Humans demonstrate the same tissue distribution, however the highest concentration of PDK2 is

found in cardiac and skeletal muscle (Gudi *et al.*, 1995). PDK2 demonstrates the highest rate of PDH-$E_1\alpha$ site 1 phosphorylation of all PDK isoforms, and approximately doubles its activity with a reduced or acetylated E_2 inner lipoyl domain compared to the oxidized form (Korotchkina & Patel, 2001).

PDK3 has the highest specific activity of all the isoforms (Korotchkina & Patel, 2001). Its tissue distribution is somewhat different than that of the other PDK isoforms. In rats, PDK3 has the highest concentrations in testis, but is also found in brain, spleen, lung, and kidney (Bowker-Kinley *et al.*, 1998), whereas in humans it is present only in heart and skeletal muscle (Gudi *et al.*, 1995).

The final PDK isoform, PDK4, has a tissue distribution similar to that of PDK2 and 3, with the exception that the relative mRNA abundancy was absent in brain, spleen, and testis of rats (Bowker-Kinley *et al.*, 1998). Like PDK2, PDK4 was also highest in concentration in cardiac and skeletal muscles (Rowles *et al.*, 1996).

Very little is known about the two PDP isoforms. The relative activities of each isoform are quite similar (Huang *et al.*, 1998). PDP1 is preferentially expressed in heart, brain and testis of rats, with detectable levels in spleen, lung, liver, skeletal muscle and kidney (Huang *et al.*, 2003). In contrast, PDP2 is abundant in heart, brain, liver, adipose tissue and kidney, lower levels detected in spleen and lung and below the level of detection in skeletal muscle and testis (Huang *et al.*, 1998;Huang *et al.*, 2003).

The regulation of PDH is complex and multifactorial. Acute or short-term regulation of PDH can be accomplished through covalent, allosteric, substrate and end-product regulation. On the other hand, more chronic or long-term regulation can occur

through stable increased protein content of individual components that make up the PDH complex, which include PDH subunits and the two regulatory proteins PDK and PDP. For the purpose of this thesis, acute or short-term modulation of PDH has been termed dynamic regulation whereas more chronic or long-term modulation has been termed stable regulation.

Dynamic Regulation

There appears to be a direct correlation between the activation of PDH and its catalytic activity. Previous human skeletal muscle studies have shown that flux through PDH is approximately equivalent to its level of activity (Gibala *et al.*, 1998;Howlett *et al.*, 1998;Putman *et al.*, 1995b;Putman *et al.*, 1995a;Watt *et al.*, 2002a) and activation of PDH through the interaction of metabolic regulators will be equal to its flux. This association is lost only in situations of limited substrate availability, which was demonstrated in human skeletal muscle during exercise after a low carbohydrate diet (Putman *et al.*, 1993). Covalently, the activity of PDH is determined by the proportion of the complex transformed from the inactive form to the active form, which is mediated by PDK and PDP. This control of PDH activation is a result of the dynamic combined activity of both PDK and PDP, with changes to either regulatory enzyme altering the activation state of PDH. Each of the covalent regulators of PDH is subject to allosteric regulation. Increased ratios of acetyl-CoA-to-CoA, ATP-to-ADP and NADH-to-NAD^+ activate PDK and inhibit PDP (Denton *et al.*, 1975;Pettit *et al.*, 1975). In addition, pyruvate inhibits PDK (Cate & Roche, 1978), whereas Ca^{2+}, insulin, and H^+ activate PDP (Chen *et al.*,

1996). The evidence of dynamic regulation by the allosteric effectors discussed below are imparted on total PDK and PDP activities and do not reflect individual isoform response, unless otherwise stated.

Calcium

Calcium (Ca^{++}) is an important regulator, responsible for PDP-mediated dephosphorylation and activation of the $E_1\alpha$ subunit, but only in the presence of magnesium (Mg^{++}). Increased Ca^{++} stimulates PDP activity, specifically PDP1 (Huang *et al.*, 1998) by 1) decreased K_m of PDP for Mg^{++} (Thomas *et al.*, 1986) and 2) assisted binding of PDP with E_2 (Pettit *et al.*, 1972), more specifically the inner lipoyl domain (Chen *et al.*, 1996;Yang *et al.*, 1998). In addition, it has been shown that Ca^{++} can also inhibit PDK activity (Cooper *et al.*, 1974).

Insulin

Much like Ca^{++}, insulin appears to act on PDP, specifically PDP2 (Huang *et al.*, 1998). Insulin has been reported to acutely activate PDH through PDP activation (Macaulay & Jarett, 1985;Nakai *et al.*, 1999;Popp *et al.*, 1980). The mode of action of insulin is through an increased sensitivity of PDP to Mg^{++} independent of changes in Ca^{++} (Thomas *et al.*, 1986). The effects of insulin may not be limited to PDP as it has been demonstrated *in vivo* that administration of insulin after 24 hours of starvation restores PDK activity similar to the fed state in rat lactating mammary gland (Baxter & Coore, 1978) and kidney (Cockburn & Coore, 1995).

ATP and ADP

The influences of ATP and ADP as allosteric modulators are directed towards PDK. Phosphorylation, and resultant inactivation, of PDH by PDK is ATP dependent. ADP competes with ATP for the catalytic binding site (Linn *et al.*, 1969), thus preventing ATP-mediated PDK activity. The apparent K_m values of the PDK isoforms for ATP were some what similar between PDK1, PDK3 and PDK4 (50-65 μM) and lower for PDK2 (10 μM; Bowker-Kinley *et al.*, 1998). For PDK2, PDK3 and PDK4, the apparent K_i for ADP were within a similar range (80-120 μM), whereas PDK1 was approximately 3 times higher (370 μM; Bowker-Kinley *et al.*, 1998).

NADH and NAD^+

The effects of NADH and NAD^+ on mammalian PDH complex *in vitro* have been well documented. NADH (0.01-0.5 mM) acts as an allosteric activator of PDK (Cate & Roche, 1978;Cooper *et al.*, 1975;Kerbey *et al.*, 1979;Pettit *et al.*, 1975) and inhibitor of PDP (Pettit *et al.*, 1975), whereas NAD^+ (0.5-2 mM) has no effect on PDK on its own but reverses the activation induced by NADH (Cooper *et al.*, 1975). NADH concentration required to achieve maximal stimulation of PDK is ~20 μM, with half-maximal at about 4 μM (Cate & Roche, 1978). PDK1-3 demonstrate similar activation response with increased [NADH] (1.1-1.3 fold increase), whereas PDK4 activity approximately doubles (Bowker-Kinley *et al.*, 1998). It has been hypothesized that the reduction of the lipoyl moiety of the PDH-E_2 subunit by NADH is responsible for the increased activation of

PDK (Cate & Roche, 1978) and inhibition of PDP (Rahmatullah & Roche, 1988). Few measurements have been made of *in vivo* [NADH] and [NAD^+], with mixed results, due to methodological differences. In addition to methodology, subcellular distribution of NADH and NAD^+ has made it difficult to estimate mitochondrial redox and its effects on PDH activation. Thus, until the development of a reliable method to determine NADH and NAD^+ in tissue, *in vivo* effects of redox on PDH remain speculative.

Acetyl CoA and CoA

The effects of acetyl-CoA and CoA on PDK occur independent of each other as well as together. Acetyl-CoA (0.01-0.5 mM) stimulates PDK activity (Cooper *et al.*, 1975;Kerbey *et al.*, 1979;Pettit *et al.*, 1975), which is increased in the presence of reducing compounds (e.g. NADH, thiols) (Cate & Roche, 1978;Pettit *et al.*, 1975). In fact, one of the most recognizable differences between PDK2 and the other isoforms is its unique response to acetyl-CoA and a high NADH/NAD^+ ratio, increasing its activity to more than 3 fold compared to control (Bowker-Kinley *et al.*, 1998;Popov, 1997). It has been hypothesized that this stimulation occurs through enhanced acetylation of the inner lipoyl moiety of PDH-E_2 subunit (Cate & Roche, 1978). On the other hand, CoA inhibits PDK activity and the strength of this inhibition is decreased with increasing NADH:NAD^+ (Cooper *et al.*, 1975;Pettit *et al.*, 1975).

Pyruvate

Pyruvate serves as both a substrate for PDH and an allosteric inhibitor of PDK. *In vitro*, pyruvate has been shown to inhibit PDK activity (Baxter & Coore, 1978;Cate & Roche, 1978;Cooper *et al.*, 1974). Comparatively, dichloroacetate (DCA), a pyruvate analogue, inhibits PDK activity in a concentration-dependent manner (Cooper *et al.*, 1974;Whitehouse *et al.*, 1974). PDK2 demonstrated the highest sensitivity to DCA of all the isoforms, whereas PDK3 was relatively insensitive and PDK1 and PDK3 were intermediate (Bowker-Kinley *et al.*, 1998). The sensitivity of PDK to either pyruvate (Pratt & Roche, 1979) or DCA (Baker *et al.*, 2000;Popov, 1997) is increased with higher [ADP].

pH

Both regulatory proteins of PDH are pH sensitive. It has been reported that bovine kidney and heart PDK have a peak optimum pH of 7.0-7.2 in the presence of either Mg^{++} or Mn^{++}, whereas that for PDP is 6.7 in the presence of Mg^{++} and 7.5-7.6 in the presence of Mn^{++} (Hucho *et al.*, 1972).

Intrinsic and free forms of PDK and PDP

PDK is known to exist in two states, intrinsic (bound) and free (unbound; Jones & Yeaman, 1991;Kerbey *et al.*, 1984;Mistry *et al.*, 1991). Intrinsic PDK binds to the innermost lipoyl domain of the E_2 component of PDH (Ravindran *et al.*, 1996) and represents only 15-30% of the total PDK activity (Kerbey *et al.*, 1984;Mistry *et al.*,

1991;Vary & Hazen, 1999). Shifts from free to intrinsic PDK may enhance the enzyme activity with little or no change in protein synthesis. In addition, intrinsic PDK activity is enhanced when associated with higher ratios of reduced or acetylated forms of the E_2-lipoyl domain compared to the oxidized form (Korotchkina & Patel, 2001), which is dependent on the intramuscular content of allosteric modulators of PDK. Although this hasn't been studied directly, it can be assumed that the same intrinsic and free forms of PDP also exist. The activity of PDP is altered through changes in intramuscular content of allosteric modulators, thus altering PDP's association with the inner lipoyl domain of the PDH-E_2 subunit.

PDH regulation in human skeletal muscle during submaximal exercise

The glucose-fatty acid (G-FA) cycle, first proposed by Randle and colleagues (see Randle, 1998 for review), suggested that carbohydrate utilization by muscle is down regulated with increased availability and utilization of fat. It was postulated that increased fat oxidation increased muscle citrate and acetyl-CoA content, resulting in inhibition of phosphofructokinase and PDH, respectively. The possibility exists that this regulatory mechanism may regulate fuel utilization in human skeletal muscle at rest, however, there has been some controversy regarding the existence of the G-FA cycle during exercise.

Numerous studies have examined the regulation of PDH activation during submaximal exercise in human skeletal muscle. These studies were carried out using various submaximal exercise intensities, altered substrate availability, induced acid-base

changes, and altered oxygen delivery in an attempt to ascertain which of the allosteric modulators are responsible for regulating the exercise-induced activation of PDH and determine the existence of the G-FA cycle in human skeletal muscle during exercise.

The activation of PDH during exercise is dependent on the intensity of exercise, with higher intensities resulting in higher PDH activation (Howlett *et al.*, 1998). This correlation may be due to exercise-dependent alteration in [Ca^{++}], glycogenolytic flux (thus pyruvate availability) and/or energy status of the cell (ADP_f), known modulators of PDH.

Acute increases in fat availability, with the infusion of Intralipid for 30 min, resulted in an attenuated activation of PDH during moderate intensity exercise (40-65% VO_{2max}; Odland *et al.*, 1998;Odland *et al.*, 2000). The increased fat availability to the muscle at the early stages of exercise stimulates fat metabolism, resulting in an increased mitochondrial [NADH], and decreases glycogenolysis, resulting in decreased pyruvate availability. NADH inhibits PDP and activates PDK (Pettit *et al.*, 1975) where as pyruvate inhibits PDK (Baxter & Coore, 1978;Cate & Roche, 1978;Cooper *et al.*, 1974), resulting in an attenuated activation of PDH. This effect is lost at higher intensities of exercise (85% VO_{2max}; Dyck *et al.*, 1993) as other modulators, such as Ca^{++} and ADP_f, may override the effects of NADH and pyruvate.

In contrast, acute increases in carbohydrate availability to skeletal muscle with nicotinic acid (Stellingwerff *et al.*, 2003) or carbohydrate (Watt *et al.*, 2002b) ingestion or epinephrine infusion (Watt *et al.*, 2001) increased PDH activation, and thus carbohydrate oxidation, during submaximal exercise. This was attributed to epinephrine-induced

increases in intramitochondrial Ca^{++} (Stellingwerff *et al.*, 2003;Watt *et al.*, 2001) or carbohydrate-induced increase in plasma insulin (Watt *et al.*, 2002b), with no changes seen with the other PDH modulators.

Increasing acetyl-CoA to CoA ratio *in vivo* with a low carbohydrate diet (Putman *et al.*, 1993;St.Amand *et al.*, 2000) or acetate infusion (Putman *et al.*, 1995b) decreased the activation of PDH at rest. The modulation of acetyl-CoA:CoA on PDH activation is lost during submaximal exercise, being overridden by PDH modulators such as pyruvate (Putman *et al.*, 1993;Putman *et al.*, 1995b). Thus, it appears that both acetyl-CoA and CoA are important regulators of PDH activation at rest but not during exercise.

Intramitochondrial [pyruvate] and carrier-mediated mitochondrial pyruvate transport have been proposed as a source of control over PDH activation (see Wieland, 1983 for review). Some studies have demonstrated that pyruvate availability in exercising human skeletal muscle correlates with the level of PDH activation (Howlett *et al.*, 1998;Odland *et al.*, 2000;Parolin *et al.*, 1999;Putman *et al.*, 1993;Putman *et al.*, 1995a;Putman *et al.*, 1999;St.Amand *et al.*, 2000). However, recent studies demonstrate an altered pyruvate flux through PDH, despite no change in skeletal muscle pyruvate content (Stellingwerff *et al.*, 2003;Watt *et al.*, 2002b;Watt *et al.*, 2002a). This discrepancy regarding the contribution of skeletal muscle pyruvate in regulating PDH activation may be attributed to; 1) intramitochondrial [pyruvate] would influence PDH activation, however, the [pyruvate] reported are of the whole muscle, 2) changes in skeletal muscle pyruvate content needed to alter PDH activation may be too small to detect as the K_i of pyruvate in human skeletal muscle is currently unknown 3) other

modulators, such as ADP_f, may be more important in regulating PDH activation, with pyruvate playing a minor role.

Alterations in systemic acid-base status lead to changes in lactate production and endogenous and exogenous skeletal muscle carbohydrate catabolism. These changes have been attributed, in part, to the differences in pH optima of the two covalent modulators of PDH, PDK and PDP. Decreased systemic pH, through metabolic acidosis, resulted in a decreased activation of PDH during incremental submaximal exercise (Hollidge-Horvat *et al.*, 1999). This is contrary to what would be expected if the activation of PDH was dependent on the pH optimums of both PDK and PDP, with an acidotic state (intramuscular pH; 6.8 in control vs. 6.6 in acidotic) that would favor PDP and inhibit PDK. Contrary to acidosis, metabolic alkalosis resulted in an increased activation of PDH during incremental submaximal exercise, however, only during 60% VO_{2max} (Hollidge-Horvat *et al.*, 2000). Again, this is contrary to what would be expected if the pH optima of PDK and PDP solely determined the activation of PDH, with an elevated pH during alkalosis favoring an increased PDK activity and inhibiting PDP. These results suggest that factors other than pH sensitivity of PDK and PDP contribute to PDH activation with induced metabolic acid-base alterations.

Elevated whole body and muscle lactate accumulation during exercise in a hypoxic environment has been attributed to delayed activation of skeletal muscle PDH (Heigenhauser & Parolin, 1999). Changes in O_2 availability to the whole body do not necessarily translate to changes in O_2 supply at the exercising muscle, thus affecting PDH activation during exercise, since alterations in muscle blood flow help maintain VO_2

(Adams & Welch, 1980;Bender *et al.*, 1988;Knight *et al.*, 1993). However, changes in O_2 availability may indirectly alter PDH activity. Hypoxia (10.9% inspired O_2) delayed the activation of PDH at the onset of exercise, with no changes in the intramuscular content of known regulators of PDH (Parolin *et al.*, 2000). Although not measured, it was hypothesized that the hyperventilation-induced increase in muscle pH contributed to the delayed activation of PDH with hypoxia. This effect was lost later in exercise as pH decreased in parallel with increased lactate accumulation.

In summary, the regulation of PDH activation during exercise in human skeletal muscle is not as previously proposed by the G-FA cycle, as the regulation of acetyl-CoA imparted on PDH is lost during exercise. During exercise, intensity-dependent changes in skeletal muscle [Ca^{++}] crudely activate PDH, whereas changes in [pyruvate], energy status of the cell (ADP_f), and redox (NADH and NAD^+) fine tune PDH activation.

Stable Regulation

PDH and its subunits

Long-term or chronic alterations to the activation state of PDH can be accomplished through stable changes in the absolute levels of the PDH complex subunits, thus increasing total PDH (PDHt). Changes in the expression of PDH subunit genes, and resultant altered PDHt, would increase maximum potential carbohydrate oxidation. Thus, alterations to overall metabolism (e.g. repeated bouts of exercise) would be reflected in adaptive changes in PDHt and expression of PDH subunits.

A few studies have examined the effects of training on human skeletal muscle PDHt activity, demonstrating no change after 8 days of aerobic exercise (Putman *et al.*, 1998) and 5 weeks of strength exercise (Ward *et al.*, 1986). However, a recent study reported an increase in PDHt activity with long-term aerobic exercise in mouse hind limb (Houle-Leroy *et al.*, 2000), suggesting that prolonged aerobic training may alter skeletal muscle PDHt activity in humans.

In contrast, no studies have been conducted to examine the relative transcriptional and translational responses of the PDH subunits in skeletal muscle during adaptive changes in PDHt. However, three studies examined PDH subunit expression in response to obesity (Amessou *et al.*, 1998), altered diet (Da Silva *et al.*, 1993), and suckling to weaning transition (Maury *et al.*, 1995) in rat non-muscle tissues. Also, increased PDH subunit expression, with concomitant increased PDHt activity, has been demonstrated in differentiating 3T3-L1 adipocytes (Hu *et al.*, 1983). Thus, long-term regulation of PDHt involves coordinated regulation of PDH subunit transcriptional and/or translational expression.

PDK and PDP

Alterations in absolute levels of either PDH regulatory protein, PDK and PDP, could also contribute to the regulation of PDH activation. The rate of activation of PDH is dependent on the ratio of active PDK and PDP, thus change in the expression of either covalent modifier would alter the rate of activation or inactivation of PDH. Skeletal muscle PDK responds to physiological and pathological perturbations, demonstrating

increased activity in rats with fasting (Fuller & Randle, 1984;Peters *et al.*, 2001a;Stace *et al.*, 1992;Sugden *et al.*, 2000a;Wu *et al.*, 1999), high-fat feeding (Holness *et al.*, 2000), chemically-induced diabetes (Fuller & Randle, 1984;Wu *et al.*, 1999), aerobic training (Nakai *et al.*, 1999), and sepsis (Vary, 1991;Vary & Hazen, 1999), along with high-fat feeding in humans (Peters *et al.*, 1998;Peters *et al.*, 2001b). In contrast, only one study examined the effects of physiological or pathological perturbations on skeletal muscle PDP, reporting no changes in PDP activity in rat hind-limb with fasting or alloxan diabetes (Fuller & Randle, 1984). The only studies to demonstrate changes in PDP activity were restricted to non-muscle tissues, reporting decreases in PDP activity during starvation in rat kidney (Cockburn & Coore, 1995) and starvation and diabetes in rat heart and kidney (Huang *et al.*, 2003). Thus, stable regulation of PDK, rather than PDP, may be central to control of the activation state of PDH in skeletal muscle.

Little information is available regarding the contribution of PDK1 and 3 isoforms in adaptive changes to skeletal muscle PDK activity due to their relative tissue distribution, since PDK2 and 4 are the most abundant isoforms present in skeletal muscle. A recent study that examined the relative response of PDK isoforms in rat skeletal muscle demonstrated no change in PDK1 protein content with fasting (Peters *et al.*, 2001a). More studies, specifically in humans, are needed to ascertain the relative importance of PDK1 and 3 to adaptive increased PDK activity.

Most of the literature has focused on the adaptive response of PDK4. Rat skeletal muscle PDK4 demonstrated increased protein expression with fasting (Peters *et al.*, 2001a;Sugden *et al.*, 2000a;Wu *et al.*, 1999), streptozotocin-induced diabetes (Wu *et al.*,

1999), high fat feeding (Holness *et al.*, 2000), and hyperthyroidism (Sugden *et al.*, 2000b). High fat feeding (Peters *et al.*, 2001a), starvation and streptozotocin-induced diabetes (Wu *et al.*, 1999) also increased skeletal muscle PDK4 mRNA levels. The effects seen with fasting and diabetes are reversed with refeeding (Sugden *et al.*, 2000a;Wu *et al.*, 1999) and insulin (Wu *et al.*, 1999), respectively. To date, only one study has examined PDK4 mRNA and protein expression together, reporting an increase in both with a high fat diet in human skeletal muscle (Peters *et al.*, 1998). Other human skeletal muscle studies only examined mRNA, reporting increased PDK4 with starvation (Pilegaard *et al.*, 2003) and recovery from an acute bout of exercise (Nordsborg *et al.*, 2003;Pilegaard *et al.*, 2000). These increases in PDK4 expression have been attributed to fatty acid activation of peroxisome proliferators-activated receptors (PPAR), specifically PPARα (Huang *et al.*, 2002;Wu *et al.*, 1999;Wu *et al.*, 2001). Thus, it appears that PDK4, the "lipid-status" responsive isoform (Sugden *et al.*, 2001), responds to acute alterations in lipid availability.

Few studies have examined the adaptive response of PDK2, reporting unresponsive expression to fasting (Peters *et al.*, 2001a;Sugden *et al.*, 2000a;Wu *et al.*, 1999) and streptozotocin-induced diabetes (Sugden *et al.*, 2000a;Wu *et al.*, 1999) in rats and to high fat diet (Peters *et al.*, 1998) in humans. However, one study examined the correlation between insulin sensitivity and PDK mRNA in skeletal muscle of a population of Pima Indians, demonstrating that PDK2, and to a lesser extent PDK4, mRNA were correlated with clinical characteristics of the onset of type II diabetes (Majer *et al.*, 1998).

Thus, the role of PDK2 may be to mediate PDH activity during more long-term perturbations (e.g. exercise training, diabetes).

Rationale for the Following Studies

The primary purpose of this thesis was to assess the control of PDH activation, and thus carbohydrate metabolism, in human skeletal muscle during exercise. This was accomplished by examining the mechanisms by which allosteric and covalent modulators regulate PDH during acute (respiratory alkalosis, short-term aerobic exercise) and chronic (long-term aerobic exercise) perturbations.

First Study – Respiratory Alkalosis

During periods of acute hypoxia, submaximal exercise at a given absolute power output results in increases in both muscle and plasma lactate concentrations compared to normoxia (Brooks *et al.*, 1992;Brooks *et al.*, 1998;Green *et al.*, 1992b;Katz & Sahlin, 1987;Parolin *et al.*, 2000). The concept of lactate production was previously ascribed to a reduction in oxygen supply, with lactate production supplementing the reduced ATP supply from oxidative phosphorylation. Recently, it has been suggested that lactate production may be due to an imbalance of pyruvate metabolism rather than limited oxygen supply (see Heigenhauser & Parolin, 1999 for review). The flux-generating enzyme of pyruvate production, through glycogenolysis, is glycogen phosphorylase (Phos), whereas pyruvate-derived acetyl-CoA availability for the TCA cycle is controlled by PDH. A mismatch between pyruvate production and pyruvate oxidation leading to

excess pyruvate will result in it being converted to lactate by the enzyme lactate dehydrogenase (LDH). This takes place simply by mass-action effects because LDH is an enzyme with an equilibrium constant that favors lactate over pyruvate in skeletal muscle (Voet & Voet, 1995). Thus, increased pyruvate production and/or decreased pyruvate oxidation will lead to an increased lactate accumulation.

Hyperventilation is a direct result of hypoxia, with ventilation rates rising to 150-200 % above normoxia (Lenfant & Sullivan, 1971). The result of hyperventilation is an associated alkalosis in the blood, with a rise in pH of 0.1-0.3 units (Davies *et al.*, 1986;Edwards & Clode, 1970;Zborowska-Sluis *et al.*, 1970). The elevated blood pH may translate into an elevated muscle pH (Heisler, 1975), which would potentially increase and decrease the activity of both PDK and PDP, respectively, resulting in a decreased activation state of PDH. Thus, extracellular alkalosis through voluntary hyperventilation may account, in part, for decreased PDH activation and result in increased lactate production seen during bouts of hypoxia.

The purpose of the first study was to examine the effects of respiratory alkalosis, through voluntary hyperventilation, on the activation state of PDH, along with its metabolic regulators, and lactate production in human skeletal muscle at rest and during exercise. The hypothesis was that hyperventilation will result in decreased activation of PDH due to the pH sensitivity of both PDK and PDP, with a resultant increase in lactate production. In addition, rates of glycogenolysis, along with Phos activity and its metabolic regulators, will also be examined, to ascertain their contribution to increased lactate production seen with respiratory alkalosis.

Second Study – Short- and Long-Term Aerobic Training, Covalent Modulators

During exercise at maximal oxygen uptake, carbohydrates are the only fuel utilized in skeletal muscle, mainly in the form of muscle glycogen (see Hultman, 1995 for review). Repeated bouts of aerobic exercise have been shown to increase maximal oxygen uptake, muscle glycogen storage, and muscle glycogen utilization during exercise at maximal oxygen uptake (see Henriksson, 1995 for review). Central to this adaptation may be PDH, where training-induced mitochondrial biogenesis may result in an increased total PDH (PDHt). To date, only two studies have examined the effects of training on human skeletal muscle PDHt, demonstrating no change after 8 days of aerobic exercise (Putman *et al.*, 1998) and 5 weeks of strength exercise (Ward *et al.*, 1986). However, a recent study reported an increase in PDHt activity with long-term aerobic exercise in mouse hind limb (Houle-Leroy *et al.*, 2000), suggesting prolonged aerobic training may alter skeletal muscle PDHt activity in humans. Also, few studies have examined the relative transcriptional and translational responses of each PDH subunit during adaptive changes in PDHt, reporting changes exclusively in non-muscle tissues (Amessou *et al.*, 1998;Da Silva *et al.*, 1993;Maury *et al.*, 1995).

Another skeletal muscle adaptive response to prolonged aerobic training is a greater reliance on fat for ATP synthesis during submaximal exercise (Henriksson, 1977;Hurley *et al.*, 1986;Martin *et al.*, 1993;Phillips *et al.*, 1996b), resulting in a net glycogen sparing effect. PDH is an important regulatory site for carbohydrate metabolism. The activation of PDH is a dynamic system and alterations in either PDK

and/or PDP activity will affect PDH activity. A training-induced attenuated activation of PDH may be due to an increased PDK or decreased PDP. Regulation of PDK may be central to control of the activation state of PDH in prolonged metabolic perturbations, including aerobic training, since skeletal muscle PDK activity responds to a number of physiological and pathological perturbations in rats (Fuller & Randle, 1984;Holness *et al.*, 2000;Nakai *et al.*, 1999;Peters *et al.*, 2001a;Sugden *et al.*, 2000a;Vary & Hazen, 1999;Wu *et al.*, 1999) and in humans (Peters *et al.*, 1998;Peters *et al.*, 2001b), whereas skeletal muscle PDP activity is unresponsive in rats (Fuller & Randle, 1984).

The complexity of PDH control by PDK is enhanced by the presence of four isoforms (PDK1-4; Bowker-Kinley *et al.*, 1998), with PDK2 and 4 being the most abundant isoforms represented in human skeletal muscle (Gudi *et al.*, 1995). Each isoform has differing concentrations, specific activities, and kinetic properties, resulting in unique responses to certain metabolic demands. PDK4, the "lipid-status" responsive isoform (Sugden *et al.*, 2001), responds to acute alterations in lipid availability whereas PDK2 demonstrates a higher sensitivity to the energy status of the cell (Bowker-Kinley *et al.*, 1998;Popov, 1997) and is highly sensitive to pyruvate (Bowker-Kinley *et al.*, 1998;Gudi *et al.*, 1995;Popov, 1997), possibly making it the "energy-status" responsive isoform and suggesting the importance of PDK2 in a training-induced attenuation of PDH during exercise post-training.

Currently, little information is available regarding the adaptive changes in PDHt and/or PDK in human skeletal muscle with short- or long-term aerobic exercise, and if these adaptations can be detected at the level of gene and/or protein expression. Thus, the

purposes of the second study were to determine if 1 and 8 weeks of aerobic exercise would: 1) increase PDHt activity, with a concomitant increase in PDH subunit mRNA and protein, 2) increase PDK activity, with a concomitant increase in PDK isoform mRNA and protein. The hypothesis was there will be no change in PDHt and PDK activity after 1 week of aerobic exercise, along with no change in protein expression. In contrast, 8 weeks of aerobic exercise will increase both PDHt and PDK activities, along with increased protein expression. Transcriptional products (mRNA) were not expected to change after 1 or 8 weeks as muscle biopsies were taken 24-48 hours after the last exercise bout to avoid the transient effects on transcriptional rate postulated to occur up to 24 hours after exercise. Measurements of citrate synthase (CS) and cytochrome oxidase (COX) maximal activities and protein expression will be used to confirm exercise training-induced alterations to skeletal muscle gene expression.

Third Study – Long-Term Aerobic Training, Allosteric Modulators

Repeated bouts of aerobic exercise carried out over several weeks (aerobic training), decreases carbohydrate utilization in skeletal muscle at the same absolute workload, resulting in a greater reliance on fat for ATP production, a net glycogen sparing effect, and less lactate accumulation (Henriksson, 1977;Hurley *et al.*, 1986;Martin *et al.*, 1993;Phillips *et al.*, 1996b). These changes have been credited to increased muscle capillarization, increased metabolic oxidative capacity due to increased mitochondrial biogenesis, and increased activity of mitochondrial enzymes (e.g. PDH, CS, β-HAD).

The training-induced increase in metabolic oxidative capacity, and resultant tighter coupling between ATP supply and demand, would result in a reduced reliance on energy sources such as phosphocreatine (PCr), with an improved ATP/(ADP + P_i) ratio in the muscle (Dudley *et al.*, 1987;From *et al.*, 1990;Henriksson, 1977;Hochachka & McClelland, 1997;Holloszy, 1967). This, in turn, would reduce the accumulation of P_i and free ADP (ADP_f) which, together with NADH and oxygen, are known regulators of oxidative phosphorylation (From *et al.*, 1990). Alterations to these metabolic variables post-training would also impart allosteric regulation on certain enzymes in skeletal muscle, more specifically glycogen phosphorylase and PDH. However, small but significant metabolic alterations have been observed in humans after 5-10 days of aerobic exercise, suggesting alterations in oxidative phosphorylation need not be linked to and occur simultaneously with changes in mitochondrial capacity and density (Cadefau *et al.*, 1994;Chesley *et al.*, 1996;Green *et al.*, 1991;Green *et al.*, 1992a;Jones & Yeaman, 1991;Mistry *et al.*, 1991;Phillips *et al.*, 1995;Phillips *et al.*, 1996a;Putman *et al.*, 1998). In the only study to examine the effects of short-term training on human skeletal muscle PDH, Putman et al. (1998) demonstrated, after 7 days of aerobic training, there was no change in PDH activation during exercise, but reported a decreased post-transformational flux through Phos. Little is known of the adaptive changes that take place after several weeks of aerobic exercise and how allosteric regulation of PDH during exercise differs from short-term aerobic training.

During exercise, the major factor determining energy requirement in contracting skeletal muscle is the rate of ATP hydrolysis. The rate of energy production, determined

by the amount of enzymes in energy-producing catabolic pathways, becomes limiting only when the energy need of the cell exceeds the maximal catalytic capacity of the rate-determining steps of these pathways (Holloszy & Coyle, 1984). As shown in Chapter 2, upregulation of PDK, thus possibly decreasing the activation of PDH, would play an important role in training-induced regulation of carbohydrate metabolism. Thus, a possible consequence of an adaptive increase in mitochondrial biogenesis, and the associated enzymatic pathways, is that the aerobic energy production pre-training becomes "submaximal" for the muscle after prolonged aerobic training (Holloszy & Coyle, 1984).

Unlike short-term, long-term aerobic training would result in larger attenuated levels of ADP_f and P_i, imparting greater post-transformational regulation on Phos during exercise. The resulting decreased glycogenolytic flux, along with increased pyruvate sensitive PDK2 demonstrated in Chapter 2, may result in a released inhibition of PDK by attenuated levels of glycogenolytically-derived pyruvate. Along with attenuated levels of ADP_f, long-term aerobic training may result in an attenuated activation state of PDH during exercise.

Thus, the purpose of the third study was to examine the effects of long-term aerobic exercise on PDH activation and its metabolic regulators in skeletal muscle during exercise. The hypothesis was that long-term aerobic exercise will decrease the activation of PDH during exercise at the same absolute workload pre-training, due to attenuated levels of glycogenolytically-derived pyruvate. In addition, rates of glycogenolysis, along

with Phos activity and its metabolic regulators, will also be examined, to ascertain their contribution to decreased glycogen utilization post-training.

CHAPTER 1. Effects of respiratory alkalosis on human skeletal muscle metabolism at the onset of submaximal exercise. (Published in *J. Physiol.* 544: 303-313)

INTRODUCTION

During acute hypoxia, submaximal exercise at a given absolute power output results in increases in both muscle and plasma lactate concentrations compared to normoxia (Katz & Sahlin, 1987; Brooks *et al.*, 1992; Green *et al.*, 1992; Brooks *et al.*, 1998; Parolin *et al.*, 2000a). These effects were thought to be due to a limitation in O_2 supply to the muscle, resulting in anaerobic lactate accumulation. A recent study has shown that the intracellular PO_2 of the exercising muscle is reduced during hypoxia, but to a level that is above the critical level at which mitochondrial respiration is compromised (Richardson *et al.*, 1998). In addition, increased muscle blood flow helps to maintain whole body $\dot{V}O_2$ (Adams & Welch, 1980), and the $\dot{V}O_2$ of the exercising leg (Bender *et al.*, 1988; Knight *et al.*, 1993) at normoxic levels during hypoxia. Thus, it seems unlikely that hypoxia-induced lactate accumulation during submaximal exercise is solely the result of a limited O_2 supply to the exercising muscle.

In contrast to steady state exercise, the increase in $\dot{V}O_2$ during the transition from rest to exercise is slowed during hypoxia (Hughson & Kowalchuk, 1995). It has been suggested that lactate accumulation during this transition in hypoxia may be due to "metabolic inertia" rather than limited O_2 supply (Timmons *et al.*, 1998). The term metabolic inertia refers to a delayed flux through metabolic pathways responsible for aerobic ATP production. Recent studies from our laboratory have shown that within the first minute of exercise, the increased lactate accumulation in hypoxia was a result of an enhanced glycogenolytic flux through glycogen phosphorylase (Phos) combined with a delayed activation (inertia) of pyruvate dehydrogenase (PDH) (Parolin *et al.*, 2000a).

These results were partially reversed when PDH was activated by administration of dichloroacetate (DCA) before exercise (Parolin *et al.*, 2000b). Decreased pyruvate oxidation, due to delayed activation of PDH, potentially reduces the availability of oxidative substrates for aerobic ATP production. As a result, there is a greater reliance on glycolysis and phosphocreatine degradation to regenerate ATP. The mismatch between pyruvate oxidation and its production results in a net lactate accumulation, because any excess pyruvate is converted to lactate by the near-equilibrium enzyme lactate dehydrogenase.

Hyperventilation usually accompanies hypoxemia, with ventilation rates rising by 150-200% above normoxic values (Hughes *et al.*, 1968; Lenfant & Sullivan, 1971). This results in arterial blood pH increasing by 0.1-0.3 units (Edwards & Clode, 1970; Zborowska-Sluis *et al.*, 1970; Davies *et al.*, 1986). Previous studies have shown an association between hyperventilation and lactate accumulation (Eldridge & Salzer, 1967; Edwards & Clode, 1970; Brice & Welch, 1985; Davies *et al.*, 1986) and it has been suggested that the change in pH, rather than an oxygen limitation is the main contributing factor that results in an increased lactate accumulation (Davies *et al.*, 1986). Thus, respiratory alkalosis may account, in part, for the increased lactate accumulation seen during exercise with acute hypoxia.

The present study was designed to determine the role of the rate-determining enzymes, PDH and Phos, and their regulatory factors in lactate accumulation during respiratory alkalosis in human skeletal muscle during exercise. Lactate accumulation is a function of adjustments in the activation of the aerobic ATP producing metabolic

pathways and/or alterations in glycogenolytic/glycolytic flux (see Spriet *et al.*, 2000 for review). Thus, we hypothesized that respiratory alkalosis might result in increased glycogenolytic flux and a delayed activation of PDH with a concomitant increase in lactate accumulation. The effects of respiratory alkalosis were examined relative to control at rest, at 1 min during the transition from rest to exercise and after 15 min of submaximal exercise.

METHODS

Subjects

Eight healthy, active males were recruited to participate in the study (age 22 ± 1.2 (SEM) yr; height 181.3 ± 2.2 cm; weight 77.6 ± 3.4 kg). Individuals were asked to consume similar diets and refrain from caffeine, alcohol, and exercise for 48 hours before each trial. Individuals served as their own control. Oral and written explanation of the experimental protocol and its attendant risks were provided and informed consent was obtained from each subject. The study was approved by the McMaster University Ethics Committee.

Pre-experimental protocol

Individuals completed an initial incremental maximal exercise test on an electro-magnetically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA.) to determine $\dot{V}O_{2max}$ and maximal work capacity using a metabolic measurement system (Quinton Q-Plex 2, Quinton Instruments, Seattle, WA.). One week later,

individuals returned to establish the degree of hyperventilation to attain an end tidal PCO_2 ($P_{ET}CO_2$) of approximately 20-25 mm Hg, to mimic the $P_{ET}CO_2$ measured during hypoxia (Parolin *et al.*, 2000a). $P_{ET}CO_2$ was monitored breath-by-breath and the subject 'targeted' the desired $P_{ET}CO_2$ displayed continuously on an oscilloscope during the equilibration period (20 minutes at rest prior to exercise) and the experimental protocol (15 minutes at 55% $\dot{V}O_{2max}$). This was repeated several times to familiarize the subject with the conditions of the study and ensure consistency of the experimental procedure. The workload required to obtain 55% $\dot{V}O_{2max}$ was confirmed through these practice trials.

Experimental protocol

The experimental protocol was conducted during normoxia (Con) or normoxia with hyperventilation (R-Alk), on two occasions separated by 1 week. The order of the Con and R-Alk trials was randomized and took place at the same time of day for each subject. Prior to the beginning of the protocol, a venous catheter was inserted in the anticubital vein of the forearm for blood sampling and was maintained patent with saline. One thigh was prepared for needle biopsies of the vastus lateralis. Incisions were made through the skin to the deep fascia under local anesthesia (2% lidocaine without epinephrine) as described by Bergström (1975). Subjects exercised for 15 min at 55% of their $\dot{V}O_{2max}$. Blood samples were taken at pre-equilibration (R-Alk only), before exercise (time 0), and after 5, 10, and 14 minutes of exercise. Muscle biopsies were taken

at pre-equilibration (R-Alk only), before exercise (time 0) and after 1 and 15 minutes of exercise.

Blood sampling and analysis

Venous blood samples (~ 7 ml) were collected in heparinized syringes (Sarstedt, Germany) and placed on ice. Blood was centrifuged at 15,900 *g* for 2 min to isolate plasma. One portion of plasma was incubated with 5 M NaCl (4:1 plasma:NaCl) at 56°C for 30 min and then frozen for later analysis of free fatty acids (Wako NEFA kit, WAKO Chemicals, Richmond, VA). A second portion of plasma was deproteinized with 0.5 M perchloric acid (PCA) (1:2 plasma:PCA) and then frozen for later analysis for glucose, lactate, and glycerol (Bergmeyer, 1983).

Muscle analysis

Muscle biopsies were immediately frozen in liquid N_2. A small piece (4-26 mg) was chipped from each biopsy (under liquid N_2) for determination of the fraction of PDH in the active form (PDH_a; Putman *et al.*, 1993). The remainder of the sample was freeze dried, dissected free of blood and connective tissue, and powdered. One aliquot was analyzed for total (a+b) and active Phos ($Phos_{a+b}$ and $Phos_a$, respectively; Young *et al.*, 1985) and the maximal velocity and mole fraction of Phos *a+b* and Phos *a* were calculated from the measured activities as described by Chasiotis et al. (1982). Phos measurements were made only on exercise samples because resting samples must be kept at room temperature for 30 seconds before freezing for accuracy, which would have

required additional biopsies (Ren & Hultman, 1988). Previous studies have reported values of ~10% for the mole fraction of Phos *a* at rest (Chesley *et al.*, 1996; Parolin *et al.*, 1999). A second aliquot was extracted in 0.5 M PCA and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$ and analyzed for acetyl-CoA, free CoA, acetylcarnitine, free carnitine, (Cederblad *et al.*, 1990), ATP, pyruvate, lactate, phosphocreatine, creatine, glucose, glucose 6-phosphate (G-6-P), glucose 1-phosphate (G-1-P), fructose 6-phosphate (F-6-P), glycerol 3-phosphate (Gly-3-P) (Bergmeyer, 1983), and glycogen (Harris *et al.*, 1974). All muscle metabolites were normalized to the highest total creatine content for a given individual to correct for non-muscle contamination.

Calculations

Arterial PCO_2 was estimated ($ePaCO_2$) from $P_{ET}CO_2$ and V_T according to Jones et al. (1979). PDH_a flux was estimated from PDH_a as measured in wet tissue and converted to dry tissue using the wet-to-dry muscle ratio of 4:1 at rest and 4.5:1 during exercise (Putman *et al.*, 1998). Pyruvate production was calculated from increases in muscle lactate and pyruvate and in blood lactate concentrations, plus the flux of pyruvate through PDH_a. Lactate accumulation was calculated from changes in muscle and blood lactate concentrations. The distribution volume of blood lactate was assumed to be 0.64 × body weight (Astrand *et al.*, 1986) and the active muscle weight was assumed to be 9 kg wet weight. This calculation does not account for lactate oxidation by other tissues and would result in a slight underestimation of lactate accumulation.

Estimates of glycogenolytic rate during the first min of exercise were derived from increases in muscle G-6-P, F-6-P, Gly-3-P, plus pyruvate production from rest to 1 min of exercise. The average glycogenolytic rate during the subsequent 14 min of exercise was calculated from the reduction in muscle glycogen concentration during 15 min of exercise minus the estimated glycogen utilization in the 1st min of exercise, divided by time.

The rate of ATP turnover from PCr was calculated from the decrease in PCr concentration. The rate of ATP turnover from glycolysis was calculated from pyruvate production (1 mmol glycosyl unit was equal to 3 mmol ATP). The rate of ATP turnover from oxidative phosphorylation originating from carbohydrate sources was calculated from total acetyl-CoA production by PDH (1 mmol of pyruvate oxidized was equal to 15 mmol ATP). These calculations were based on the mean data.

Statistical analysis

All data are presented as means ± SEM. A two-way analysis of variance (ANOVA) (Steel and Torrie, 1980) with repeated measures was used to establish differences between conditions and time. Tukeys post hoc test was used to determine significance ($p<0.05$). Assumptions for normality were verified by generating appropriate residual plots. Data transformations (log, square root, and inverse square root) were used when appropriate to meet the above assumption.

RESULTS

Cardiorespiratory measurements

Mean $\dot{V}O_{2max}$ for the group was 3.4 ± 0.2 l/min. Hyperventilation was attained by a 2.1- and 1.8-fold increase in expiratory ventilation ($\dot{V}_E$) at rest and exercise, respectively, and was accompanied by similar $\dot{V}O_2$, $\dot{V}CO_2$, and RER and lower $P_{ET}CO_2$, and $ePaCO_2$ in R-Alk compared to Con (Table 1.1).

Muscle measurements

After the 20 min pre-equilibration in R-Alk, all values were similar to those of Con (Tables 1.2-1.5 and Fig 1.1 and 1.3), with the exception of muscle [acetylcarnitine], which was higher in R-Alk compared to Con (Table 1.4).

After 1 min of exercise, PDH_a increased during both conditions compared with rest but was lower in R-Alk (Fig 1.1). After 15 min of exercise, there was no difference in PDH_a between conditions. The mole fraction of Phos *a* was unaffected by R-Alk after 1 and 15 min of exercise (Fig 1.2).

Resting muscle [glycogen] was similar between conditions (Table 1.2). At the end of 15 min of exercise, muscle [glycogen] decreased by 24 and 36% during both Con and R-Alk, respectively. Muscle [G-1-P] and [pyruvate] were unaltered by exercise and were similar between conditions. Muscle [G-6-P] was also similar between conditions, however muscle [G-6-P] in R-Alk increased above resting values after 15 min of exercise. Muscle [F-6-P] did not differ between conditions at rest but after 1 min of exercise,

muscle [F-6-P] in Con increased above resting values and was higher than R-Alk. After 15 min of exercise, [F-6-P] in Con returned to levels similar to rest and did not differ from R-Alk, which was higher than resting levels. [Gly-3-P] was unaltered by exercise in Con but increased above resting levels in R-Alk during exercise and was higher than in Con after 15 min of exercise.

Resting muscle [lactate] was similar between conditions. After 1 min of exercise, muscle [lactate] increased in R-Alk and was higher than in Con (Table 1.2, Fig 1.3). After 15 min of exercise, muscle [lactate] was higher than rest in both conditions, but was not different between conditions.

Muscle [ATP] was unaltered by exercise and similar in the two conditions (Table 1.3). Muscle [PCr] decreased after 1 and 15 min of exercise compared with rest in both conditions. Muscle [Cr] similarly increased after 1 min of exercise in both conditions and did not change after 15 min of exercise compared with the first minute.

Muscle [free CoA] was similar between conditions and did not change with exercise (Table 1.4). In contrast, [acetyl-CoA] increased after 15 min of exercise in both conditions. Muscle [free carnitine] in Con decreased after 15 min of exercise compared with rest and 1 min of exercise. [Free carnitine] in R-Alk decreased after 1 min of exercise and decreased even further after 15 min of exercise. Muscle [acetylcarnitine] was similar between conditions and increased after 15 min of exercise.

Table 1.1. Respiratory parameters at rest and during exercise in control and respiratory alkalosis.

	Rest		Exercise	
	Con	R-Alk	Con	R-Alk
$\dot{V}O_2$ (l/min)	0.40 ± 0.03	0.39 ± 0.02	1.94 ± 0.08*	1.94 ± 0.07*
$\dot{V}CO_2$ (l/min)	0.37 ± 0.03	0.40 ± 0.05	2.01 ± 0.08*	1.95 ± 0.11*
RER	0.99 ± 0.08	1.03 ± 0.13	1.04 ± 0.01	1.00 ± 0.02
$P_{ET}CO_2$ (mm Hg)	33.4 ± 1.8	17.0 ± 0.9†	41.1 ± 1.7*	19.2 ± 0.5*†
$ePaCO_2$ (mm Hg)	35.6 ± 1.6	20.8 ± 0.9†	42.5 ± 1.5*	22.8 ± 0.4*†
$\dot{V}_E$ (BTPS) (l/min)	15.3 ± 1.7	31.5 ± 2.6†	53.3 ± 2.4*	95.7 ± 5.2*†
V_T (BTPS) (l)	0.85 ± 0.05	0.89 ± 0.24	2.26 ± 0.18*	2.10 ± 0.47*

Values are means ± SEM; ND, not determined; $\dot{V}O_2$, O_2 uptake; $\dot{V}CO_2$, CO_2 production; RER, respiratory exchange ratio; $P_{ET}CO_2$, end tidal partial pressure of CO_2; $ePaCO_2$, estimated arterial concentration of CO_2; $\dot{V}_E$, ventilation; V_T, tidal volume; * denotes significance from rest; † denotes significance from control. Parameters during exercise were obtained from the mean of the last 5 min of exercise for each subject.

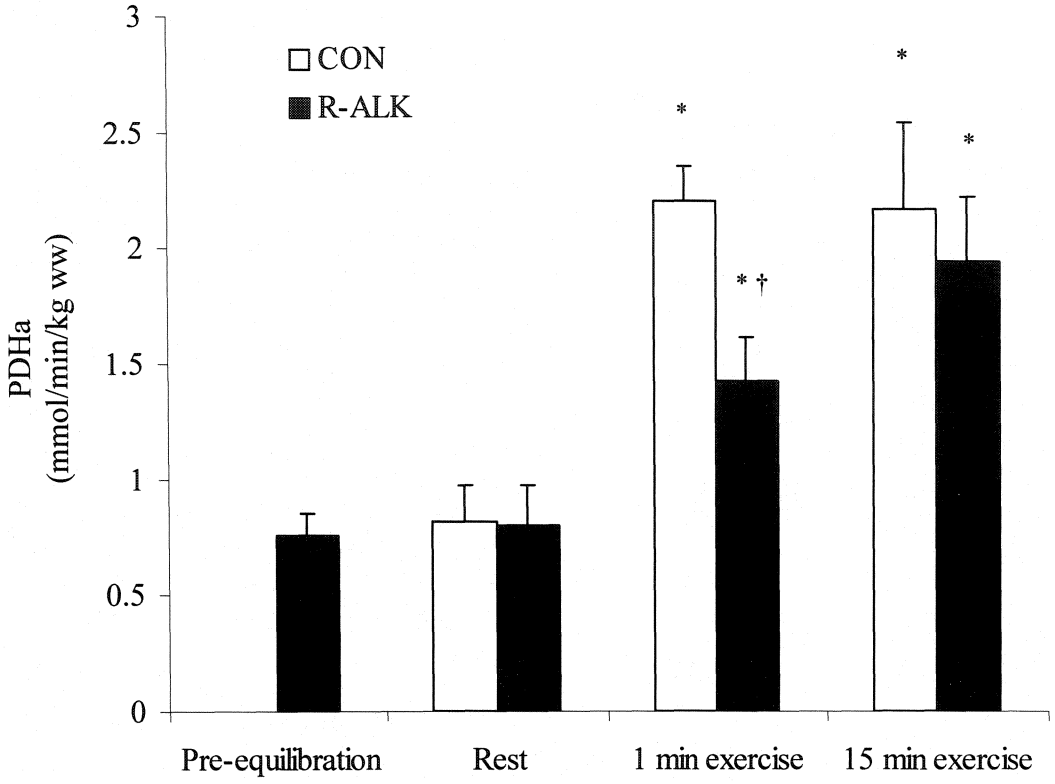

Figure 1.1. Muscle pyruvate dehydrogenase in its active form (PDHa) at rest and during exercise in control and respiratory alkalosis. Values are means ± SEM; * denotes significance from rest; † denotes significance from control.

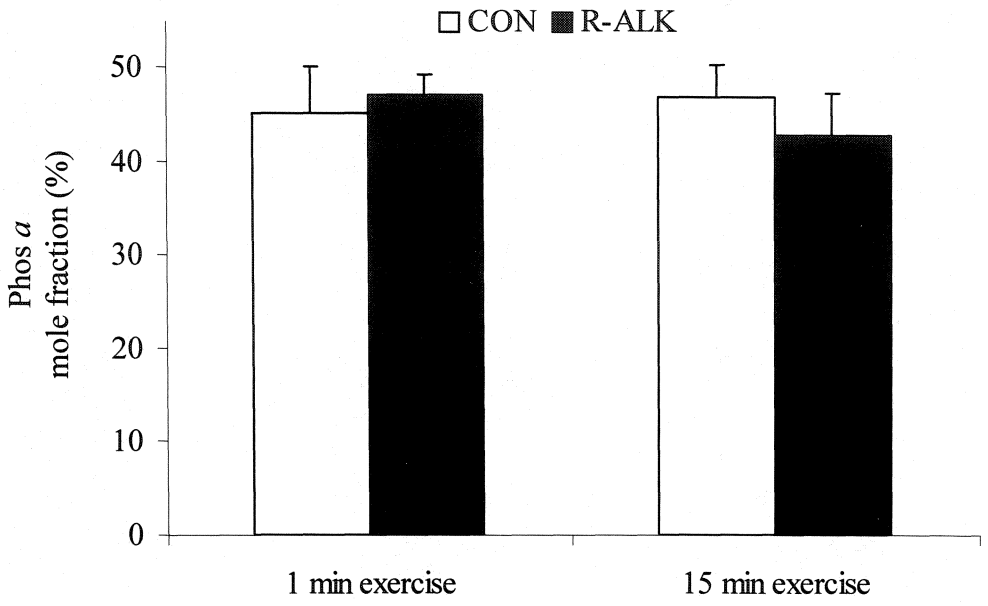

Figure 1.2. Muscle phosphorylase a mole fraction during exercise in control and respiratory alkalosis. Values are means ± SEM.

Table 1.2. Muscle contents of glycolytic intermediates at rest and during exercise in control and respiratory alkalosis.

Metabolite	Condition	Pre-equilibration	Rest	Exercise	
				1 min	15 min
Glycogen	Con	ND	439 ± 41	ND	333 ± 38*
	R-Alk	ND	538 ± 47	ND	346 ± 70*
G-1-P	Con	ND	0.17 ± 0.09	0.08 ± 0.02	0.30 ± 0.13
	R-Alk	0.13 ± 0.11	0.09 ± 0.05	0.11 ± 0.07	0.12 ± 0.10
G-6-P	Con	ND	0.95 ± 0.14	2.03 ± 0.39	2.23 ± 0.42
	R-Alk	1.01 ± 0.13	0.90 ± 0.12	1.18 ± 0.22	4.33 ± 0.82*
F-6-P	Con	ND	0.20 ± 0.05	0.63 ± 0.20*	0.30 ± 0.06
	R-Alk	0.16 ± 0.05	0.05 ± 0.06	0.19 ± 0.07†	0.47 ± 0.09*
Gly-3-P	Con	ND	0.47 ± 0.01	0.53 ± 0.01	0.51 ± 0.01
	R-Alk	0.51 ± 0.01	0.50 ± 0.01	0.61 ± 0.11*	0.53 ± 0.07*†
Pyruvate	Con	ND	0.49 ± 0.07	0.53 ± 0.06	0.43 ± 0.06
	R-Alk	0.43 ± 0.05	0.58 ± 0.09	0.51 ± 0.10	0.49 ± 0.15
Lactate	Con	ND	6.4 ± 1.5	11.3 ± 1.8	13.9 ± 2.2*
	R-Alk	3.6 ± 0.7	8.1 ± 1.6	20.1 ± 3.5*†	18.7 ± 4.3*

Values are means ± SEM and are expressed in mmol/kg dry wt; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; Gly-3-P, glycerol 3-phosphate; ND, not determined; * denotes significance from rest; † denotes significance from control.

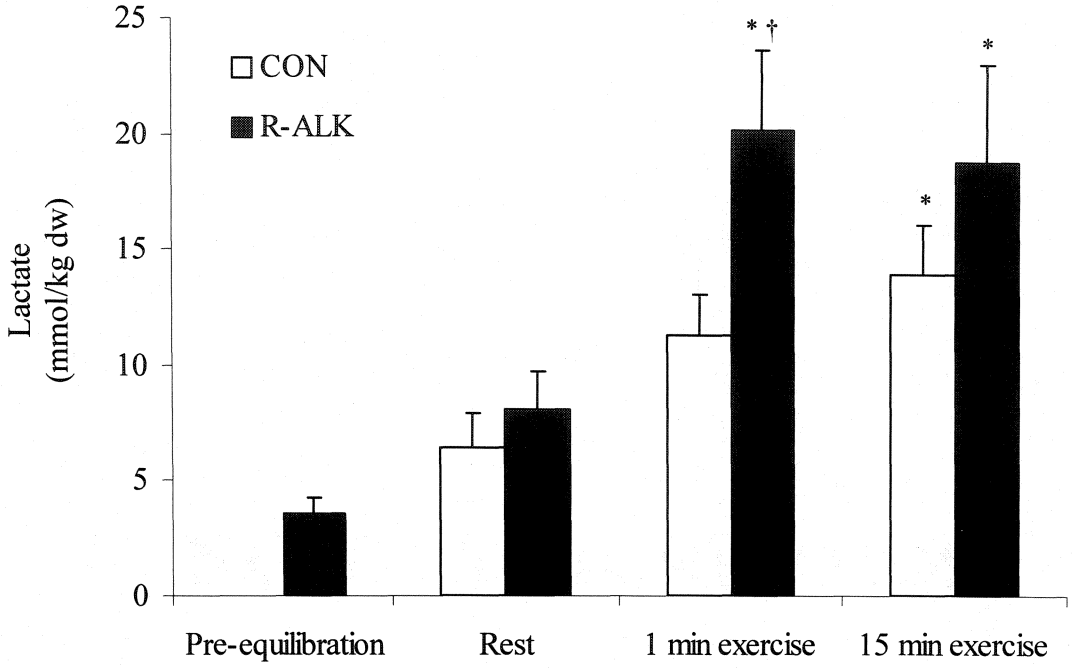

Figure 1.3. Muscle lactate concentration at rest and during exercise in control and respiratory alkalosis. Values are means ± SEM; * denotes significance from rest; † denotes significance from control.

Table 1.3. Muscle contents of ATP, creatine, and phosphocreatine at rest and during exercise in control and respiratory alkalosis.

Metabolite	Condition	Pre-equilibration	Rest	Exercise	
				1 min	15 min
ATP	Con	ND	25.1 ± 2.2	24.6 ± 1.7	21.5 ± 1.8
	R-Alk	26.6 ± 1.8	27.0 ± 1.7	25.5 ± 1.6	22.7 ± 1.8
Creatine	Con	ND	30.8 ± 3.0	44.0 ± 3.2*	52.9 ± 3.2*
	R-Alk	38.0 ± 2.4	33.5 ± 2.2	45.3 ± 4.0*	56.4 ± 6.4*
Phosphocreatine	Con	ND	94.3 ± 2.0	75.3 ± 4.1*	71.9 ± 4.2*
	R-Alk	98.3 ± 6.0	97.2 ± 4.5	80.9 ± 5.1*	63.9 ± 4.3*

Values are means ± SEM and are expressed as mmol/kg dry wt; ND, not determined; * denotes significance from rest.

Table 1.4. Muscle contents of CoA, carnitine, and their acetylated forms at rest and during exercise in control and respiratory alkalosis.

Metabolite	Condition	Pre-equilibration	Rest	Exercise	
				1 min	15 min
Acetyl-CoA	Con	ND	8.0 ± 0.9	11.1 ± 2.2	15.8 ± 1.1*
	R-Alk	8.3 ± 1.1	8.5 ± 0.7	11.2 ± 1.0	18.4 ± 2.3*
Free CoA	Con	ND	80.9 ± 7.3	74.7 ± 10.2	69.5 ± 3.5
	R-Alk	82.3 ± 17.7	85.5 ± 9.1	66.9 ± 11.2	73.7 ± 12.4
Acetyl CoA/CoA	Con	ND	0.11 ± 0.02	0.16 ± 0.04	0.23 ± 0.03*
	R-Alk	0.11 ± 0.02	0.11 ± 0.01	0.23 ± 0.07*	0.24 ± 0.03*
Acetylcarnitine	Con	ND	1.7 ± 0.2	4.1 ± 0.6	12.6 ± 1.3*
	R-Alk	2.8 ± 0.6	4.3 ± 0.9†	5.4 ± 1.0	13.4 ± 2.2*
Free carnitine	Con	ND	17.2 ± 1.6	15.2 ± 1.1	8.3 ± 0.7*‡
	R-Alk	19.1 ± 1.2	17.1 ± 1.8	13.3 ± 1.2*	9.1 ± 1.6*‡

Values are means ± SEM; acetyl-CoA and free CoA measures are expressed in μmol/kg dry wt; all other measurements are expressed as mmol/kg dry wt; acetyl-CoA/CoA, ratio of acetyl-CoA to CoA; ND, not determined; * denotes significance from rest; † denotes significance from control; ‡ denotes significance from 1 minute.

Table 1.5. Plasma lactate, glucose, glycerol, and free fatty acid (FFA) concentration at rest and during exercise in control and respiratory alkalosis.

				Exercise		
Metabolite	Condition	Pre-equilibration	Rest	5 min	10 min	14 min
Lactate	Con	ND	1.2 ± 0.1	2.8 ± 0.3*	3.6 ± 0.3*	3.7 ± 0.4*
	R-Alk	1.2 ± 0.1*	2.4 ± 0.4†	4.4 ± 0.6*†	5.1 ± 0.6*†	5.3 ± 0.5*†
Glucose	Con	ND	4.1 ± 0.2	3.8 ± 0.2	3.8 ± 0.1	3.9 ± 0.1
	R-Alk	4.1 ± 0.2	4.3 ± 0.2	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3
Glycerol	Con	ND	38.0 ± 4.9	36.4 ± 2.3	42.6 ± 7.1	30.8 ± 4.9
	R-Alk	27.8 ± 1.3	33.9 ± 6.0	47.1 ± 4.0	56.3 ± 4.7	49.7 ± 6.5
FFA	Con	ND	0.36 ± 0.07	0.38 ± 0.07	0.38 ± 0.07	0.40 ± 0.07
	R-Alk	0.38 ± 0.07	0.50 ± 0.05	0.47 ± 0.06	0.47 ± 0.08	0.55 ± 0.09

Values are means ± SEM; glycerol is expressed in μM; all other measurements are expressed in mM; FFA, free fatty acid; ND, not determined; * denotes significance from rest; † denotes significance from control.

Blood metabolites

Blood [glucose], [glycerol], and [free fatty acids] did not change with exercise and were similar between conditions (Table 1.5). Blood [lactate] increased with exercise and was higher in R-Alk compared with Con at rest and during exercise.

Glycogenolysis

During the first minute of exercise, the glycogenolytic rate was higher in R-Alk compared to Con (Fig 1.4). During the subsequent 14 min of exercise, glycogenolytic rates were similar between conditions and did not differ from the first min.

Pyruvate production and oxidation and lactate accumulation

In Con during the first min of exercise there was a lower rate of pyruvate production compared with the subsequent 14 min of exercise, with a lower rate of pyruvate oxidation and no difference in lactate accumulation (Fig 1.5). In contrast, a higher rate of pyruvate was produced in R-Alk during the first minute compared with the subsequent 14 min of exercise, with a lower rate of pyruvate oxidized and more lactate accumulation. During the first minute of exercise, there was a higher pyruvate production and lactate accumulation and lower pyruvate oxidation in R-Alk compared with Con. Between the first and fifteenth minute of exercise, were no differences in pyruvate production and lactate accumulation between conditions and pyruvate oxidation was lower in R-Alk compared to Con.

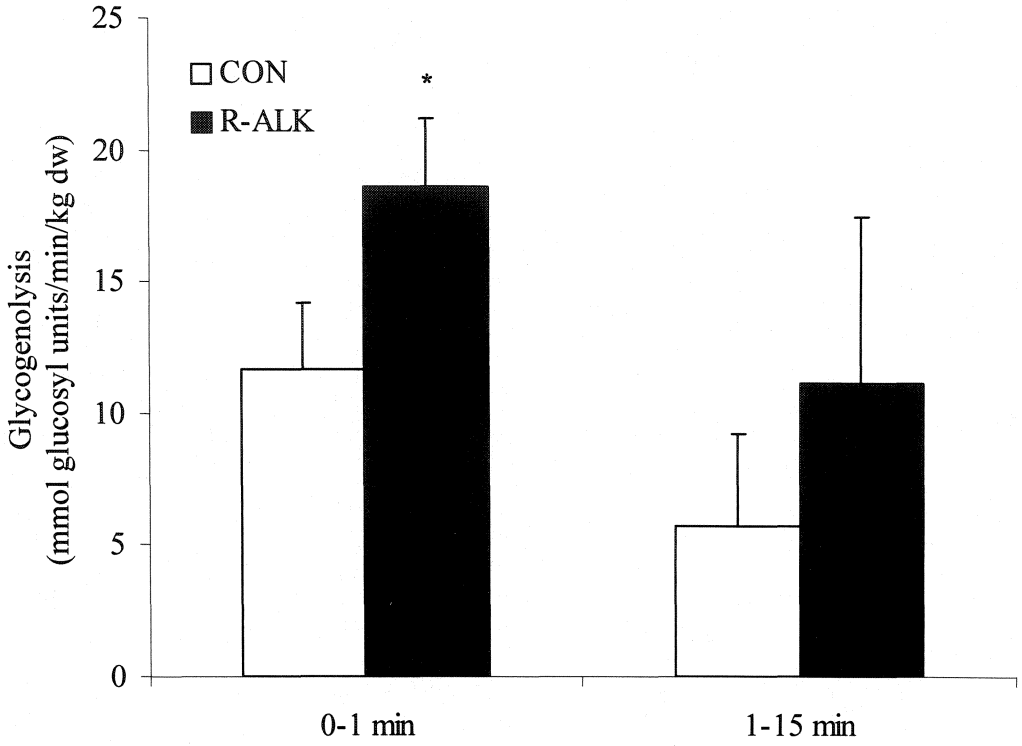

Figure 1.4. Estimated rates of muscle glycogen use during exercise in control and respiratory alkalosis. Values are means ± SEM; * denotes significance from control.

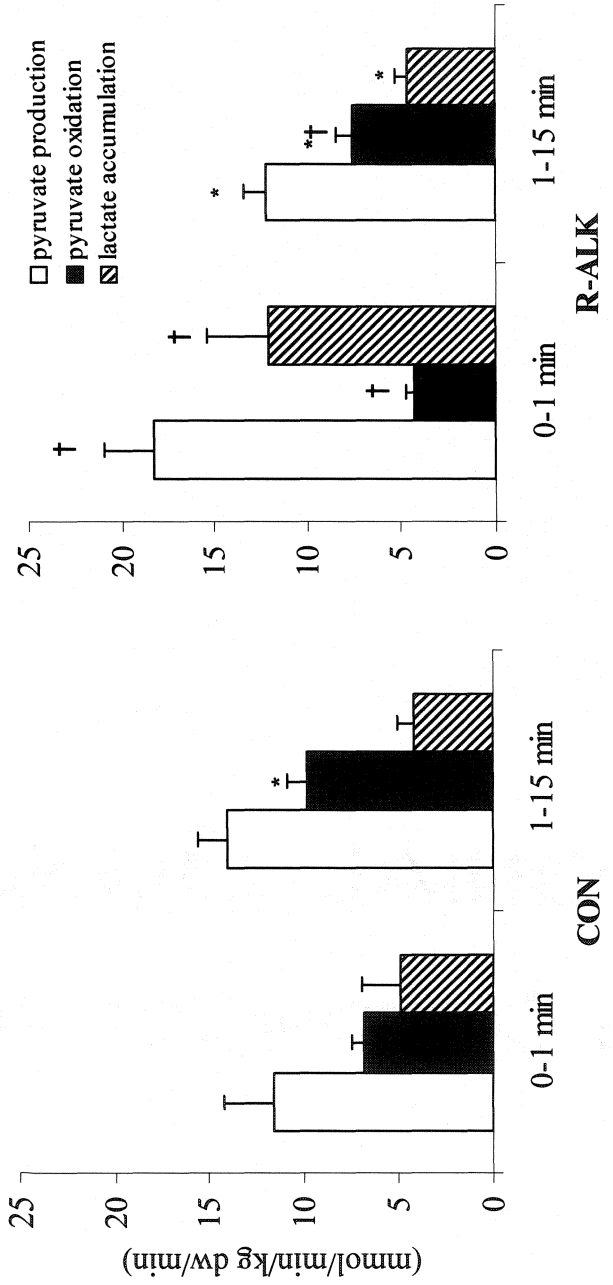

Figure 1.5. A comparison of the rates of pyruvate production and oxidation and lactate accumulation during exercise in control and respiratory alkalosis. * denotes significance from 0-1 min; † denotes significance from control.

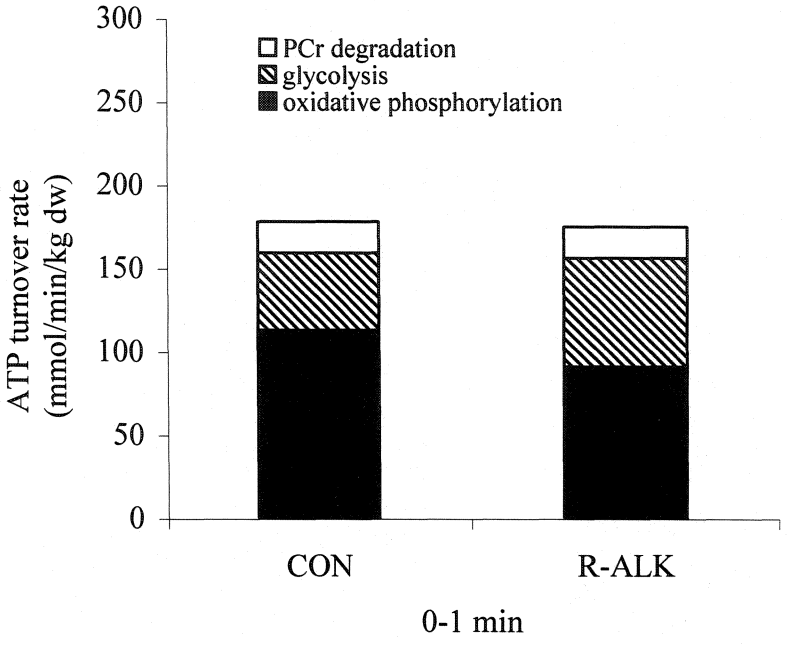

Figure 1.6. ATP turnover rates from PCr degradation, glycolysis, and oxidative phosphorylation during the transition from rest to exercise in control and respiratory alkalosis.

ATP turnover at the onset of exercise

During the first minute of exercise in Con, 37% of the total ATP turnover was attributed to glycolysis (26%) and PCr degradation (11%; Fig 1.6). ATP regeneration through the TCA cycle/electron transport chain (oxidative phosphorylation) accounted for the remaining 63% of total ATP turnover. In R-Alk, 48% of the total ATP turnover was attributed to glycolysis (37%) and PCr degradation (11%). Oxidative phosphorylation accounted for the remaining 52% of total ATP turnover.

DISCUSSION

The present study examined the acute effects of respiratory alkalosis, through voluntary hyperventilation, on the regulation of skeletal muscle metabolism at rest, during the transition from rest to exercise and during steady-state submaximal exercise. At rest, no effects on muscle metabolism were observed in response to R-Alk. During the first min of exercise, R-Alk resulted in a greater pyruvate production, despite no difference in the mole fraction of Phos *a* or glycolytic intermediates. In addition to increased pyruvate production in R-Alk, a delayed activation of PDH resulted in lower pyruvate oxidation. This mismatch in pyruvate production and oxidation resulted in greater lactate accumulation in both muscle (Fig 1.3) and plasma (Table 1.5) in R-Alk during the transition from rest to exercise. In contrast, there was no additional accumulation of muscle and plasma [lactate] during steady state exercise. Hence, an elevated lactate accumulation associated with respiratory alkalosis during exercise was

explained by the differing fluxes through the rate-determining enzymes, Phos and PDH, at the onset of exercise.

Respiratory alkalosis

Voluntary hyperventilation resulted in a reduction in $ePaCO_2$ from 35.6 to 20.8 mmHg at rest (Table 1.1). A drop of approximately 15 mmHg in $PaCO_2$ would translate to a rise in plasma pH from 7.42 to 7.59 (Siggard-Anderson, 1963). A change in extracellular pH (ΔpH_e) induced by respiratory alkalosis has been associated with a 0.67 increase in intracellular pH (ΔpH_i) of rat diaphragm (Heisler, 1975). Thus, assuming a muscle pH of 7 in Con and a ΔpH_e of 0.17, intramuscular pH for R-Alk can be estimated at 7.11. This increase in intramuscular pH may be the contributing factor to the changes seen in muscle metabolism.

Oxidative phosphorylation

Mitochondrial oxidative phosphorylation is represented by the following equation:

$$NADH + 0.5O_2 + H^+ + 3ADP + 3P_i \rightleftharpoons NAD^+ + H_2O + 3ATP$$

and the rate of ATP production is regulated by O_2 availability, $[NADH]/[NAD^+]$, and $[ADP][P_i]/[ATP]$ (Wilson, 1994). Changes in any of the parameters result in a compensatory change in the others to drive oxidative phosphorylation.

PDH is a mitochondrially bound rate-determining enzyme that regulates the entrance of glycolytically produced acetyl units into oxidative metabolism. Two

enzymes covalently modify PDH, PDH kinase (PDK) and PDH phosphatase (PDP). PDK catalyzes the phosphorylation, and resulting inactivation, of PDH whereas PDP dephosphorylates the enzyme, thereby activating PDH (Denton *et al.*, 1975; Stansbie, 1976). The amount of PDH in its active form (PDH_a) determines its activity. In turn, both PDK and PDP are allosterically modulated by increased ratios of acetyl-CoA to CoA and NADH to NAD^+, with increased ratios activating PDK and inhibiting PDP (Denton *et al.*, 1975; Pettit *et al.*, 1975; Stansbie, 1976). Other modulators include pyruvate (Cate & Roche, 1978) and ATP to ADP ratio (Denton *et al.*, 1975), which inhibits and activates PDK respectively, and Ca^{2+} and H^+, which activate PDP (Chen *et al.*, 1996).

During the transition from rest to exercise, Ca^{2+} release from the sarcoplasmic reticulum is probably the primary stimulus for the activation of PDH. Ca^{2+} release stimulates PDP and dephosphorylates PDH, rendering it more active. In the present study, PDH activation increased during the first min of exercise in Con with no further increase in PDH activity after the subsequent 14 min of exercise, which is consistent with previous studies (Howlett *et al.*, 1999; St.Amand *et al.*, 2000; Parolin *et al.*, 2000a). In R-Alk there was a delayed activation of PDH during the first min of exercise, similar to the effects of hypoxia (Parolin *et al.*, 2000a). During the transition from rest to exercise, none of the typical allosteric modulators were affected by R-Alk, with the exception of $[H^+]$. Both PDK and PDP have pH optimums of 7.0-7.2 and 6.7-7.1, respectively (Hucho *et al.*, 1972). The rate of activation of PDH is dependent on the ratio of active PDK and PDP, thus a slight change in the activity of either covalent modifier would alter the rate

of activation or inactivation of PDH. An increase in intramuscular pH of 7 in Con to 7.11 in R-Alk may slow the activity of PDP and potentially enhance the activity of PDK, resulting in a delayed activation of PDH.

A delayed activation of PDH may cause a mismatch between ATP utilization and aerobic ATP production. Reduced flux through PDH during the transition from rest to exercise in R-Alk may result in metabolic inertia, delaying the availability of carbohydrate-derived substrate to the TCA cycle and the subsequent production of reducing equivalents for oxidative phosphorylation. In R-Alk, a similar [ATP] and O_2 availability during the transition from rest to exercise and a lower [NADH] and $[H^+]$ may require higher free [ADP] and $[P_i]$ to drive oxidative phosphorylation and maintain the same ATP turnover rate as Con. Free [ADP], calculated from the near-equilibrium reaction of creatine kinase (Dudley *et al.*, 1987), was higher in R-Alk compared to Con (130 ± 29 and 92 ± 13 μmol/kg dry wt respectively). As a result, the increase in free [ADP] may stimulate glycogenolysis in R-Alk.

Previous studies have shown there is a delayed O_2 uptake response during respiratory alkalosis during the transition from rest to exercise (Ward *et al.*, 1983; Hayashi *et al.*, 1999). It has been hypothesized that this delay may be due to impaired O_2 off-loading from hemoglobin (Hayashi *et al.*, 1999) or decreased blood flow (Brice & Welch, 1985; Gustafsson *et al.*, 1993; Karlsson *et al.*, 1994) through exercising muscles. We cannot disregard the fact that respiratory alkalosis in the present study may impair both diffusive and/or conductive delivery of oxygen to the exercising muscle. However, the present study demonstrated similar effects seen during hypoxia (Parolin *et al.*, 2000a),

suggesting the importance of metabolic inertia, at the level of PDH, on oxidative phosphorylation at the onset of exercise.

After 15 min of exercise, the activation of PDH in R-Alk was similar to Con and none of the typical allosteric modulators were affected by R-Alk. Previous studies of steady state submaximal exercise during acute hypoxia (Parolin *et al.*, 2000a) and metabolic alkalosis (Hollidge-Horvat *et al.*, 2000) have shown an elevated PDH_a and intramuscular [pyruvate], which exerted a feed-forward effect on PDH_a. The present study did not show any differences in intramuscular [pyruvate] between conditions. Similar PDH_a activities between conditions may be attributed to alterations in the acid-base status of the muscle. The alkalizing effects of hyperventilation are transient and lactate accumulation during the first min of exercise in R-Alk may have reduced the estimated pH differences between conditions. However, the time course of pH changes in the muscle is difficult to determine during this transition from rest to exercise.

Phosphocreatine degredation and glycolysis

The regeneration of ATP is also possible through phosphocreatine (PCr) degradation and glycolysis. PCr degradation was similar between conditions during exercise and did not differ in regeneration of ATP during the transition from rest to exercise. However, glycogenolytic rate was higher in R-Alk compared to Con during the first min of exercise and may have been the major contributor that maintained ATP turnover rates similar between conditions.

Phos is a flux-generating enzyme of glycogenolysis in skeletal muscle and is subject to both covalent and allosteric regulation. Phos *a*, the more active form, is active in the absence of AMP whereas Phos *b* requires AMP. Covalent transformation from Phos *b* to *a* is mediated by Phos kinase *a*, which in turn is allosterically regulated by epinephrine and cytosolic Ca^{2+}. Post-transformational allosteric regulation of Phos *b* is mediated by AMP and IMP, and inhibited by ATP and G-6-P. Of equal importance in regulating both Phos *a* and *b* is one of the substrates, inorganic phosphate (P_i; Chasiotis *et al.*, 1982). R-Alk compared to Con resulted in an elevated pyruvate production during the first min of exercise, in the absence of any change in the percent mole fraction of Phos *a*. This would suggest that enhanced glycogenolysis in R-Alk, with no change in Phos *a* transformation, is likely a result of post-transformational modulation.

The levels of intramuscular G-6-P did not differ between conditions and although not estimated in the present study, free [AMP] and free [P_i] have been shown to be similar during the first min of exercise during acute hypoxia (Parolin *et al.*, 2000a). However, an increase in pH, due to respiratory alkalosis, may influence the concentration of the monoprotonated form of P_i, HPO_4^{2-}, over the diprotonated form, $H_2PO_4^-$. The monoprotonated form is considered to be the only active substrate for Phos (Kasvinsky & Meyer, 1977). Estimated intramuscular free [P_i] at the onset of exercise in Con increased from the assumed resting concentration of 10.8 mmol/kg dry wt to a calculated level of 25.5 ± 5.2 after 1 min of exercise. The concentration of free P_i during the first min of exercise was calculated as the difference between resting and exercise [PCr], less the accumulation of G-6-P, F-6-P, and Gly-3-P, plus the assumed resting concentration of

10.8 mmol/kg dry wt (Dudley *et al.*, 1987). A change in estimated intramuscular pH from 7 in Con to 7.11 in R-Alk prior to the onset of exercise would result in an increase of HPO_4^{2-} from 15.4 to 16.9 mmol/kg dry wt, assuming a pK_a of 6.82 (Voet & Voet, 1995) and no change in free P_i. This 10% increase in substrate availability for Phos may account for some of the increased pyruvate production, through increased glycogenolytic flux, seen during the transition from rest to exercise in R-Alk. Another factor contributing to increased glycogenolytic flux in R-Alk may be a result of the previously mentioned elevated intramuscular free [ADP], which may have led to an increased [IMP], which in turn may have activated Phos *b*.

The elevated pH in R-Alk would also affect another key enzyme that regulates glycolysis, phosphofructokinase (PFK). PFK catalyzes the conversion of F-6-P to fructose 1,6-bisphosphate with the use of ATP (Voet & Voet, 1995), and is subject to allosteric modulation by a number of metabolites. ATP and H^+ inhibit whereas ADP, AMP, P_i, and F-6-P activate the enzyme complex. During the transition from rest to exercise in R-Alk compared to Con, elevated pH, [ADP], and [P_i] and lower F-6-P would stimulate PFK. As a consequence, a better match between PFK and glycogenolytically produced ATP would partially compensate for the decreased oxidative ATP production, due to the delayed activation of PDH.

After 15 min of exercise, the percent mole fraction of Phos *a*, along with the glycogenolytic rate and pyruvate production, were all similar between conditions. This is contrary to what has been shown in previous studies of both acute hypoxia and metabolic alkalosis. During steady state exercise, hypoxia (Parolin *et al.*, 2000a) and metabolic

alkalosis (Hollidge-Horvat *et al.*, 2000) resulted in significant decreases in the mole fraction of Phos *a* and an increase in glycogen utilization and pyruvate production. The increased glycogenolytic rate was a result of post-transformational regulation of Phos through increases in intramuscular free AMP and free P_i (Hollidge-Horvat *et al.*, 2000; Parolin *et al.*, 2000a). The absence of these metabolic changes in the present study may be attributed to alterations in the acid-base status of the muscle. As previously mentioned, intramuscular lactate accumulation in R-Alk during exercise would have decreased the pH of the muscle, thereby potentially reversing the effects of respiratory alkalosis seen prior to the transition from rest to exercise.

Lactate accumulation

At rest, no differences were seen in intramuscular [lactate] between conditions. However, it is interesting to note that plasma [lactate] was higher in R-Alk after 20 min of hyperventilation compared to Con. This is consistent with previous studies examining the effects of hyperventilation on plasma lactate in humans (Eldridge & Salzer, 1967; Edwards & Clode, 1970; Davies *et al.*, 1986). Plasma [lactate] represents the balance between lactate uptake and release from tissues. Thus, the elevated plasma lactate that accompanies hyperventilation may be related to increased lactate appearance and/or decreased lactate removal from the plasma. An increased lactate appearance may be due to increased lactate production of respiratory muscles due to increased work of breathing. However, it has been shown that isocapnic hyperventilation is not associated with a rise in blood lactate (Huckabee, 1957; Edwards & Clode, 1970; Martin *et al.*, 1984; Engelen

et al., 1995). Increased lactate appearance in plasma may also be due to glycolysis in erythrocytes, which is stimulated by low PCO_2 or high pH (Murphy, 1960; Zborowska-Sluis & Dossetor, 1967) and may contribute to the elevated plasma lactate at rest.

Several studies have demonstrated that respiratory alkalosis decreases the clearance of lactate from plasma by active and inactive tissues (Eldridge *et al.*, 1974; Druml *et al.*, 1991). It has been demonstrated previously that inactive muscle during exercise responds in much the same way as active muscle, demonstrating an increase in PDH_a (Putman *et al.*, 1999). Thus, PDH_a activity in inactive muscle during respiratory alkalosis may also be delayed, resulting in less lactate oxidation in inactive muscle and less lactate clearance from plasma.

Intramuscular lactate accumulation is also a function of the rate of efflux from the muscle. Lactate movement across the muscle occurs via a monocarboxylate lactate-proton cotransport protein (MCT), and as such is the rate-determining step in lactate efflux (Bonen *et al.*, 1997). The increased intramuscular [lactate] in R-Alk compared to Con at the onset of exercise may have resulted in a slight increase in the rate of lactate appearance in the blood from rest to 5 min of exercise. MCT is known to be sensitive to changes in concentration of the transport components, lactate and H^+, demonstrating increased lactate movement out of the cell with increased lactate in the cell and increased pH outside the cell (Juel & Halestrap, 1999). The combined respiratory alkalosis and elevated intramuscular [lactate] may have resulted in an enhanced movement of lactate out of the muscle.

Summary and conclusions

This study demonstrates that respiratory alkalosis has a profound impact on muscle metabolism at the onset of exercise, resulting in increased lactate accumulation. This supports the claims from previous studies that hyperventilation-induced respiratory alkalosis during hypoxia plays an important role in increased lactate accumulation during exercise (Edwards & Clode, 1970; Adams & Welch, 1980; Davies *et al.*, 1986; Parolin *et al.*, 2000a).

In the present study, respiratory alkalosis resulted in metabolic effects that are found during the transition from rest to exercise and are similar to the effects seen during acute hypoxia (Parolin *et al.*, 2000a). Hyperventilation prior to and during constant-load exercise resulted in a delayed activation of PDH during the transition from rest to exercise. This delayed activation of PDH was presumably a consequence of a hyperventilation-induced increase in muscle pH, as end-tidal PCO_2 (and presumably $PaCO_2$) was reduced from approximately 33 to 17 mmHg, and may be related to the pH sensitivity of the covalent modifiers PDK and PDP. A delayed activation of PDH would result in a concomitant decrease in carbohydrate-derived substrate availability for oxidative ATP production and increased lactate production. The cellular energetics dictate that to maintain a similar ATP production in R-Alk compared to Con, a higher [ADP] and [P_i] are needed to drive oxidative phosphorylation. As a result, a higher intramuscular [ADP] and/or [P_i] would stimulate glycogenolysis through Phos in the absence of covalent modification. Phos is further stimulated with an increased concentration in the monoprotonated form of P_i due to an increase in pH. The mismatch

in pyruvate production and oxidation with respiratory alkalosis possibly explains the lactate accumulation seen in acute hypoxia during the transition from rest to exercise.

In contrast, the pH effects seen during the transition from rest to exercise are lost during steady state exercise as a greater increase in lactate accumulation in R-Alk early in exercise presumably resulted in a greater metabolic acidosis to offset the effects of the respiratory alkalosis. This is contrary to what has been shown during acute hypoxia, where the rate of pyruvate production continued to exceed the rate of pyruvate oxidation, resulting in further lactate accumulation (Parolin *et al.*, 2000a). The regulation of lactate accumulation is multifactorial and more work is required to ascertain what factors are responsible for lactate accumulation during steady state exercise in acute hypoxia.

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CHAPTER 2. Effects of aerobic training on pyruvate dehydrogenase and pyruvate dehydrogenase kinase in human skeletal muscle. (Submitted to *J. Physiol.* Nov. 2003)

INTRODUCTION

During exercise at maximal oxygen uptake, carbohydrates are the dominant fuel utilized in skeletal muscle, mainly in the form of muscle glycogen (see Hultman, 1995 for review). Repeated bouts of aerobic exercise have been shown to increase maximal oxygen uptake and muscle glycogen storage (see Henriksson, 1995 for review). In addition, training has also shown to increase glycogen utilization during exercise at workloads eliciting maximal oxygen uptake (see Henriksson, 1995 for review). Central to this adaptation may be pyruvate dehydrogenase (PDH), which modulates carbohydrate metabolism by regulating the entrance of carbohydrate-derived acetyl units into the tricarboxylic acid cycle. To date, only two studies have examined the effects of training on human skeletal muscle total PDH (PDHt) activity, demonstrating no change after 7 days of aerobic exercise (Putman *et al.*, 1998) and 5 weeks of strength exercise (Ward *et al.*, 1986). However, a recent study reported an increase in PDHt activity with long-term aerobic exercise in mouse hind limb (Houle-Leroy *et al.*, 2000), suggesting prolonged aerobic training may alter skeletal muscle PDHt activity in humans.

PDH is a multienzyme complex, comprised of pyruvate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3; Denton *et al.*, 1975; Wieland, 1983), along with a tightly-associated protein (E_3bp), anchoring E_3 subunits to the E_2 core (Harris *et al.*, 1997), and two regulatory enzymes, PDH kinase (PDK) and PDH phosphatase (PDP; Denton *et al.*, 1975; Wieland, 1983). To date, few studies have examined the relative transcriptional and translational responses of each

PDH subunit during adaptive changes in PDHt, reporting changes exclusively in non-muscle tissues (Da Silva *et al.*, 1993; Maury *et al.*, 1995; Amessou *et al.*, 1998).

Another skeletal muscle adaptive response to prolonged aerobic training, during submaximal exercise, is a greater reliance on fat for ATP synthesis (Henriksson, 1977; Hurley *et al.*, 1986; Martin *et al.*, 1993; Phillips *et al.*, 1996b), resulting in a net glycogen sparing effect. In a recent study, we demonstrated an attenuated rise in PDH activation during the same absolute submaximal exercise after 7 weeks of prolonged aerobic exercise (LeBlanc *et al.*, 2003). Thus, the control of PDH appears to play a central role in the training-induced shift from carbohydrate to fat metabolism in skeletal muscle during exercise, however, the mechanism is currently unknown. The activation of PDH is covalently regulated by PDK, which phosphorylates and inactivates, and PDP, which dephosphorylates and activates PDH. The activation of PDH is a dynamic system and alterations in either PDK and/or PDP activity will affect PDH activity. With prolonged aerobic training, an attenuated activation of PDH may be due to a stable increased or decreased protein, and thus intrinsic activity, of PDK and PDP, respectively. Regulation of PDK may be central to control of the activation state of PDH in prolonged metabolic perturbations, including aerobic training, since skeletal muscle PDK activity responds to a number of physiological and pathological perturbations in rats (Fuller & Randle, 1984; Nakai *et al.*, 1999; Vary & Hazen, 1999; Wu *et al.*, 1999; Holness *et al.*, 2000; Sugden *et al.*, 2000; Peters *et al.*, 2001a) and in humans (Peters *et al.*, 1998; Peters *et al.*, 2001b), whereas skeletal muscle PDP activity is unresponsive (Fuller & Randle, 1984).

The complexity of PDH control by PDK is enhanced by the presence of four isoforms (PDK1-4; Bowker-Kinley *et al.*, 1998), with PDK2 and 4 being the most abundant isoforms represented in human skeletal muscle (Gudi *et al.*, 1995). Each isoform has differing concentrations, specific activities, and kinetic properties, resulting in unique responses to certain metabolic demands. PDK4 responds to acute alterations in lipid availability and has been termed the "lipid-status" responsive isoform (Sugden *et al.*, 2001) where as PDK2 demonstrates a higher sensitivity to the energy status of the cell (Popov, 1997; Bowker-Kinley *et al.*, 1998) and is highly sensitive to pyruvate (Gudi *et al.*, 1995; Popov, 1997; Bowker-Kinley *et al.*, 1998), suggesting the importance of PDK2 in a training-induced attenuation of PDH activation during exercise post training.

Currently, little information is available regarding the adaptive changes in PDHt and/or PDK in human skeletal muscle with short- or long-term aerobic exercise, and if these adaptations can be detected at the level of gene and/or protein expression. Thus, the purposes of the study were to determine if 1 and 8 weeks of aerobic exercise would: 1) increase PDHt activity, with a concomitant increase in PDH subunit mRNA and protein, 2) increase PDK activity, with a concomitant increase in PDK isoform mRNA and protein. We hypothesized there will be no change in PDHt and PDK activity after 1 week of aerobic exercise, along with no change in mRNA or protein. In contrast, 8 weeks of aerobic exercise will increase both PDHt and PDK activities, with a concomitant increase in transcriptional and translational products, thus allowing for greater potential carbohydrate flux through PDH yet greater metabolic control of carbohydrate utilization through inactivation of PDH by PDK. Measurements of citrate

synthase (CS) and cytochrome oxidase (COX) maximal activities and protein expression were used to confirm training-induced alterations to skeletal muscle gene expression.

METHODS

Subjects

Eight healthy, active men were recruited to participate in the study (age 22 ± 1 yr; height 181.6 ± 2.2 cm; weight 83.3 ± 2.6 kg). Individuals were asked to consume similar diets and refrain from caffeine, alcohol, and exercise for 48 hours before each trial. Oral and written explanation of the experimental protocol and its attendant risks were provided and informed written consent was obtained from each subject. The study was approved by the McMaster University Ethics Committee in accordance with the Declaration of Helsinki.

Pre-experimental protocol

Individuals completed an initial incremental maximal exercise test on an electro-magnetically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA.) to determine maximal work capacity and maximal oxygen uptake ($\dot{V}O_{2max}$) using a metabolic measurement system (Quinton Q-Plex 2, Quinton Instruments, Seattle, WA.).

Exercise Training Protocol

Individuals were aerobically trained on a cycle ergometer at a power output that reflected 75% of their maximal O_2 uptake for 1 hour per day, five days a week for a total

of 8 weeks. For the first week, the 1 hour of training was broken up into four 15 min intervals with 5 min rest in between. For the second and third weeks, the 1 hour of training was broken up into three 20 minute intervals with 5 and 2.5 minutes rest in between, respectively. For the fourth to eighth week, the 1 hour of training was broken up into three 20 minute intervals with 1 minutes rest in between. On the fourth and eighth week, individual subjects completed an incremental maximal exercise test to determine changes in maximal work capacity and O_2 uptake so adjustments could be made to the workload to reflect 75% of their maximal O_2 uptake, and to determine the adaptive response in maximal O_2 uptake, respectively.

Muscle sampling

Two muscle biopsies (~80-120 mg each) were obtained at rest before and after 1 and 8 weeks of aerobic exercise. Studies were performed at the same time of day. Individuals served as their own control. One thigh was prepared for needle biopsies of the vastus lateralis muscle as described by Bergström (1975). Incisions were made through the skin to the deep fascia under local anesthesia (2% lidocaine without epinephrine). Resting muscle biopsies were taken 24-48 hours after the last exercise bout, to avoid the transient effects on transcriptional rate postulated to occur up to 24 hours after the last exercise bout (Neufer *et al.*, 1998; Pilegaard *et al.*, 2000). The first biopsy was immediately processed for mitochondrial isolation. The second biopsy was immediately frozen by plunging the needle into liquid nitrogen.

Enzyme activities

Intact mitochondria (Makinen & Lee, 1968; Peters *et al.*, 1998) were extracted from 45-110 mg fresh muscle and an aliquot was used to determine PDK and PDHt activities (Fatinia *et al.*, 1986; Peters *et al.*, 1998) with the exception that PDK activities were determined at 37°C. The final mitochondrial suspension required an incubation time of 20 min at 30°C with 10 μM carbonyl cyanide m-chlorophenyl-hydrazone, which decreased ATP concentration to zero and resulted in complete conversion of PDH to the active form (Peters *et al.*, 1998). As such, during the PDK activity assay, the time point that represents "zero time" also represents "total PDH activity" (Peters *et al.*, 1998). Fractional recovery of intact mitochondria (25 ± 2%) was used to convert mitochondrial activities of PDHt to mmol min^{-1} kg wet wt^{-1} (Peters *et al.*, 1998). CS and COX maximal enzyme activities were measured on 17-40 mg frozen muscle (Carter *et al.*, 2001).

Western blotting

Mitochondria were diluted to a final protein concentration of 1 μg μl^{-1} as previously described (Peters *et al.*, 2001b). Standard SDS/PAGE electrophoresis was performed as previously described (Peters *et al.*, 2001b) with the exception of a 12 % separating gel and 10 μg of mitochondrial protein loaded per lane. Electrophoretically separated proteins were transferred onto Protran nitrocellulose membranes (0.45 μm pore size, Schleicher & Schuell, NH, USA) using the Trans-blot semi-dry electrophoretic transfer cell (BioRad, CA, USA) with a transfer buffer containing 34.8 mM Tris base, 31.2 mM glycine, 0.03% (w/v) SDS, and 20% (v/v) absolute ethanol. Membranes were

incubated in TBST buffer (20 mM Tris base, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) with 5% (w/v) non fat dry milk for 1 hour to block all non-specific binding sites. Membranes were then incubated for 1 hour in 5% milk/TBST containing monoclonal antibodies against PDH subunits ($E_1\alpha$, E_2, and E_3bp; Molecular Probes, OR, USA), CS (Chemicon, CA, USA), or COX-II and IV subunits (Molecular Probes, OR, USA), or polyclonal antibodies against PDK isoforms (PDK1-4; Santa Cruz Biotechnology, CA, USA). The membranes were washed and then incubated for 1 hour in 5% milk/TBST containing either goat anti-mouse IgG (peroxidase conjugated, Sigma, Ont, CAN) or bovine anti-goat IgG (peroxidase conjugated, Santa Cruz Biotechnology, CA, USA). Membranes were again washed and antibody-antigen complexes were visualized on autoradiography film (Hyperfilm ECL, Amersham Biosciences, NJ, USA) after addition of chemiluminesent substrate (ECL, Amersham Biosciences, NJ, USA). Relative densities were quantified using Scion Image (Scion Corporation, MD, USA). Blots were washed, stained with DB-71 (Hong *et al.*, 2000) and total protein per lane was used to normalize loading between lanes on each blot. To correct for differences in blotting efficiency between gels, a rat standard was included in every gel. PDK antibodies were tested for cross-reactivity against purified PDK isoform proteins and did not cross-react with each other under the loading and detection conditions used for Western-blot analysis (data not shown).

Total RNA isolation and reverse transcription

Total RNA from 6-27 mg of frozen muscle was isolated using the FastRNA Kit-Green (Q-BIOgene, CA, USA) protocol and reagents. Total RNA concentrations were determined spectrophotometrically at 260 nm and purity at 280 nm. First strand cDNA was generated from 0.5 μg RNA using AMV Reverse Transcriptase (Promega, WI, USA) as previously described by Wadley et al. (2001). The cDNA was stored at –80°C for subsequent analysis.

Real-time PCR analysis

Primers were designed using Primer Express™ software package version 1.0 (Applied Biosystems, CA, USA) from gene sequences obtained from GenBank (CS, AF047042; PDH-$E_1\alpha$, NM_000284; PDH-E_2, NM_001931; PDH-E_3bp, NM_003477; PDK1, NM_002610; PDK2, NM_002611; PDK3, NM_005391; PDK4, NM_002612). The primer sequences were validated using BLAST (Altschul *et al.*, 1990) to ensure homology between primer and desired mRNA of human skeletal muscle. Primer sequences are shown in Table 1. Quantification of mRNA expression was performed (in triplicate) by real-time PCR using the GeneAmp® 5700 sequence detection system (Applied Biosystems, CA, USA) as described previously (Wadley *et al.*, 2001; Tunstall *et al.*, 2002). To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-actin (GenBank, NM_001101) mRNA was quantified, and results were normalized to these values as described previously (Wadley *et al.*, 2001; Tunstall *et*

Table 2.1. Gene primer sequences.

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')
β-actin	GAC AGG ATG CAG AAG GAG ATT ACT	TGA TCC ACA TCT GCT GGA AGG T
CS	GTG CCC ATA CCA GCC ACT TG	CTG CCA GCC CGT TCA TG
PDH-E1α	TGT GGA AGA ACT AAA GGA AAT TGA TGT	TTC CAA AGG TGG CTC AGG AT
PDH-E2	CTC CCA CAG GTC CTG GAA TG	GTC CAA TAA CCC GCA GAA TGT
PDH-E3bp	CTG AGG ATG AAG AGG GAA ATG C	TCA TCA ACC ACT CGA CTC TCA CT
PDK1	CCG CTC TCC ATG AAG CAG TT	TTG CCG CAG AAA CAT AAA TGA G
PDK2	CCG CTG TCC ATG AAG CAG TT	TGC CTG AGG AAG GTG AAG GA
PDK3	CAA GCA GAT CGA GCG CTA CTC	CGA AGT CCA GGA ATT GTT TGA TG
PDK4	CCC GAG AGG TGG AGC ATT T	GCA TTT TCT GAA CCA AAG TCC AGT A

al., 2002). β-actin mRNA levels did not change in response to 1 or 8 weeks of training (results not shown), similar to previous aerobic training studies in rat (Murakami *et al.*, 1994) and human skeletal muscle (Wadley *et al.*, 2001).

Statistical analysis

All data are presented as means ± SEM. A two-way analysis of variance (ANOVA) with repeated measures was used to establish differences between condition and time. Tukeys post hoc test was used to determine significance. Assumptions for normality and independence were verified by generating appropriate residual plots. Data transformations (log, square root, and inverse square root) were used when appropriate to meet the above assumptions.

RESULTS

The effects of the exercise training program are evident by a significant increased maximal oxygen uptake after 8 weeks, increasing from 3.51 ± 0.15 to 4.05 ± 0.15 l min^{-1} ($p<0.05$). This was accompanied by a 40 and 41% increase in CS and COX maximal activity, respectively, which was not seen after 1 week (Table 2.2). Proteins of CS and both COX subunits did not change after 1 week of aerobic training (Fig 2.1). CS protein increased 54% after 8 weeks of training, as did COX-II and IV protein, demonstrating a 12 and 62% increase, respectively. CS mRNA were unchanged after 1 (1.11 ± 0.20 fold) and 8 (0.98 ± 0.15 fold) weeks of training compared to pre-training.

Table 2.2. Maximal enzyme activities before and after 1 and 8 weeks of aerobic training.

	Training (weeks)		
	0	1	8
CS	20.2 ± 1.6	23.3 ± 1.6	28.2 ± 1.7*†
COX	12.4 ± 2.0	8.4 ± 1.1	17.5 ± 1.4*†
PDHt	3.75 ± 0.24	3.65 ± 0.35	4.93 ± 0.38*†
PDK	0.09 ± 0.01	0.11 ± 0.02	0.18 ± 0.03*†

Values are means ± SEM; enzyme activity expressed in mmol/min/kg wet wt. except for PDK which is expressed min^{-1}; CS, citrate synthase; COX, cytochrome c oxidase; PDHt, total pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase * denotes significance from pre-training; † denotes significance from 1 week.

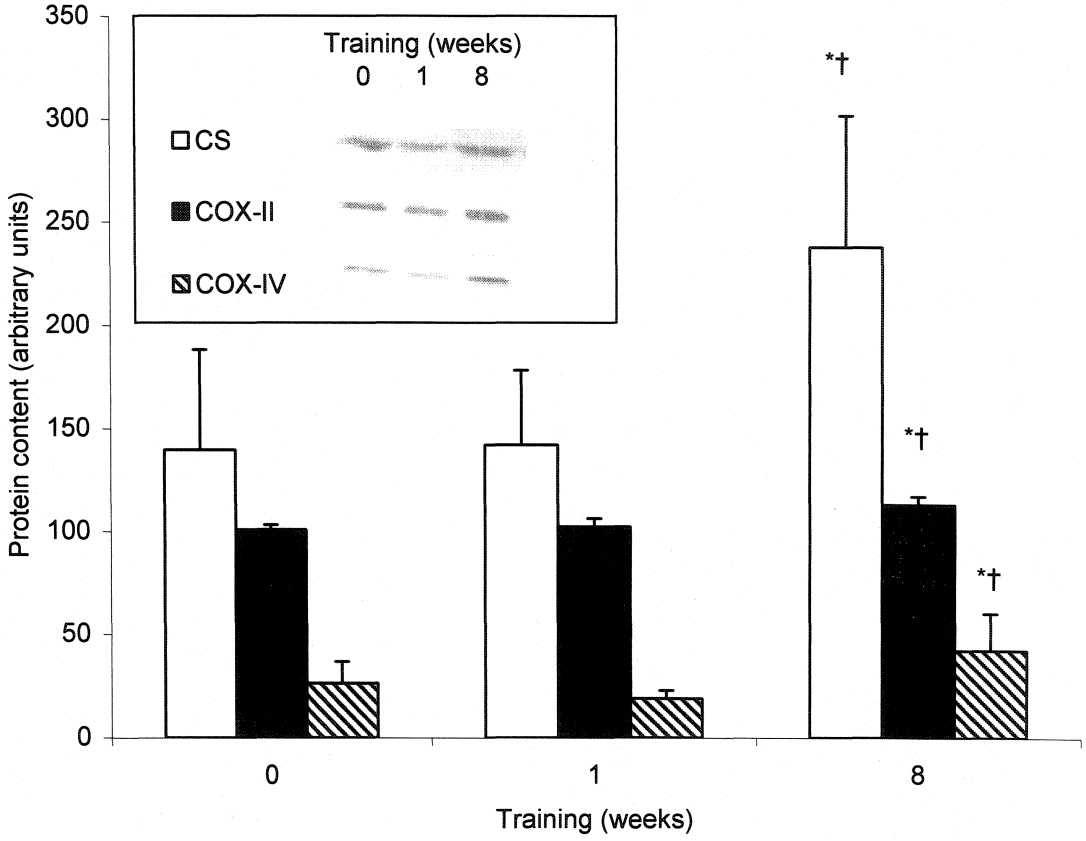

Figure 2.1. Mitochondrial protein content of CS, COX-II, and COX-IV before and after 1 and 8 weeks of aerobic training. * denotes significance from rest; † denotes significance from 1 week.

PDHt did not differ after 1 week of exercise but increased by 31% after 8 weeks (Table 2.2). There was no change in the protein expression of PDH subunits after 1 week of exercise and only PDH-$E_1\alpha$ increased 1.3 fold after 8 weeks (Fig 2.2). Both PDH-$E_1\alpha$ and PDH-E_3bp mRNA were unaffected by exercise training and PDH-E_2 was lower after 8 weeks of exercise compared to 1 week (Table 2.3).

PDK activity did not differ after 1 week of exercise but approximately doubled after 8 weeks (Table 2.2). There was no change in the protein expression of PDK isoforms after 1 week of exercise and only PDK2 increased 1.3 fold after 8 weeks (Fig 2.3). PDK3 was undetectable in the present study. PDK isoform mRNA levels were unaffected by exercise training (Table 2.3).

DISCUSSION

The highly adaptive nature of skeletal muscle with repeated bouts of exercise allows for metabolic remodeling, leading to enhanced functional capacity. To our knowledge, this is the first study to examine the effects of short- and long-term aerobic training on enzyme activity and gene and protein expression of both PDH and PDK in human skeletal muscle. The major novel findings from the present study were that 8 weeks, but not 1 week, of aerobic training in human skeletal muscle resulted in a; 1) 31% increase in PDHt activity, thus increasing the maximum potential capacity to utilize carbohydrates, and 2) 2 fold increase in PDK activity, thus potentially attenuating the activation of PDH during submaximal exercise. Increased PDHt activity was accompanied by increased PDH-$E_1\alpha$ protein expression whereas increased PDK activity

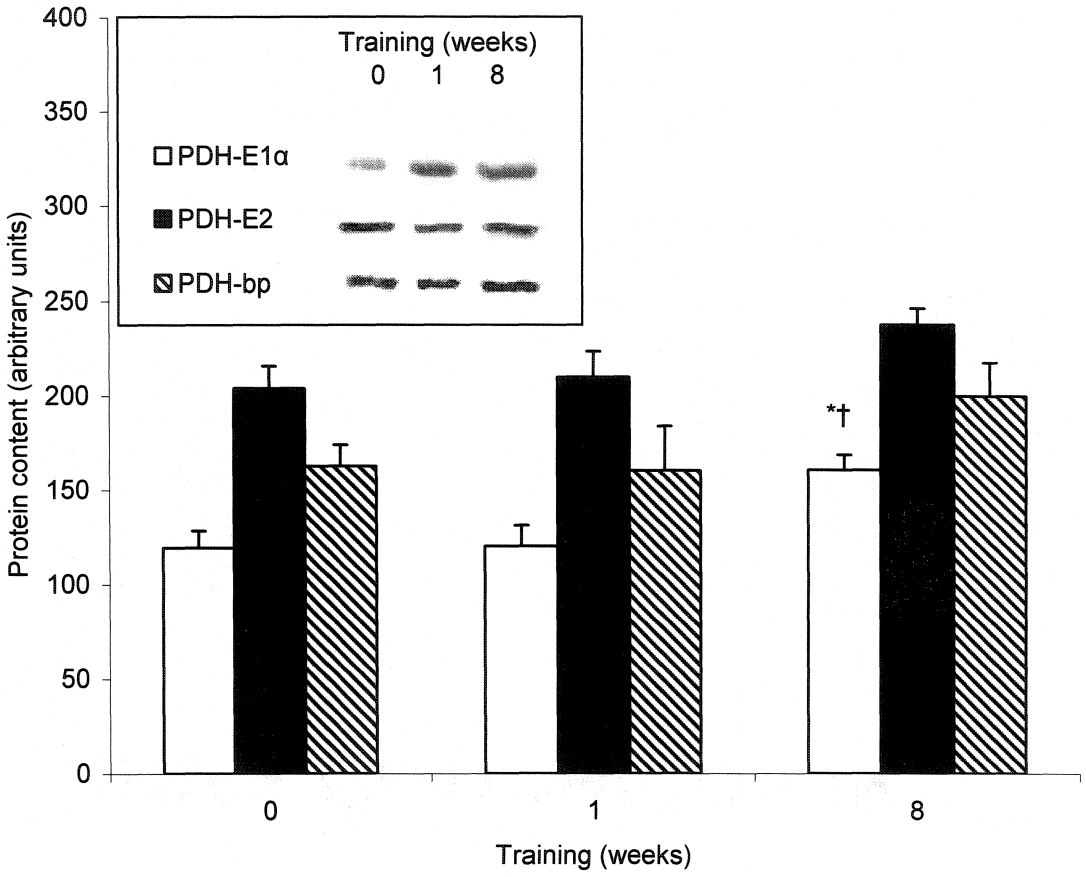

Figure 2.2. Mitochondrial protein content of PDH-E1α, PDH-E2, and PDH-E3bp before and after 1 and 8 weeks of aerobic training. Legend demonstrating protein for a representative subject. * denotes significance from pre-training; † denotes significance from 1 week.

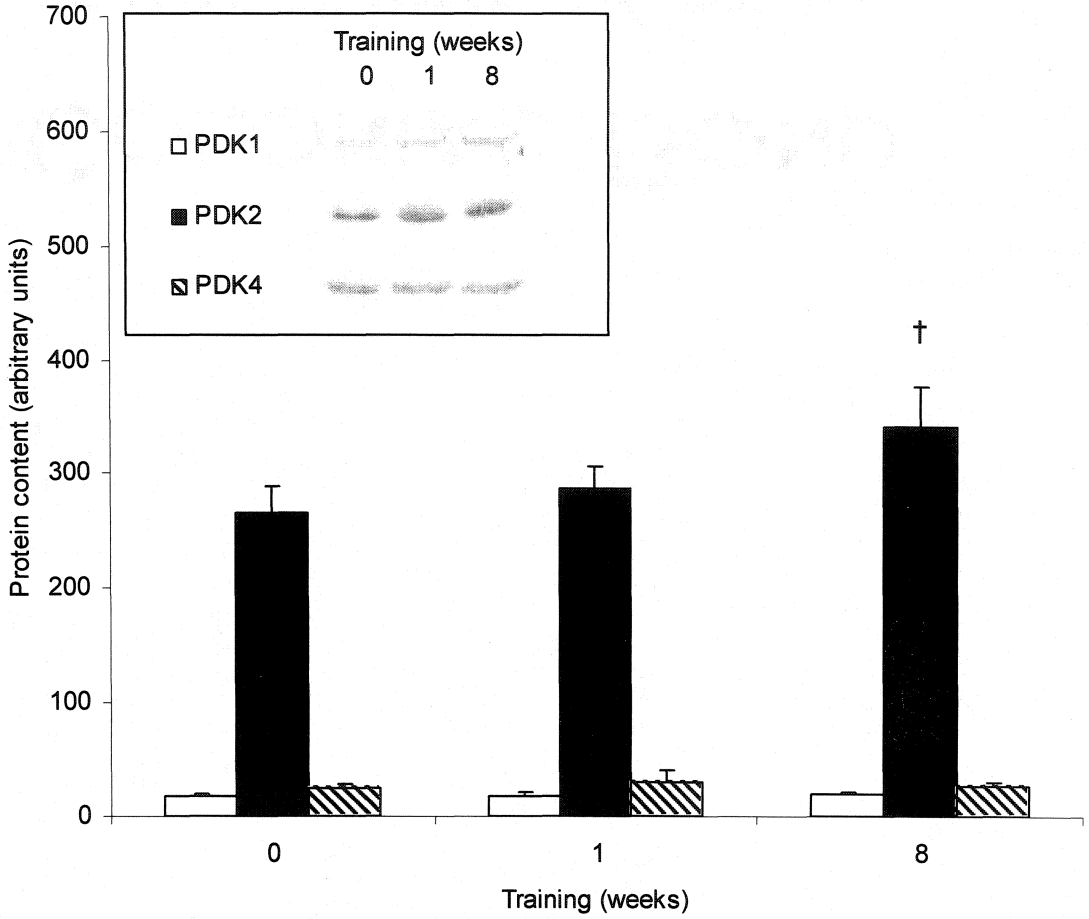

Figure 2.3. Mitochondrial protein content of PDK1, PDK2, and PDK4 before and after 1 and 8 weeks of aerobic training. † denotes significance from pre-training. Legend demonstrating protein for a representative subject.

Table 2.3. Skeletal muscle mRNA content of PDH-$E_1\alpha$, PDH-E_2, PDH-E_3bp and PDK1-4 before and after 1 and 8 weeks of aerobic training.

	Training (weeks)		
	0	1	8
PDH-$E_1\alpha$	1.296 ± 0.146	1.547 ± 0.209	1.277 ± 0.119
PDH-E_2	0.092 ± 0.009	0.119 ± 0.020	0.076 ± 0.009†
PDH-E_3bp	0.076 ± 0.008	0.102 ± 0.016	0.073 ± 0.009
PDK1	0.004 ± 0.001	0.005 ± 0.001	0.003 ± 0.001
PDK2	0.207 ± 0.038	0.269 ± 0.057	0.150 ± 0.017
PDK3	0.002 ± 0.0002	0.002 ± 0.0002	0.002 ± 0.0001
PDK4	0.005 ± 0.002	0.005 ± 0.002	0.005 ± 0.001

Values are means ± SEM with n=8; mRNA content expressed in arbitrary untis; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase. † denotes significance from 1 week.

was accompanied by increased PDK2 protein expression. Despite changes in activity and protein expression of PDHt and PDK, mRNA levels did not differ with training.

Mitochondrial oxidative capacity

An important by-product of repeated bouts of aerobic exercise is mitochondrial biogenesis (Hood, 2001), defined by increases in mitochondrial content following training. Training also increases the mitochondrial capacity to produce ATP, resulting in tightly coupled oxidative phosphorylation and higher level of respiratory control. Depending on the training protocol and the pre-exercise trained state of subjects, mitochondrial adaptations may require 1-6 weeks before reaching a higher steady-state level (Hood, 2001). Thus the maximal activity and gene expression of representatives of the TCA cycle (CS) and electron transport chain (COX) will give insight into the adaptation to the training program.

In the present study, 1 week of exercise did not alter the maximal activity or gene expression of CS and COX. This is consistent with the majority of previous studies, reporting that 5-12 days of aerobic exercise in humans did not change maximal activities of trained skeletal muscle mitochondrial enzymes (Green *et al.*, 1991; Green *et al.*, 1992; Phillips *et al.*, 1995a; Phillips *et al.*, 1996a; Putman *et al.*, 1998). However, a couple of studies have demonstrated an increased CS maximal activity after 7-10 days of aerobic training (Chesley *et al.*, 1996; Spina *et al.*, 1996). The metabolic adaptations to short-term aerobic training remain controversial and will not be discussed in this paper. In contrast, after 8 weeks of exercise, the present study demonstrated an approximate 40%

increase in maximal activities of CS and COX. This is consistent with another human study using a similar training program (Carter *et al.*, 2000). There was also a concomitant increase in gene expression of these two enzymes at the level of translation. The increases seen in CS and COX II and IV proteins are similar to previously reported values in exercise trained skeletal muscle of rats (Booth, 1991; Samelman *et al.*, 2000) and humans (Dubouchaud *et al.*, 2000; Bengtsson *et al.*, 2001). Therefore, the present study demonstrates a clear adaptation in skeletal muscle typically seen with long-term aerobic exercise.

Short-term training – PDHt and PDK

Five days of aerobic exercise did not alter PDHt activity or PDH subunit expression in human skeletal muscle. This is consistent with a recent study which demonstrated that PDHt activity was unresponsive to 7 days of aerobic exercise in humans (60% $\dot{V}O_{2max}$ for 2 h daily; Putman *et al.*, 1998), however, PDH subunit expression was not measured. In addition, numerous studies have shown no difference in $\dot{V}O_{2max}$, and thus maximal skeletal muscle carbohydrate oxidative capacity, after 5-10 days of aerobic exercise (Green *et al.*, 1991; Green *et al.*, 1992; Green *et al.*, 1995; Phillips *et al.*, 1995b; Phillips *et al.*, 1996a).

As hypothesized, 5 days of aerobic exercise was not enough to elicit changes in PDK activity or the expression of any of the PDK isoforms. This is similar to a previous study, despite no measurements of PDK activity or isoform protein expression, which

reported no change in PDK4 transcriptional rate or mRNA content in human skeletal muscle after 5 days of aerobic exercise (Pilegaard *et al.*, 2000). Previous studies of short-term aerobic exercise in humans (5-10 days) have shown that metabolic adaptations precede changes to mitochondrial capacity, demonstrating reductions in lactate production and phosphocreatine and glycogen utilization (Green *et al.*, 1991; Green *et al.*, 1992; Green *et al.*, 1995; Phillips *et al.*, 1995b; Phillips *et al.*, 1996a; Putman *et al.*, 1998). It was hypothesized that these adaptations were possibly mediated through allosteric modulation of PDH and glycogen phosphorylase (Putman *et al.*, 1998). Thus any regulation of PDH with short-term aerobic exercise would be through acute regulators acting on PDK and PDP rather than changes to PDK or PDP maximal activities.

Long-term training - PDHt

The effect of long-term aerobic exercise on skeletal muscle carbohydrate utilization is an intricate balance between the control of carbohydrate utilization, through the activation of PDH, and the maximum potential capacity to utilize carbohydrates. In addition to increases in muscle mitochondrial volume, prolonged aerobic exercise training enhances the maximum potential carbohydrate flux in exercise trained compared to untrained skeletal muscle (see Goodyear & Kahn, 1998; Holloszy *et al.*, 1998; Wojtaszewski & Richter, 1998; Henriksen, 2002 for review). This increased maximum potential for carbohydrate utilization may be at the level of PDH, through stable increases in PDHt activity. This is the first study to report an increased PDHt activity (31%) in

human skeletal muscle after long-term aerobic exercise. In contrast, the only other study to examine the effects of repeated bouts of exercise on PDHt in humans reported no change in activity in triceps muscle after 5 weeks of strength exercise (Ward *et al.*, 1986). Due to differences in muscle adaptation with strength and aerobic exercise, it is difficult to compare these studies.

It was hypothesized that the increased skeletal muscle PDHt activity after 8 weeks of exercise would be paralleled by increased PDH subunit protein expression. This was true only for PDH-$E_1\alpha$, which increased 1.3 fold after 8 weeks of exercise. This is consistent with previous studies examining tissues other than skeletal muscle. Maury et al. (Maury *et al.*, 1995) demonstrated an increased PDHt activity and PDH-$E_1\alpha$ protein content in rat adipose tissue during the suckling-weaning transition, in which milk, a high-fat low-carbohydrate diet, is progressively replaced at weaning by an adult low-fat high-carbohydrate diet. In addition, livers of genetically obese (Amessou *et al.*, 1998) and fat-free fed (Da Silva *et al.*, 1993) rats demonstrate increased PDHt activity and PDH-$E_1\alpha$ gene expression, with little or no change in the other subunits measured. This is of importance because the PDH-E_1 tetramer ($\alpha_2\beta_2$) carries out the non-reversible decarboxylation of pyruvate and the $E_1\alpha$ subunit has three phosphorylation sites which, when phosphorylated, inactivate the PDH complex (Patel & Korotchkina, 2001). Although not measured in the present study, it would be expected that PDH-$E_1\beta$ would increase in parallel to PDH-$E_1\alpha$. In addition, a lack of significant increases in the other PDH subunits measured suggests that the amount of the $E_1\alpha$ subunit, and possibly the E_1 heterotetramer, is rate-determining for the formation of new PDH complexes, as

previously suggested for other tissues (Maury *et al.*, 1995). Thus, it appears that an increased expression of the E_1 subunit accommodates an increased PDHt activity in human skeletal muscle with long-term aerobic exercise.

Despite the increased PDHt activity and PDH-$E_1\alpha$ with long-term aerobic exercise, no differences were detected in PDH subunit mRNA. Altered mRNA content is a product of the rate of mRNA production (gene transcription), degradation (mRNA stability), and subsequent protein production (translation). It is known that a single bout of exercise results in a transient increase in transcriptional rate of genes in skeletal muscle during the recovery period, believed to return to pre-exercise levels after 24 hours (Neufer *et al.*, 1998; Pilegaard *et al.*, 2000). In addition, studies on cytochrome oxidase gene expression in rats have reported that increased mRNA in skeletal muscle with chronic electrical stimulation is a result of time-dependent increased mRNA stability (Freyssenet *et al.*, 1999). There also appears to be translational and/or post-translational regulation of gene expression that occurs with exercise training in skeletal muscle of rats (Booth, 1991). The adaptation to repeat bouts of exercise may be due to the cumulative effects on any of these factors that contribute to gene expression, thus altering the level of skeletal muscle mRNA. As a result, it becomes difficult to interpret the mRNA results from the present study until further studies are done to determine what effect aerobic exercise training has on skeletal muscle mRNA stability and translational rates.

Long-term training - PDK

Long-term aerobic training results in a greater reliance on fat for ATP synthesis during submaximal exercise (Henriksson, 1977; Hurley *et al.*, 1986; Martin *et al.*, 1993; Phillips *et al.*, 1996b), resulting in less reliance on carbohydrates and a net glycogen sparing effect. Activities of PDK and PDP, and resultant activation state of PDH, play important role in this adaptation to training. In the current study, 8 weeks of aerobic exercise increased PDK activity approximately 2 fold. It is important to note that this increased PDK activity persisted after a rigorous mitochondrial preparation, and thus was not due to altered mitochondrial effectors known to increase PDK activity. The increased PDK post training reported in the present study is consistent with aerobic training in rats, demonstrating an increased PDK after 8 weeks of voluntary wheel running (Nakai *et al.*, 1999). Increased PDK activity with long-term aerobic training would result in greater phosphorylation, and resulting inactivation, of the aforementioned 31% increased PDHt.

The long-term aerobic training-induced adaptations in skeletal muscle metabolism are not limited to the carbohydrate side. Previous studies have demonstrated greater adaptive increases in fat metabolism relative to carbohydrate metabolism in aerobically trained skeletal muscle (Davies *et al.*, 1981; Wibom *et al.*, 1992; Bizeau *et al.*, 1998). Thus although aerobic training increases the maximal capacity to oxidize carbohydrates, there is a greater relative increase in enzymes that suppress carbohydrate flux (e.g. PDK) and enhance fat metabolism during submaximal exercise post training.

The increased PDK activity reported in the current study may be attributed to a 1.3 fold increase in PDK2 protein, with no change detected in protein of the other

isoforms measured. To our knowledge, this is the first study to examine the effects of prolonged aerobic exercise training on the expression of PDK isoforms. Previous studies, focusing on dietary manipulation and acute disease states, reported an increased PDK4 protein in skeletal muscle during high fat diet (Holness *et al.*, 2000), fasting (Wu *et al.*, 1999; Sugden *et al.*, 2000; Peters *et al.*, 2001a), and insulin resistance (Wu *et al.*, 1999) in rats and high fat diet in humans (Peters *et al.*, 2001b). It appears that the adaptive response of skeletal muscle PDK4 is to acute alterations in fat availability, termed the "lipid status"-responsive PDK isoform (Sugden *et al.*, 2001). In contrast, previous studies examining the less active but more abundant isoform PDK2, report no change to acute physiological (Wu *et al.*, 1999; Sugden *et al.*, 2000; Peters *et al.*, 2001a) or pathological (Wu *et al.*, 1999) perturbations. PDK2 is important because it has a strong synergistic inhibition by ADP and dichloroacetate (DCA; Gudi *et al.*, 1995; Bowker-Kinley *et al.*, 1998), a unique response to high NADH/NAD ratio plus acetyl-CoA, increasing its activity to more than 300% of the control (Bowker-Kinley *et al.*, 1998), and appears to respond to chronic perturbations, such as non-insulin-dependent diabetes mellitus (Majer *et al.*, 1998). This evidence, along with the finding of the present study, suggests that chronic alterations to energy metabolism and energy status of the cell favor an upregulation of PDK2, possibly making this the "energy status"-responsive PDK isoform.

A recent study conducted in our laboratory demonstrated a significantly lower activation of PDH during steady state submaximal exercise in individuals after 7 weeks of aerobic exercise (LeBlanc *et al.*, 2003). We concluded that the altered flux through

glycogen phosphorylase, influenced by cellular energy status, attenuated the exercise-induced increase in muscle [pyruvate], thus releasing inhibition on PDK. In the present study, the training-induced adaptive increase in skeletal muscle PDK2, which is highly sensitive to pyruvate, may be important in regulating PDH activation during submaximal exercise post training.

With only an approximate 25% increase in PDK2 protein content in skeletal muscle, there appears to be other factors responsible for a two fold increase in PDK activity. One possible contributor may be the other PDK isoforms found in the muscle, but in lower quantities. Both PDK1 and 3 demonstrate 2-25 times higher specific activity compared to PDK2 and 4 (Bowker-Kinley *et al.*, 1998). Thus, small changes in training-induced protein expression of PDK1 and 3 would result in large changes in relative contribution to total PDK activity. Other possible contributors other than increased protein synthesis may be the interaction between PDK and the PDH complex. PDK is known to exist in two states, intrinsic (bound) and free (unbound; Kerbey *et al.*, 1984; Jones & Yeaman, 1991; Mistry *et al.*, 1991). Intrinsic PDK binds to the innermost lipoyl domain of the E_2 component of PDH (Ravindran *et al.*, 1996) and represents only 15-30% of the total PDK activity (Kerbey *et al.*, 1984; Mistry *et al.*, 1991; Vary & Hazen, 1999). Shifts from free to intrinsic PDK may enhance the enzyme activity with little or no change in protein synthesis. In addition, intrinsic PDK activity is enhanced when associated with higher ratios of reduced or acetylated forms of the E_2-lipoyl domain (Korotchkina & Patel, 2001). More specifically, the activity of PDK2 approximately doubled with reduced/acetylated E_2 compared to the oxidized form (Korotchkina & Patel,

2001). To date, no study has examined the effects of long-term aerobic training on the expression of intrinsic and free PDK and the interaction between these two forms of PDK and the PDH-E_2 lipoyl domain.

Similar to PDH mRNA, PDK isoform mRNA was unaltered with exercise training, which is consistent with a recent study that examined high-intensity training in human skeletal muscle (Nordsborg *et al.*, 2003). Again, this may be due to the transient nature of gene expression of these proteins with exercise, which may not be detectable during the sampling time frame (24-48 hours after last exercise bout). Despite no change in PDK mRNA with aerobic exercise training, the reported increase in PDK2 protein would suggest an increased gene expression of this protein. It is possible that the cumulative effect of transient increases in PDK2 mRNA following each bout of exercise may have contributed to the stable increase in PDK2 protein after 8 weeks of exercise. Previous studies, possibly representing more short-term sustained perturbations, reported increased PDK4 mRNA with fasting (Wu *et al.*, 1999; Peters *et al.*, 2001a) and streptozotocin-induced diabetes (Wu *et al.*, 1999) in rats and during recovery from a prolonged bout of exercise in 5 day trained humans (Pilegaard *et al.*, 2000), with little or no change to PDK2 mRNA. It is believed that potential fat-dependent promoters, such as peroxisome proliferators-activated receptor α (see Sugden *et al.*, 2001 for review), are responsible for the increased PDK4 transcription. To date, no study has examined which exercise-induced promoters may stimulate gene expression of PDK isoforms, more specifically PDK2. There are numerous mechanical, metabolic, neuronal, and hormonal factors (see Fluck & Hoppeler, 2003 for review) that could contribute to an upregulation

of PDK2 expression with exercise training. Future experiments will have to determine which regulatory parameters are linked to altered PDK2 transcription and translation.

We have demonstrated in the present paper that prolonged aerobic training up regulated the amount and activity of PDHt and one of its regulatory proteins, PDK. The other covalent regulator of PDH, PDP, was not measured in this study, however, it may be speculated that it also increased with exercise training to accommodate the overall increase in the enzyme complex. To date, there are two known isoforms of PDP in mammals; PDP1 and PDP2. PDP1 is Ca^{++}-sensitive and preferentially expressed in skeletal muscle, whereas PDP2 is relatively unaffected by the absence or presence of Ca^{++}, is insulin sensitive, and found more abundantly in liver and adipose tissue (Huang *et al.*, 1998). Few studies have examined the effects of physiological or pathological perturbations on PDP, reporting mixed results. The only study to examine skeletal muscle reported no change with acute starvation and streptozotocin-induced diabetes in rats (Fuller & Randle, 1984). More work is needed to establish the relative contribution of PDP to skeletal muscle adaptations to more prolonged physiological (e.g. exercise training) or pathological (e.g. obesity, diabetes) conditions.

Summary and conclusion

The present study clearly demonstrates that long-term aerobic training influences carbohydrate utilization in human skeletal muscle. The alterations are evident at the level of PDH and its control. Eight weeks of aerobic exercise increased PDHt activity, thus increasing the maximal potential capacity to oxidize carbohydrates. This was mainly due

to increased expression of PDH subunit genes, more specifically the $E_1\alpha$ subunit. Also, aerobic exercise training increased PDK activity, thus increasing the level of control on PDH. This was in part due to increased expression of PDK2. As a result, exercise trained skeletal muscle would have an increased maximal capacity to utilize carbohydrates during maximal oxygen uptake, with an increased metabolic control sensitivity to pyruvate during submaximal exercise through increased PDK2.

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CHAPTER 3. Seven weeks of aerobic training attenuates the exercise-induced activation of pyruvate dehydrogenase in human skeletal muscle. (Submitted to *Amer. J. Physiol.* Nov. 2003)

INTRODUCTION

Carbohydrates and fat are the major metabolic fuels for ATP production in skeletal muscle at rest and during exercise (see 11 for review). At workloads above 65-70% of maximal oxygen uptake, carbohydrates, primarily in the form of muscle glycogen, become the dominant fuel source (43, 45). Exercise at the same absolute workload, thus similar ATP turnover rates, after several weeks of repeated bouts of aerobic exercise (aerobic training) result in a greater reliance on fat for ATP synthesis (18, 24, 31, 35), with less reliance on carbohydrates, a net glycogen sparing effect, and less lactate accumulation in skeletal muscle.

A common theory to explain the regulation of fat and carbohydrate use in skeletal muscle with prolonged aerobic training centers around the importance of mitogenesis, and the resulting increased muscle oxidative potential and tighter coupling between ATP supply and demand (9, 18, 21). During exercise, the major factor determining energy requirement in contracting skeletal muscle is the rate of ATP hydrolysis. The rate of ATP production, determined by the amount of enzymes in energy-producing catabolic pathways, becomes limiting only when the energy need of the cell exceeds the maximal catalytic capacity of the rate-determing steps of these pathways. Thus, a possible consequence of an adaptive increase in mitochondria, and the associated enzymatic pathways, is that the aerobic energy production pre-training becomes "submaximal" for the muscle after prolonged aerobic training (22). This improved capacity of aerobic energy production results in a greater respiratory control sensitivity, with smaller relative changes in cytosolic modulators of oxidative phosphorylation (9). This is a result of

reduced reliance on energy sources such as phosphocreatine (PCr), with an added improvement ATP/(ADP + P_i) ratio in the muscle, and reduced accumulations of P_i and free ADP (ADP_f). Both P_i and ADP_f, together with NADH and oxygen, are known regulators of oxidative phosphorylation (9). Alterations to these metabolic parameters also influence enzyme systems in skeletal muscle, more specifically glycolysis. Two important enzymes of the glycogenolytic/glycolytic pathway are glycogen phosphorylase (Phos), a flux-generating enzyme of glycogenolysis, and pyruvate dehydrogenase (PDH), a rate-determing enzyme that regulates the entrance of glycogenolytically/glycolytically produced acetyl units into oxidative metabolism. Since both Phos and PDH play important roles in dictating the rate of carbohydrate-derived acetyl units for ATP production suggest that the response of these enzymes, and their regulators, to prolonged aerobic training may provide insight into aerobic training-induced regulation of skeletal muscle substrate utilization.

Previous studies have demonstrated that 3-12 days of aerobic training can induce changes in skeletal muscle fuel utilization, energy metabolism, and lactate accumulation prior to changes in oxidative capacity (12, 13, 15, 33, 34), with no changes in PDH activation (37). However, the question remains whether extending the training duration to increase the exercising muscle respiratory capacity, and possible alterations to PDH and Phos, will exaggerate or replace the metabolic adaptations observed early in training.

To date, no studies have examined the effects of prolonged aerobic training on the activation states of Phos and PDH in human skeletal muscle, or the roles that these two enzymes may play in regulating the shift from carbohydrate to fat utilization post-

training. Thus, the aim of the present study was to investigate potential changes in Phos and PDH activity, along with the regulatory factors which control the activation states of these enzymes, during submaximal exercise in human skeletal muscle after 7 weeks of aerobic training. We hypothesized that prolonged aerobic training would increase mitochondrial oxidative capacity and reduce disturbances to the energy status of the cell, resulting in less glycogen breakdown, due to reduced flux through Phos and PDH, during submaximal exercise at the same absolute workload.

METHODS

Subjects

Eight healthy, active men were recruited to participate in the study (age 22 ± 1.0 (SEM) yr; height 178.9 ± 2.0 cm; weight 91.6 ± 4.3 kg). Individuals were asked to refrain from caffeine, alcohol, and exercise for 48 hours before each trial. Individuals also consumed the same diet 24 hours prior to each trial, confirmed by subsequent nutritional analyses of dietary records (Nutritionist 5, ver. 1.7, First DataBank Inc., CA). The dietary analysis revealed that the diets consisted of 2751 ± 168 kcal (53 ± 2% carbohydrates, 30 ± 2% fat, and 17 ± 1% protein). Prior to each experimental protocol, subjects consumed a standardized high carbohydrate meal (711 kcal; 87% carbohydrate, 3% fat and 10% protein) that was provided for them. Verbal and written explanation of the experimental protocol and its attendant risks were provided and informed consent was obtained from each subject. The study was approved by the Hamilton Health

Sciences/Faculty of Health Sciences Research Ethics Board in accordance with the Declaration of Helsinki.

Pre-experimental protocol

Individuals completed an initial incremental maximal exercise test on an electro-magnetically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA.) to determine maximal work capacity and $\dot{V}O_{2max}$ using a metabolic measurement system (Moxus Modular VO2 System, AEI Technologies, Inc., Pittsburgh, PA).

Training Protocol

Individuals were trained on a cycle ergometer at a workload that elicited 75% of their maximal O_2 uptake for 1 hour per day, five days a week for a total of 7 weeks. For the first week, the 1 hour of training was broken up into four 15 min intervals with 5 min rest in-between. For the second and third weeks, the 1 hour of training was broken up into three 20 minute intervals with 5 and 2.5 minutes rest in-between, respectively. For the third to eighth week, the 1 hour of training was broken up into three 20 minute intervals with 1 minutes rest in-between. On the fourth week, individual subjects completed an incremental maximal exercise test to determine changes in maximal work capacity and O_2 uptake so that adjustments could be made to the workload in order to maintain a training intensity equivalent to 75% of each subject's maximal O_2 uptake.

Experimental protocol

Each subject participated in two experimental trials, before (PRE) and after (POST) 7 weeks of submaximal aerobic training. Studies were performed at the same time of day. Individuals served as their own control. Prior to the beginning of the exercise protocol, a venous catheter was inserted in an anticubital vein for blood sampling and was maintained patent with saline. One thigh was prepared for the extraction of needle biopsy samples from the vastus lateralis as described by Bergstrom (2). Three incisions were made through the skin to the deep fascia under local anesthesia (2% lidocaine without epinephrine). Subjects cycled to exhaustion at the same absolute workload (206 ± 5 W), eliciting 80% of their peak power output pre-training. Expired gases were collected during steady state exercise pre- (25-30 min) and post- (20-30 and 55-70 min) training for the determination of $\dot{V}O_2$ and RER and averaged over the sampling time. Blood samples were taken at rest and after 10 min of exercise. Muscle biopsies were obtained at rest and after 5 and 15 min of exercise.

Blood sampling and analysis

Venous blood samples (~ 7 ml) were collected in heparinized syringes (Sarstedt, Germany) and placed on ice. Blood was centrifuged at 15,900 *g* for 2 min to isolate plasma. One portion of plasma was incubated with 5 M NaCl (4:1 plasma:NaCl) at 56°C for 30 min and then frozen for later analysis of free fatty acids (Wako NEFA kit, WAKO Chemicals). A second portion of plasma was deproteinized with 500 mM perchloric acid (PCA; 1:2 plasma:PCA) and then frozen for later analysis for glucose and lactate (1).

Muscle analysis

Muscle biopsies were immediately frozen by plunging the needles into liquid N_2. Small pieces were chipped from each biopsy (under liquid N_2) for determination of the activity of PDH in the active form (PDH_a; 40) and citrate synthase (CS; 20). The remainder of the sample was freeze dried, dissected free of blood and connective tissue, and powdered. One aliquot was analyzed for total (a+b) and active Phos (Phos *a*+*b* and Phos *a* respectively; 48) and the maximal velocity and mole fraction of Phos *a*+*b* and Phos *a* were calculated from the measured activities as described by Chasiotis et al. (5). Phos measurements were made only on exercise samples because accurate resting samples are obtainable only if the biopsy is held from liquid nitrogen freezing for 30 seconds, due to Ca^{++} release during muscle sampling, which artificially increases the transformation of Phos *b* to *a* (42). Thus it was deemed unethical to obtain extra samples for resting Phos and previous studies have reported values of ~10% for the mole fraction of Phos *a* at rest in human skeletal muscle (42). A second aliquot was extracted in 500 mM PCA and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$ and analyzed for acetyl-CoA, free CoA, acetylcarnitine, free carnitine (4), ATP, pyruvate, lactate, phosphocreatine, creatine, glucose, glucose 6-phosphate (G-6-P), glucose 1-phosphate (G-1-P), fructose 6-phosphate (F-6-P), and glycerol 3-phosphate (Gly-3-P; 1). The final aliquot was used to measure muscle glycogen (17). All muscle metabolites, along with Phos, PDH and CS enzyme measurements, were normalized to the highest total creatine content for a given individual to correct for non-muscle contamination.

Calculations

The concentrations of ADP_f and free AMP (AMP_f) were calculated from the near-equilibrium reactions of creatine kinase and adenylate kinase, respectively (7), however these calculations do not provide information as to the exact location of these metabolites within the cell. The concentration of free inorganic phosphate (P_i) was calculated as the difference between resting and exercise [PCr], less the accumulation of G-6-P, F-6-P, and Gly-3-P, plus the assumed resting concentration of 10.8 mmol/kg dry wt (7).

Previous studies have shown that *in vivo* flux through PDH is similar to its *in vitro* level of activity at varying exercise power outputs (10, 23, 38, 39). Thus pyruvate oxidation, estimated by PDH_a flux, was calculated from PDH_a activity as measured in wet tissue and converted to dry tissue using the wet-to-dry muscle ratio of 4:1 at rest and 4.5:1 during exercise (37).

Pyruvate production was calculated from the sum of the measured rate of muscle lactate and pyruvate accumulation, plus the rate of blood lactate accumulation (distribution volume of blood lactate was assumed to be 0.64*body weight), plus the flux of pyruvate through PDH_a. The calculation of blood lactate accumulation does not account for lactate oxidation by other tissues and would result in a slight underestimation of lactate accumulation.

Statistical analysis

All data are presented as means ± SE. A two-way analysis of variance (ANOVA) with repeated measures was used to establish differences between condition and time. Tukeys post hoc test was used to determine significance ($p<0.05$). Assumptions for normality and independence were verified by generating appropriate residual plots. Data transformations (log, square root, and inverse square root) were used when appropriate to meet the above assumptions.

RESULTS

Cardiorespiratory measurements

$\dot{V}O_{2max}$ increased 5% after 7 weeks of aerobic training (PRE, 3.90 ± 0.15 l/min; POST, 4.13 ± 0.13 l/min; $p<0.05$). $\dot{V}O_2$ during exercise was unchanged before and after training (PRE, 3.03 ± 0.08 l/min; POST, 2.88 ± 0.07; $p=0.17$). RER decreased post-training (PRE, 1.07 ± 0.03; POST, 0.98 ± 0.01; $p<0.05$).

Muscle enzymes

Training resulted in increased resting muscle CS maximal activity from 26.8 ± 1.4 to 34.1 ± 3.4 mmol/min/kg wet wt ($p<0.05$). The activity and V_{max} of Phos and mole fraction of Phos *a* were unaffected by training after 5 and 15 min of exercise (Table 3.1, Fig 3.1). PDH_a increased during exercise compared to rest in both conditions, with no

Table 3.1. Glycogen phosphorylase activity during exercise before and after 7 weeks of aerobic training.

Phos	Pre-training		Post-training	
	5 min	15 min	5 min	15 min
Total a + b	81.8 ± 7.9	83.9 ± 9.1	75.9 ± 5.3	76.0 ± 6.8
A	20.5 ± 3.6	22.3 ± 4.6	18.0 ± 1.8	20.5 ± 2.0
V_{max} a + b	110 ± 10	120 ± 13	96 ± 9	102 ± 9
V_{max} a	47 ± 8	51 ± 10	39 ± 5	47 ± 5

Values are means ± SEM and are expressed in mmol glycosyl units kg dry wt^{-1} min^{-1}.

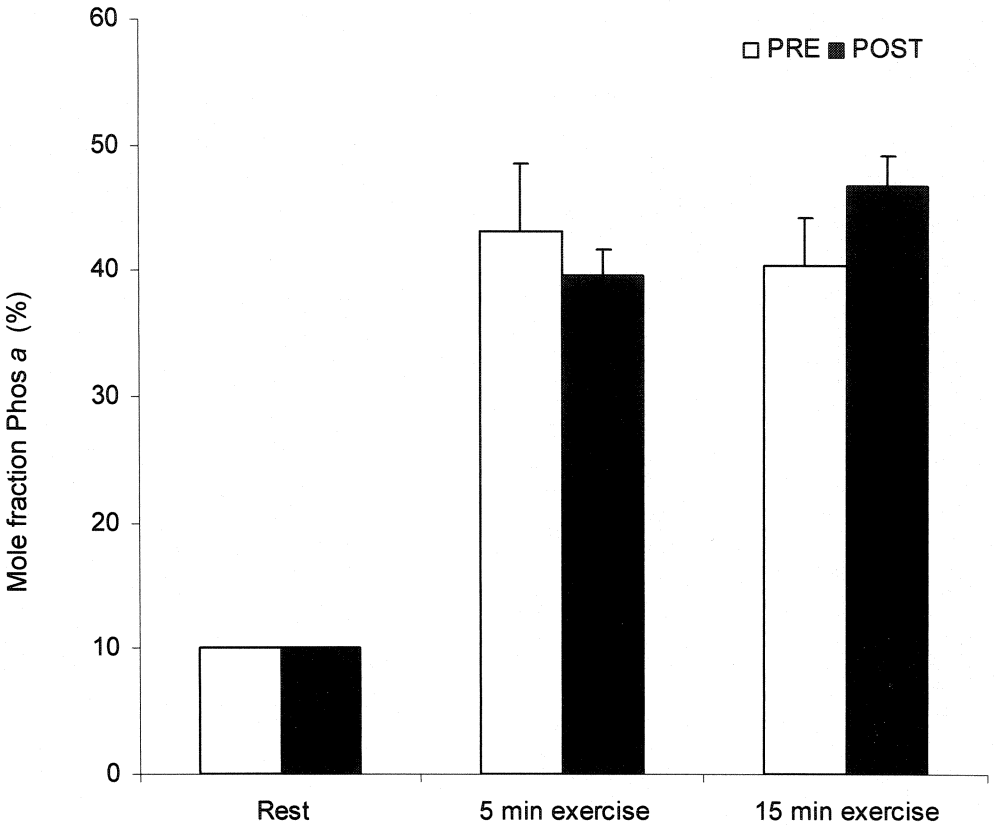

Figure 3.1. Muscle phosphorylase a mole fraction during exercise before and after 7 weeks of aerobic training. Values are means ± SEM. Note: resting values are based on previous studies (see Methods for details).

further increase after 5 min of exercise (Fig 3.2). After 15 min of exercise, PDH_a was lower in POST compared to PRE.

Muscle metabolites

Resting muscle [glycogen] was higher POST compared to PRE at all time points (Table 3.2). At the end of 15 min of exercise, muscle [glycogen] decreased by 49 and 17% during PRE and POST, respectively. Glycogenolytic rate was lower POST compared to PRE (Fig 3.3). At rest, all glycolytic intermediates were similar between conditions (Table 3.2). G-1-P was not detectable. After 5 min of exercise, muscle [glucose] was elevated compared to rest but there was no difference between conditions. After 5 min of exercise muscle [G-6-P], [F-6-P], and [Gly-3-P] were lower POST compared to PRE. With the exception of muscle [G-6-P], all glycolytic intermediates were lower after the last 10 min of exercise POST compared to PRE.

Resting muscle [pyruvate] and [lactate] were similar between both conditions (Table 3.2). During exercise, both muscle [pyruvate] and [lactate] increased from rest. After 5 min of exercise, muscle [pyruvate] was similar between conditions, however, after 15 min of exercise, [pyruvate] was lower POST compared to PRE. After both 5 and 15 min of exercise, muscle [lactate] was lower POST compared to PRE.

Muscle [ATP] was unchanged during exercise in both conditions (Table 3.3). Muscle [PCr] decreased during PRE after 5 min of exercise, with no further change during the last 10 min of exercise. In comparison, muscle [PCr] decreased during POST only after 15 min of exercise and remained higher during exercise compared to PRE.

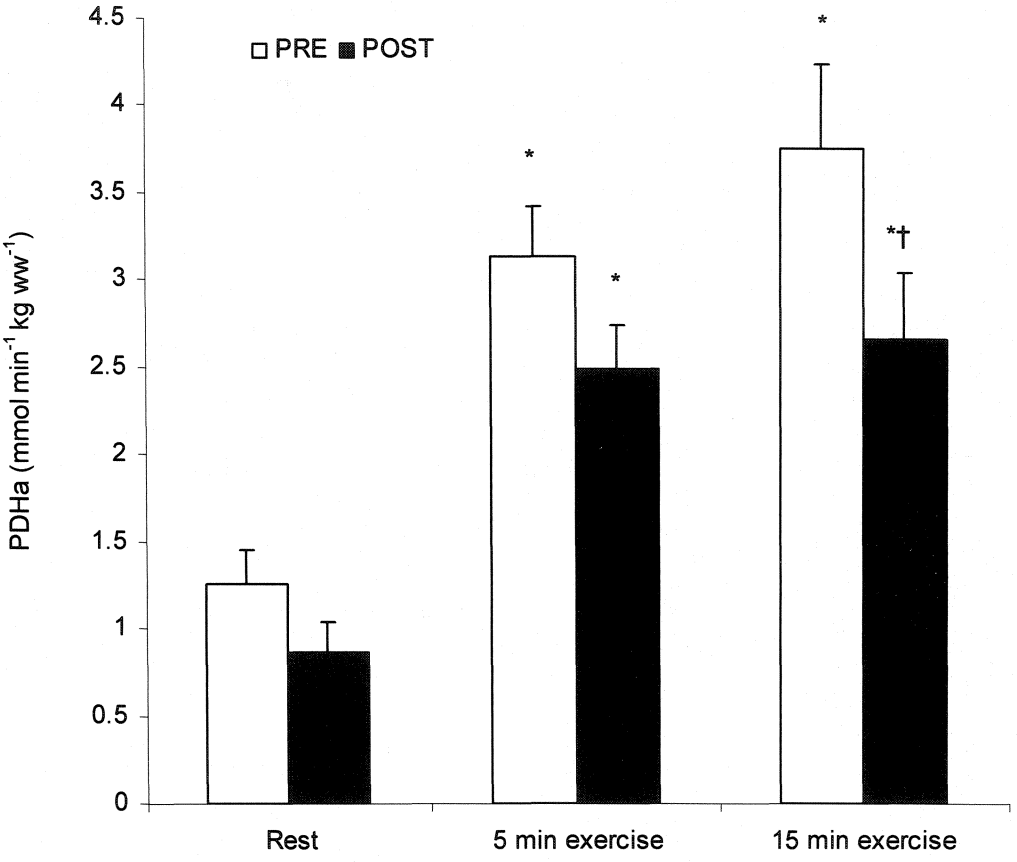

Figure 3.2. Muscle pyruvate dehydrogenase in its active form (PDHa) at rest and during exercise before and after 7 weeks of aerobic training. Values are means ± SEM; * denotes significance from rest; † denotes significance from pre-training.

Table 3.2. Muscle contents of glycolytic intermediates at rest and during exercise before and after 7 weeks of aerobic training.

Metabolite	Condition	Rest	Exercise	
			5 min	15 min
Glycogen	PRE	405 ± 17	345 ± 22	207 ± 23*‡
	POST	640 ± 30†	611 ± 37†	530 ± 39*†
Glucose	PRE	4.1 ± 1.5	14.6 ± 3.0*	11.6 ± 2.0*
	POST	4.9 ± 1.6	15.6 ± 5.7*	6.1 ± 1.2†
G-6-P	PRE	0.65 ± 0.14	2.94 ± 0.41*	2.51 ± 0.47*
	POST	0.32 ± 0.07	1.16 ± 0.26*†	2.04 ± 0.38*‡
F-6-P	PRE	0.49 ± 0.08	0.97 ± 0.19*	1.20 ± 0.19*
	POST	0.36 ± 0.04	0.50 ± 0.06†	0.72 ± 0.07*†
Gly-3-P	PRE	1.20 ± 0.36	6.34 ± 0.95*	3.03 ± 1.19*
	POST	0.61 ± 0.24	3.17 ± 0.68*†	1.39 ± 0.47†
Pyruvate	PRE	0.16 ± 0.03	0.58 ± 0.04*	0.94 ± 0.14*‡
	POST	0.11 ± 0.02	0.47 ± 0.02*	0.46 ± 0.03*†
Lactate	PRE	4.4 ± 0.5	56.4 ± 6.5*	72.8 ± 9.3*
	POST	4.2 ± 0.4	16.7 ± 1.6*†	21.4 ± 3.3*†

Values are means ± SEM and are expressed in mmol kg dry wt^{-1}; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; Gly-3-P, glycerol 3-phosphate; * denotes significance from rest; † denotes significance from pre-training; ‡ denotes significance from 5 min.

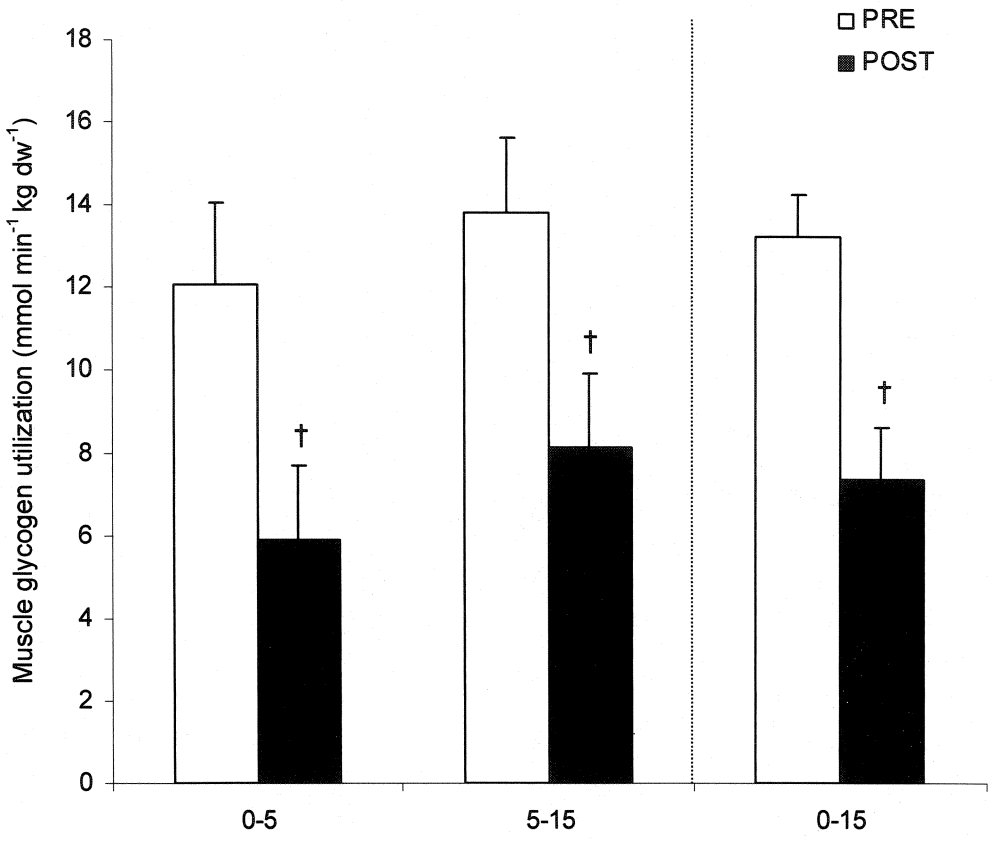

Figure 3.3. Estimated rates of muscle glycogen use during exercise before and after 7 weeks of aerobic training. † denotes significance from pre-training.

Table 3.3. Muscle contents of high energy phosphates at rest and during exercise before and after 7 weeks of aerobic training.

Metabolite	Condition	Rest	Exercise	
			5 min	15 min
ATP	PRE	25.3 ± 0.4	26.6 ± 0.5	24.0 ± 0.6
	POST	24.4 ± 0.6	25.5 ± 1.3	23.0 ± 1.3
Phosphocreatine	PRE	97.7 ± 2.2	56.0 ± 5.0*	53.0 ± 8.1*
	POST	95.5 ± 2.1	86.8 ± 4.9†	78.1 ± 7.2*†
Creatine	PRE	35.5 ± 2.5	77.1 ± 4.0*	64.3 ± 4.7*‡
	POST	37.6 ± 1.7	46.3 ± 2.5†	49.4 ± 4.9*†
Free ADP	PRE	61 ± 5	154 ± 13*	111 ± 16*‡
	POST	64 ± 3	81 ± 5†	84 ± 10†
Free AMP	PRE	0.15 ± 0.02	0.89 ± 0.14*	0.59 ± 0.20*‡
	POST	0.16 ± 0.01	0.27 ± 0.05†	0.32 ± 0.06
ATP/ADP	PRE	0.43 ± 0.03	0.18 ± 0.02*	0.24 ± 0.03*
	POST	0.39 ± 0.02	0.33 ± 0.04†	0.30 ± 0.04
Free P_i	PRE	10.8	44.7 ± 5.2*	57.1 ± 7.9*
	POST	10.8	16.5 ± 3.9†	28.6 ± 5.6*†

Values are means ± SEM; free ADP and AMP are expressed as μmol kg dry wt^{-1} and all others are expressed as mmol kg dry wt^{-1}; * denotes significance from rest; ‡ denotes significance from 5 min; † denotes significance from pre-training.

Calculated [ADP_f], [AMP_f], and [P_i] increased in PRE after 5 and 15 min of exercise and were higher than POST at both time points. In contrast, [ADP_f] and [AMP_f] were unaffected by exercise in POST and free [P_i] was higher at 15 min of exercise compared to 5 min and rest.

With the exception of muscle [acetylcarnitine] being lower POST compared to PRE after 5 min of exercise, muscle [acetyl-CoA], [free CoA], and [free carnitine] at rest and during exercise were unaffected by training (Table 3.4).

Blood metabolites

Blood [glucose] and [free fatty acids] did not change with exercise and were similar between conditions (Table 3.5). Blood [lactate] increased with exercise and was higher PRE compared to POST at 10 min of exercise.

Pyruvate production and oxidation and lactate accumulation

In the first 5 min of exercise, less pyruvate was produced and less lactate accumulated POST compared to PRE, with no change in pyruvate oxidation (Fig 3.4). During the subsequent 10 min of exercise, there was less pyruvate produced and oxidized and less lactate accumulation POST compared to PRE.

DISCUSSION

This is the first study to examine the effects of prolonged aerobic training on the activation state of both Phos and PDH in human skeletal muscle at rest and during

Table 3.4. Muscle contents of CoA, carnitine, and their acetylated forms at rest and during exercise before and after 7 weeks of aerobic training.

Metabolite	Condition	Rest	Exercise	
			5 min	15 min
Acetyl-CoA	PRE	10.4 ± 2.1	27.2 ± 2.3*	40.1 ± 9.0*
	POST	9.8 ± 1.7	21.7 ± 3.2*	36.1 ± 4.5*‡
Free CoA	PRE	81.4 ± 7.8	68.8 ± 9.6	77.3 ± 6.3
	POST	105.1 ± 7.4	83.4 ± 10.0	84.4 ± 8.6
Acetyl-CoA/CoA	PRE	0.14 ± 0.03	0.43 ± 0.04*	0.50 ± 0.07*
	POST	0.09 ± 0.01	0.29 ± 0.05*	0.48 ± 0.09*‡
Acetylcarnitine	PRE	2.5 ± 0.6	13.7 ± 1.5*	17.3 ± 3.7*
	POST	2.8 ± 1.1	7.4 ± 1.2*†	10.8 ± 1.7*
Free carnitine	PRE	13.2 ± 1.4	4.9 ± 1.0*	4.2 ± 0.9*
	POST	12.4 ± 1.3	6.7 ± 0.8*	5.1 ± 1.0*

Values are means ± SEM; acetyl-CoA and free CoA measures are expressed in μmol kg dry wt^{-1}; all other measurements are expressed as mmol kg dry wt^{-1}; acetyl-CoA/CoA, ratio of acetyl-CoA to CoA; * denotes significance from rest; ‡ denotes significance from 5 min; † denotes significance from pre-training.

Table 3.5. Plasma lactate, glucose, and free fatty acid (FFA) concentration at rest and during exercise before and after 7 weeks of aerobic training.

Metabolite	Condition	Rest	Exercise
Lactate	PRE	0.73 ± 0.15	5.11 ± 0.39*
	POST	0.80 ± 0.13	3.13 ± 0.25*†
Glucose	PRE	4.39 ± 0.31	4.02 ± 0.34
	POST	4.70 ± 0.35	4.09 ± 0.13
FFA	PRE	0.19 ± 0.02	0.18 ± 0.04
	POST	0.13 ± 0.03	0.13 ± 0.02

Values are means ± SEM; exercise samples were taken after 10 min of exercise; all measurements are expressed in mM; FFA, free fatty acid; * denotes significance from rest; † denotes significance from pre-training.

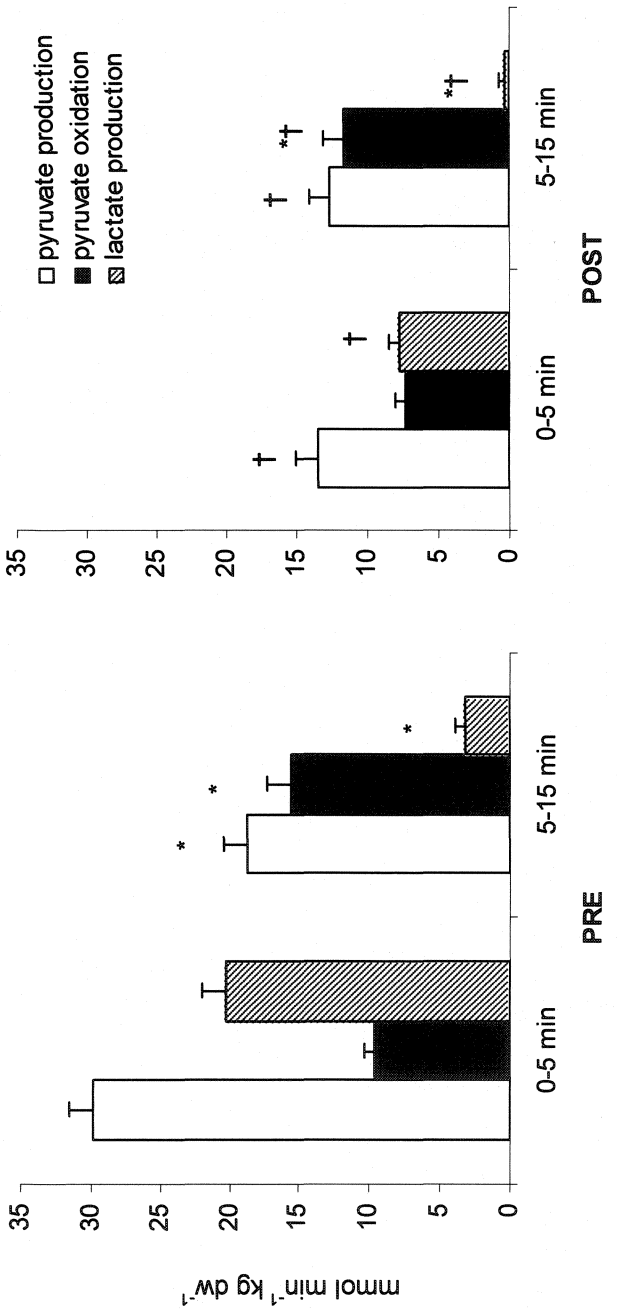

Figure 3.4. A comparison of the fate of pyruvate produced during exercise before and after 7 weeks of aerobic training; * denotes significance from 0-5 min; † denotes significance from pre-training.

submaximal exercise. The effects of aerobic training were evidenced by improved maximal O_2 uptake, 27% increase in citrate synthase maximal activity and an approximate doubling of endurance capacity (results not shown). The major novel finding from the present study was that the acute exercise-induced activation of PDH was attenuated after 7 weeks of aerobic training during submaximal exercise. These effects were not seen at rest. This attenuation may be due to a series of events resulting from prolonged aerobic training. First, the 7 week training program significantly increased the oxidative capacity of skeletal muscle, evident with an increased maximal CS activity. This resulted in an improved coupling of ATP supply and demand during exercise with less reliance on PCr stores for ATP resynthesis, resulting in lower skeletal muscle [ADP_f], [AMP_f], and [P_i], and higher [PCr] post-training. Despite no change in the mole fraction of Phos in the more active form (Phos *a*), attenuated levels of its allosteric regulators (ADP_f, AMP_f, and P_i) resulted in decreased glycogenolytic flux through Phos due to post-transformational regulation. Finally, the decreased glycogenolytic rate resulted in less pyruvate production from glycogenolysis, in addition to attenuated levels of ADP_f, resulted in a possible stimulation of PDK and decreased activation of PDH.

Control of glycogenolysis

In the present study, glycogenolytic rate and resulting pyruvate production during exercise was lower in POST compared to PRE. Previous studies have shown a conservation of glycogen content in trained skeletal muscle (14, 18, 24, 25, 29). The changes in glycogenolytic flux through Phos during exercise demonstrated in the present

study with prolonged aerobic training took place in the absence of any change in total Phos activity or the percent mole fraction of the more active form of Phos (Phos *a*). This is similar to what has previously been shown in short-term aerobically trained human skeletal muscle (6). Previous studies have demonstrated that exercise-induced covalent transformation of Phos *b* to *a* is always well in excess of glycogenolytic flux at submaximal exercises (6, 23, 34). As a result, the decreased glycogenolytic rate in POST compared to PRE is more likely due to post-transformational regulators linked to energy balance.

Post-transformationally, Phos *b* is up regulated by AMP_f and IMP_f, and inhibited by ATP and G-6-P whereas Phos *a* is up regulated only by AMP_f (8, 26). Recently, it has been demonstrated *in vitro* that Phos *a* is also up regulated by ADP_f (44). In addition, the availability of one of the substrates, P_i, is critical in increasing the catalytic rate of Phos (5). After 5 and 15 min of exercise in POST, there was a decreased reliance on PCr, resulting in 50-63% reduced availability of P_i, and a 46-70% and 24-47% attenuated increase of AMP_f and ADP_f, respectively. These results are consistent with what has been previously demonstrated with long-term aerobic training (14, 34) and similar, but of a greater magnitude, that those demonstrated with short-term aerobic training (12, 13, 34). The increased muscle oxidative capacity POST, with attenuated levels of ADP_f, AMP_f, and P_i, may represent potentiated effects seen before changes in training-induced increases in muscle mitochondrial content. Thus long-term aerobic training further attenuates exercise-induced disturbances to the energy status of the cell, resulting in further decreased flux through Phos and larger net glycogen sparing.

Control of pyruvate utilization

The present study demonstrated an attenuation of the exercise-induced activation of PDH. This is the first study to report changes in human skeletal muscle PDH activation with training, with a previous study demonstrating no change in PDH activation during exercise after 7 days of aerobic exercise (37). Thus, it appears that the regulation of PDH during exercise POST represents an adaptation that replaces the underlying mechanisms responsible for metabolic adjustments observed early in training.

PDH activity is controlled by the relative activities of PDH kinase (PDK) and PDH phosphatase (PDP) which inhibit and activate PDH, respectively. Increased ratios of ATP/ADP and NADH/NAD^+ stimulate PDK and inhibit PDP. In addition, pyruvate inhibits PDK, whereas Ca^{2+} and H^+ activate PDP. The increased [Ca^{++}] at the onset of exercise stimulates PDP and crudely activates PDH where as the other allosteric modulators fine tune PDH activation. The effects of allosteric modulators on PDK activity are mediated by altering the association of PDK with the PDH complex, more specifically the E_2 component. PDK exist in two states, intrinsic (bound) and free (unbound; 27, 28, 32). Intrinsic PDK binds to the innermost lipoyl domain of PDH-E_2 (41) and represents only 15-30% of the total PDK activity (28, 32, 46). Thus shifts from free to intrinsic PDK, which is dependent on the concentration of allosteric modulators, would increase total PDK activity.

The apparent mechanisms regulating the attenuated exercise-induced activation of PDH post-training are potentially mediated by two allosteric modulators. The improved

mitochondrial oxidative potential with POST attenuated the increased ADP_f during exercise, thus increasing ATP/ADP ratio, stimulating PDK and attenuating PDH activation. Also, reduced glycogenolysis/glycolysis during exercise POST was associated with attenuated skeletal muscle [pyruvate], which in turn may have released inhibition on PDK and reduced activation of PDH after 15 min of exercise. The inhibition constant (K_i) of pyruvate for PDK has been reported in cardiac muscle to be in the range of 0.08-2 mM (see 47 for review). Assuming the fluid volume of muscle is 75% of the total volume, muscle [pyruvate] after 15 min of exercise decreased from 0.31 mM PRE to 0.15 mM POST. Although the K_i of pyruvate in human skeletal muscle has yet to be confirmed, the possibility remains that it may be similar in magnitude to the pyruvate concentrations we observed pre-training in the present study. Another important regulatory mechanism of pyruvate-mediated inhibition of PDK is its integration with ADP, where an increased ATP/ADP ratio decreases the sensitivity of PDK to pyruvate (36). Thus, an increased ATP/ADP ratio, in combination with a decreased pyruvate content, would decrease the activation of PDH possibly due to increased PDK activity. It is important to note that the pyruvate and ADP_f concentrations reported here are of the whole muscle cell and further research is needed to ascertain the subcellular distribution of these modulators since the inner mitochondrial concentration would influence the activity of PDK.

PDH control can also be accomplished through stable alterations to PDK. The complexity of PDH control by PDK is enhanced by the presence of four isoforms (PDK1-4; (3), with PDK2 and 4 being the most abundant isoforms represented in human skeletal

muscle (16). Each isoform has differing concentrations, specific activities, and kinetic properties from each other, resulting in unique responses to certain metabolic demands. A recent study conducted in our laboratory demonstrated that 8 weeks of aerobic training resulted in a 2-fold increase in PDK activity, mainly due to increased pyruvate-sensitive PDK2 (30). Thus, this evidence supports the idea of a pyruvate-mediated feed-forward regulation of PDH during exercise post-training.

It has been shown that the level of PDH activity is similar to flux through this enzyme at varying exercise power outputs (10, 23, 38, 39). As a result, the decreased PDH activation, and resultant pyruvate oxidation, may be compensated through increased fatty acid metabolism. Previous human studies have shown that prolonged aerobic training induces an increased fat oxidation, more specifically intramuscular lipolysis, in trained skeletal muscle compared to pre-trained during submaximal exercise (see 11, 19, 22 for review). Since the exercise bout was done at the same absolute workload, thus the same ATP turnover rate, the amount of fatty acid (palmitate) that would be needed to account for this difference in pyruvate-derived oxidative ATP production would be approximately 10 mmol of palmitate for each leg during the 15 min of exercise post-training. With an estimated 91 mmol of glucosyl units oxidized per leg during the 15 min of exercise post-training, this would equate to approximately 90% carbohydrates and10% fat.

Summary and conclusions

The present study clearly demonstrates that long-term aerobic exercise influences skeletal muscle carbohydrate utilization during exercise. This study supports the theory that training-induced mitochondrial biogenesis improves the energy status of the cell, thus regulating carbohydrate metabolism partially through metabolic regulators of both Phos and PDH. This is in contrast to short-term training that imparts regulation solely on Phos. Improved control of aerobic energy production, with a decreased reliance on substrate level phosphorylation, led to an attenuation of ADP_f, AMP_f, and P_i, known post-transformational activators of Phos. Reduced flux through the glycogenolytic pathway reduced pyruvate availability and, in addition to attenuated levels of ADP_f, resulted in an attenuated exercise-induced activation of PDH. This was possibly due to allosteric modulator-induced and/or training-induced increase in PDK activity.

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General Conclusions

A majority of the information in the literature regarding dynamic regulation of PDH has utilized *in vitro* analysis of rodent, bovine, and swine tissue homogenates or isolated proteins (see Denton *et al.*, 1975;Wieland, 1983 for review). With regards to *in vivo* PDH regulation, the focus has been mainly on rodents in a variety of tissues (see Harris *et al.*, 2002 for review). However, recently the importance of PDH regulation of human skeletal muscle *in vivo* has been examined in relation to oxygen availability (hypoxia and hyperoxia), acute and chronic dietary manipulation (high fat, high carbohydrate), acid-base perturbations (metabolic acidosis and alkalosis), acute hormonal alterations (epinephrine), and short-term training at rest and during exercise at differing intensities and durations (Heigenhauser & Parolin, 1999;Spriet & Heigenhauser, 2002). The studies outlined in the preceding chapters add new information concerning the dynamic and stable regulation of PDH in human skeletal muscle *in vivo* and how this regulation may influence fuel selection and lactate production at rest and during exercise.

The first study (Chapter 1) examined the effect of hyperventilation-induced respiratory alkalosis on the dynamic regulation of PDH activity and lactate metabolism at rest and during exercise in human skeletal muscle. This study reinforces the idea that PDH is a significant contributor to metabolic inertia and lactate accumulation during hypoxia. This study also identifies respiratory alkalosis as a potential source of hypoxia-related attenuation of PDH at the onset of exercise.

The final two studies examined aerobic training-induced adaptations in PDH and PDK activities (Chapter 2) and subsequent alterations in the regulation of PDH activation

during submaximal exercise (Chapter 3). These studies demonstrate the adaptive response of the PDH enzyme complex to aerobic training, both at a dynamic and stable regulatory level. These studies also expand our knowledge of the role different PDK isoforms play in regulating PDH.

The present chapter collectively summarizes the important findings from these studies and makes recommendations for future research initiatives into the topic of PDH regulation in human skeletal muscle.

PDH regulation by hyperventilatory-induced respiratory alkalosis

Results from the study outlined in Chapter 1 demonstrated the relative role of hyperventilatory-induced changes in pH that contributed to lactate production during hypoxia. Voluntary hyperventilation resulted in an increased calculated pH in plasma and muscle. This resulted in a possible pH-induced stimulation of PDK and/or inhibition of PDP, resulting in a delayed activation of PDH during exercise. The importance of this study is in demonstrating that respiratory alkalosis accounted for approximately 57% of muscle lactate produced at the onset of exercise during hypoxia (Parolin *et al.*, 2000). Also, the delayed activation of PDH was similar in magnitude, as demonstrated during respiratory alkalosis in Chapter 1 and during hypoxia (Parolin *et al.*, 2000). This suggests that O_2 delivery to the working muscle at the onset of exercise was not solely responsible for the delayed activation of PDH and resultant lactate accumulation.

Future studies

Despite the convincing evidence of the important role respiratory alkalosis plays in skeletal muscle and whole body lactate production at the onset of exercise during hypoxia, there are certain limitations to the study that warrant future research.

The argument for pH-induced regulation of PDK and PDP is based on two assumptions; 1) calculated respiratory alkalosis-induced elevated pH in skeletal muscle, based on isolated rat diaphragm (Heisler, 1975), reflect increases seen *in vivo* and 2) PDK and PDP pH sensitivity demonstrated *in vitro* for bovine kidney and heart (Hucho *et al.*, 1972) are similar to human skeletal muscle *in vivo*. These limitations need to be addressed to verify the assumptions and ensure the elevated pH seen with respiratory alkalosis is an underlying mechanism of hypoxia-induced skeletal muscle lactate production at the onset of exercise in humans.

The isoform expressions of PDK and PDP in human skeletal muscle may contribute to altered activities with respiratory alkalosis. It is known that PDK2 and PDK4 are the most abundant isoforms in human skeletal muscle (Gudi *et al.*, 1995), however, there is no information regarding the PDP isoform distribution in human skeletal muscle. In addition, there have not been any studies regarding the relative pH sensitivities of each of the PDK and PDP isoforms. Thus, PDK and PDP individual isoform isolation and *in vitro* determination of pH sensitivity, along with PDP skeletal muscle isoform distribution, are required to ascertain the importance of isoform expression and distribution in respiratory alkalosis-induced regulation of skeletal muscle PDH activation and resulting lactate production.

Aerobic training-induced alterations to the PDH enzyme complex and implications during exercise

PDHt

It is known that aerobic training increases the maximal carbohydrate oxidative capacity of skeletal muscle. The results of Chapter 2 demonstrate that the training-induced increase in maximal oxygen uptake may, in part, be linked to the adaptive increase in total PDH complex, and not on representative enzymes of the tricarboxylic acid cycle (citrate synthase) and electron transport chain (cytochrome oxidase) in human skeletal muscle. This was evident by a closer association between PDHt and maximal O_2 uptake, demonstrated by a slight excess (1.3 times) of PDHt activity in contrast to considerable excess for citrate synthase (9 times) and cytochrome oxidase (6 times) maximal activities. These observations follow the concept of metabolic symmorphosis proposed by Hochachka et al. (1998), in which enzymes operating far from equilibrium are regulated the least where as equilibrium enzymes are adjusted the most. The aerobic training-induced increase in PDHt was accompanied by a change in only one of the PDH subunits measured, a 1.3 fold increase in PDH-$E_1\alpha$.

Despite a 31% increase in PDHt seen in Chapter 2, the results of Chapter 3 demonstrate an attenuated submaximal exercise-induced increase in PDH post-training, which may be at the level of PDK. These results are similar to a study that examined branched-chain 2-oxoacid dehydrogenase (BCOAD), another mitochondrial enzyme that requires dephosphorylation for activation, in human skeletal muscle with long-term

aerobic training. McKenzie and colleagues (2000) demonstrated that 38 days of endurance exercise training resulted in a 50% increase in total BCOAD yet the percent activation during exercise at the same absolute submaximal workload was 2 fold lower in post-training compared to pre-training. The aerobic training-induced changes seen in Chapters 2 and 3 emphasize the adaptive capacity of skeletal muscle to utilize more carbohydrates at maximal oxygen uptake, evident by an increased PDHt, yet down-regulate PDH during submaximal exercise so as to use more fat post-training.

PDK

Another skeletal muscle adaptive response to aerobic training is a switch in fuel utilization from carbohydrate to fat during submaximal exercise. Activation of PDH, a key regulatory step for carbohydrate metabolism, may be central to this adaptation. The results of Chapter 2 demonstrate that aerobic training approximately doubled PDK activity, representing a stable increase rather than changes to allosteric modulators. The aerobic training-induced increase in PDK was accompanied by a change in only one of the PDK isoforms measured, a 1.3 fold increase in PDK2.

The importance of a two-fold increase in PDK activity seen in Chapter 2 is reflected in the results of Chapter 3, which demonstrate an attenuated exercise-induced PDH activation post-training. The aerobic training-induced increase in PDK2, the pyruvate-sensitive PDK isoform, coincides with an attenuated skeletal muscle [pyruvate] during submaximal exercise post-training. Thus, aerobic training-induced down

regulation of skeletal muscle PDH during submaximal exercise appears to be at the level of PDK.

Future studies

Link between exercise and increased PDH complex proteins

There is convincing evidence in Chapter 2 that demonstrates long-term aerobic exercise stimulates a stable increase in PDK and PDHt activities and protein expression. The importance of an increased PDK2 protein expression in skeletal muscle with aerobic training was discussed in Chapters 2 and 3, however, the significance of a 1.3 fold increase in PDH-$E_1\alpha$ subunit remains elusive. It is known that the PDH-$E_1\alpha$ subunit is a component of the rate-determining, non-reversible step of the PDH reaction and is the regulatory component of the heterotetrameric E_1 subunit, which when phosphorylated inactivates the PDH complex. Although this specific increase in the $E_1\alpha$ subunit, with no changes in any of the other subunits, has been previously demonstrated in non-muscle tissue (Amessou *et al.*, 1998;Da Silva *et al.*, 1993;Maury *et al.*, 1995), the importance of this remains unanswered. It has been postulated that the amount of the $E_1\alpha$ subunit, and possibly the E_1 heterotetramer, is the rate limiting step in the formation of new PDH complexes. Further studies are needed to address this possibility.

Despite an increased PDH-$E_1\alpha$ and PDK2 protein expression, the regulatory signals responsible for this training-induced increase are currently unknown. There are a number of exercise- and training-linked stimuli that may contribute to the aerobic training-induced increase in gene expression. These include stretch and muscle tension,

calcium fluxes during muscle contraction, energy status of the cell, substrate availability, and circulating hormones (Hargreaves & Cameron-Smith, 2002). These stimuli result in activation of kinases (e.g. protein kinase C, AMP kinase) and phosphatases (e.g. calcinurin) with exercise, which in turn stimulate transcription factors (e.g. nuclear respiratory factor, c-Jun, c-Fos) that alter transcription and translation of targeted genes of interest during the recovery period from exercise (see Hood, 2001 for review). Currently, it is unknown as to which transcription factor(s) or signaling pathway(s) link repeated bouts of exercise with increased protein expression of the PDH enzyme complex in human skeletal muscle. As a result, more work is needed to establish which stimuli contribute to the adaptive phenotypic changes seen in skeletal muscle with repeated bouts of exercise.

Skeletal muscle regional adaptation

The results of Chapter 2 reflect an aerobic training-induced adaptation to the PDH complex, an intermitochondrial enzyme. Two populations of mitochondria exist within a skeletal muscle cell: subsarcolemmal (SS), which are near the sarcolemma, and intermyofibrillar (IMF), which are between the myofibrils (Hoppeler *et al.*, 1973). The mitochondrial isolation technique used in Chapter 2 preferentially extracted SS mitochondria and thus reported alterations to activities of PDK and PDHt would reflect that population. This is in contrast to PDH measurements in Chapter 3 which were done on whole muscle, which would reflect both populations. Previous studies examining the differential response of SS and IMF to endurance training have shown somewhat similar

responses, although different in magnitude, between the populations (Bizeau *et al.*, 1998;Chilibeck *et al.*, 2002;Roussel *et al.*, 2000). However, no studies have been conducted to examine the potential differential PDHt and/or PDK responses of each mitochondrial population. Thus, further research is needed in this area to assess the relative contribution of both mitochondrial subpopulations to exercise-induced attenuation of PDH activation post-training.

Association between PDK protein and activity

With only an approximate 25% increase in PDK2 protein content in skeletal muscle post-training, there appear to be other factors responsible for an aerobic training-induced two fold increase in PDK activity. As discussed in Chapter 2, this discrepancy may be due to; 1) contributions by other PDK isoforms and/or 2) interaction between PDK and PDH (bound vs. unbound).

Despite no changes seen in skeletal muscle protein content of PDK1 and 4 pre- and post-training, and undetectable levels of PDK3, we can not rule out the contribution of these isoforms to the aerobic training-induced increase in PDK activity. Specifically, PDK3 demonstrates approximately 2-25 times higher specific activity compared to the other PDK isoforms (Bowker-Kinley *et al.*, 1998), and small changes in training-induced protein expression of PDK3 would result in large changes in relative contribution to total PDK activity. Thus, more sensitive methods are required to ascertain the important relative contribution of all PDK isoforms, specifically PDK3, to the aerobic training-induced increase in PDK activity.

Increased PDK activity can occur in the absence of increased PDK protein expression. The association of PDK with the innermost lipoyl domain of PDH-E_2 determines its two states, intrinsic (bound) and free (unbound), with all PDK isoforms demonstrating increased activity in the intrinsic form (Korotchkina & Patel, 2001). To date, no study has examined the effects of long-term aerobic exercise on the expression of intrinsic and free PDK and the interaction between these two forms of PDK and the PDH-E_2 lipoyl domain. Utilizing immunoprecipitation methodology, one may quantify the relative intrinsic-to-free ratio of each PDK isoform in response to exercise and training.

Contribution of PDP to aerobic training-induced PDH regulatory adaptation

Changes in protein or activity to either PDK or PDP would alter the regulation of PDH activation. Starvation, high fat feeding, and chemically-induced diabetes result in an enhanced phosphorylated, and inactivated, state of PDH in most tissues of the rat (Holness *et al.*, 2000;Peters *et al.*, 2001a;Sugden *et al.*, 2000a;Wu *et al.*, 1998;Wu *et al.*, 1999;Wu *et al.*, 2000). Although increased PDK activity could account for the inactive state of PDH during starvation, high fat feeding, and diabetes, a much greater effect would be achieved with a concomitant decreased PDP activity. In fact, starved and chemically-induced diabetic rats demonstrated decreased PDP activity in heart and kidney (Huang *et al.*, 2003). Thus, changes in PDP opposite to changes in PDK may occur to maximize the inactivation of PDH.

Currently, there is little information regarding PDP activity in mammalian skeletal muscle, with only one study demonstrating no change in PDP activity in starved and

chemically-induced diabetic rats (Fuller & Randle, 1984). To date, no studies have measured PDP in human tissues, let alone in skeletal muscle. The possibility of opposite changes in PDK and PDP, along with the results of Chapter 3, demonstrate the need for further investigation into the relative contribution of altered PDP activity in attenuated exercise-induced PDH activation post-training in human skeletal muscle.

There are two known isoforms of PDP in mammalian tissues; PDP1 and PDP2. PDP1 is Ca^{++}-sensitive and preferentially expressed in skeletal muscle, whereas PDP2 is relatively unaffected by the absence or presence of Ca^{++}, is insulin sensitive, and found more abundantly in liver and adipose tissue, with small amounts present in skeletal muscle (Huang *et al.*, 1998). The relative PDP isoform distribution is currently unknown in human skeletal muscle and more research is needed to ascertain which, if not both, isoforms of PDP may be altered with aerobic training.

Role of aerobic training-induced adaptation of the PDH enzyme complex in metabolic disorders

The benefits of aerobic training on improving whole body and skeletal muscle health are numerous. Physically active individuals are at a reduced risk of developing chronic degenerative disorders such as type 2 diabetes, obesity, coronary heart disease and hypertension. Physical activity has also been shown to improve skeletal muscle metabolism, including fat oxidation, insulin sensitivity, glucose disposal, and lactate handling, to name a few. The importance of the adaptive response of PDH regulation in these metabolic disorders has been emphasized using a rodent model (see Harris *et al.*,

2002;Randle, 1994;Sugden *et al.*, 2001;Sugden & Holness, 2003 for review). However, care must be taken when extrapolating these PDH enzyme complex-adaptive responses found in rodents to a human model. An attempt to understand the role of the PDH enzyme complex in the aerobic training-induced adaptive response in healthy human skeletal muscle, as evident by the results demonstrated in Chapters 2 and 3, will assist in establishing the relative contribution PDH enzyme complex adaptation plays in the improvements in skeletal muscle post-training with specific metabolic disorders associated with the lack of physical activity.

Summary

The studies outlined in this thesis offer new insight into the dynamic and stable regulation of PDH activation in human skeletal muscle. The results of the first study reinforce the importance of dynamic regulation of PDH in modulating lactate production in exercising human skeletal muscle. It also identifies PDH as a key contributor to the delayed onset of catabolic fuel utilization. The second study demonstrated that the training-induced increase in maximal oxygen uptake may be dependent on the adaptive increase in total PDH complex, and not on representative enzymes of the tricarboxylic acid cycle and electron transport chain. In addition, this study represents only the third human study to measure PDK activity in skeletal muscle and the first to show an adaptive response of PDK2 to a physiological perturbation (training). The combined determinations of mRNA, protein, and enzyme activity of this study provide a complete picture of exercise-induced regulation of PDH, something that is not often done in the

literature. The third study expands our knowledge of the importance of dynamic regulation of PDH activation in metabolic fuel selection during exercise post-training, demonstrating the significance of the training-induced increase in PDH and PDK2 from the previous study. Both aerobic training studies identify the role different PDK isoforms may play in regulating the activity of PDH in response to different metabolic perturbations and provide a complete examination, from molecular to whole body, of the adaptive response and plasticity of skeletal muscle.

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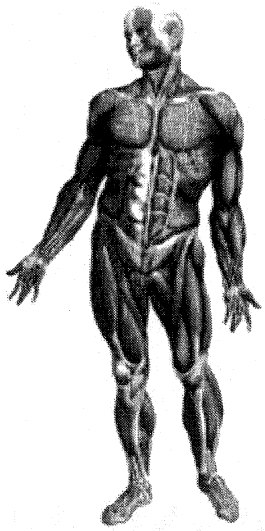

"Listen to my muscle memory"

M.J. Keenan, 1996