EFFECTS OF HYPEROXIA AND ACETATE INFUSION ON SUBSTRATE PHOSPHORYLATION DURING THE ONSET OF MODERATE EXERCISE

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ABSTRACT

EFFECTS OF HYPEROXIA AND ACETATE INFUSION ON SUBSTRATE PHOSPHORYLATION DURING THE ONSET OF MODERATE EXERCISE

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This study investigated whether increased muscle acetylcarnitine provision or hyperoxia would reduce the reliance on substrate phosphorylation during exercise onset. Eight subjects underwent three randomized trials: 1) saline infusion over 1hr prior to exercise while breathing room air for 20 min prior to and during 120s of cycling at 65% VO_2max (CON), 2) saline infusion and breathing 100% O_2 (HYP) and 3) sodium acetate infusion and breathing room air (ACE). Muscle biopsies were sampled at rest, 30s, and 120s of exercise. ACE significantly increased muscle acetyl-CoA and acetylcarnitine at rest vs. CON and HYP (22.9 ± 2.8 vs. 8.9 ± 2.4 and 10.5 ± 1.8 µmol/kg dm; 11.0 ± 1.2 vs. 3.5 ± 1.3 and 4.0 ± 1.2 mmol/kg dm) and after 30s of exercise (20.0 ± 2.7 vs. 12.3 ± 1.6 and 12.1 ± 1.0 µmol/kg dm; 9.4 ± 1.4 vs. 4.2 ±1.2 and 4.6 ± 0.7 mmol/kg dm). However, there was no significant difference in pyruvate dehydrogenase activity or reliance on substrate phosphorylation between treatments at rest or during exercise. In summary, increasing acetylcarnitine availability or inspired O_2 did not enhance the activation of aerobic metabolism.

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INTRODUCTION

The main source of chemical energy that fuels muscular contractions during exercise is adenosine-5'-triphosphate (ATP). At the onset of exercise the demand for ATP can increase up to 100-fold compared to rest (Hochachka and Matheson 1992). However, the amount of ATP stored in human skeletal muscle (25-30 mmol/kg dry muscle) is only sufficient to sustain a few seconds of intense exercise if not replenished. Despite this small amount, it is known that in most exercise situations the concentration of ATP in skeletal muscle remains relatively constant and thus, there is a precise matching of ATP demand with ATP supply.

The large demand for ATP during exercise is due to the activity of specific enzymes called adenosine triphosphatases (ATPases). Actomyosin ATPase is the primary consumer of ATP in muscle. This enzyme uses the high-energy phosphate bond in ATP for shortening of the sarcomere and accounts for approximately 70% of the ATP utilized during muscular contractions. Ca^{2+}-ATPase, which is responsible for Ca^{2+} reuptake into the sarcoplasmic reticulum, accounts for 25 – 30% of ATP utilization. The Na^{+} K^{+}-ATPase, responsible for maintaining cellular membrane potential is also active during exercise but contributes minimally (up to 5%) to ATP consumption (as reviewed by Spriet and Howlett 1999). ATPase activation is triggered by Ca^{2+} release from the sarcoplasmic reticulum at the onset of exercise. Since Ca^{2+} release is directly proportional to exercise intensity, the rate of ATP utilization by ATPases is also dependent on exercise intensity (Hochachka and Matheson 1992).

The processes responsible for ATP provision include substrate level phosphorylation and oxidative phosphorylation. Substrate level phosphorylation encompasses phosphocreatine (PCr) breakdown via the creatine kinase reaction and glycogen (and minimal blood glucose) breakdown through anaerobic glycogenolysis/glycolysis (defined as glycolytic activity ending in lactate production). Both of these systems operate independent of oxygen (non-oxidative ATP production) and provide the majority of energy required during short-term high intensity exercise. PCr breakdown acts as an immediate ATP buffer at exercise onset with the net result being accumulation of creatine (Cr) and inorganic phosphate (P_i). The creatine kinase reaction is driven by the increase in adenosine-5'-diphosphate (ADP) and decrease in ATP resulting from ATP breakdown. Anaerobic glycolysis involves the breakdown of muscle glycogen and/or blood glucose to pyruvate with resultant production of lactate. The degree of reliance on glycogen and blood glucose varies with exercise intensity and duration. However, muscle glycogen is the dominant source of glucose during exercise onset as blood glucose uptake occurs at a slower rate. The main regulatory sites of anaerobic glycolysis are glycogen phosphorylase (PHOS) and phosphofructokinase (PFK). Another fate of pyruvate is transport into the mitochondria and oxidation to acetyl-CoA. Acetyl-CoA, derived from the oxidation of both fat and carbohydrate, is then further metabolized in the TCA cycle producing NADH and $FADH_2$ for oxidation and resultant ATP production via oxidative phosphorylation.

An important feature distinguishing substrate level phosphorylation and oxidative phosphorylation is energy yield. ATP production from anaerobic glycolysis is 3 mmol ATP/mmol glucose from glycogen breakdown compared to oxidative phosphorylation, which yields 38 – 39 mmol ATP/mmol glucose or glycogen, respectively. Despite less ATP production/mmol glucose, substrate level phosphorylation has the unique ability of being able to produce ATP at a rate 5-6 times that of oxidative phosphorylation. Thus, it is the main source of ATP during the transition from rest to exercise, the transition from one power output to a higher power output, and at exercise intensities > 100% VO_2max (maximal oxygen uptake). However, substrate level phosphorylation has a limited capacity due to a finite store of PCr and the accumulation of byproducts of anaerobic glycolysis (lactate and H^+). Conversely, oxidative phosphorylation, while operating at a slower rate, can operate continuously and therefore, is the main energy producing pathway during prolonged exercise.

The ATP turnover rate and thus, the requirement for ATP provision necessary to maintain ATP concentration is dictated by power output. While the rate of ATP turnover is constant upon exercise onset at a maintained power output, aerobic metabolism (oxidative phosphorylation) increases monoexponentially until a metabolic steady state (the rate of ATP utilization equals the rate of ATP synthesis by oxidative phosphorylation) is achieved (Figure 1) (Barstow 1994). Thus, there is an initial phase where the rate of ATP utilization is greater than the rate of ATP production by oxidative phosphorylation. During this transition period, ATP demand is partially met through substrate level

phosphorylation. This is a result of the key characteristic of non-oxidative ATP production in being able to provide ATP at a faster rate than oxidative phosphorylation. The non-oxidative ATP production required during this transition is termed the oxygen (O_2) deficit.

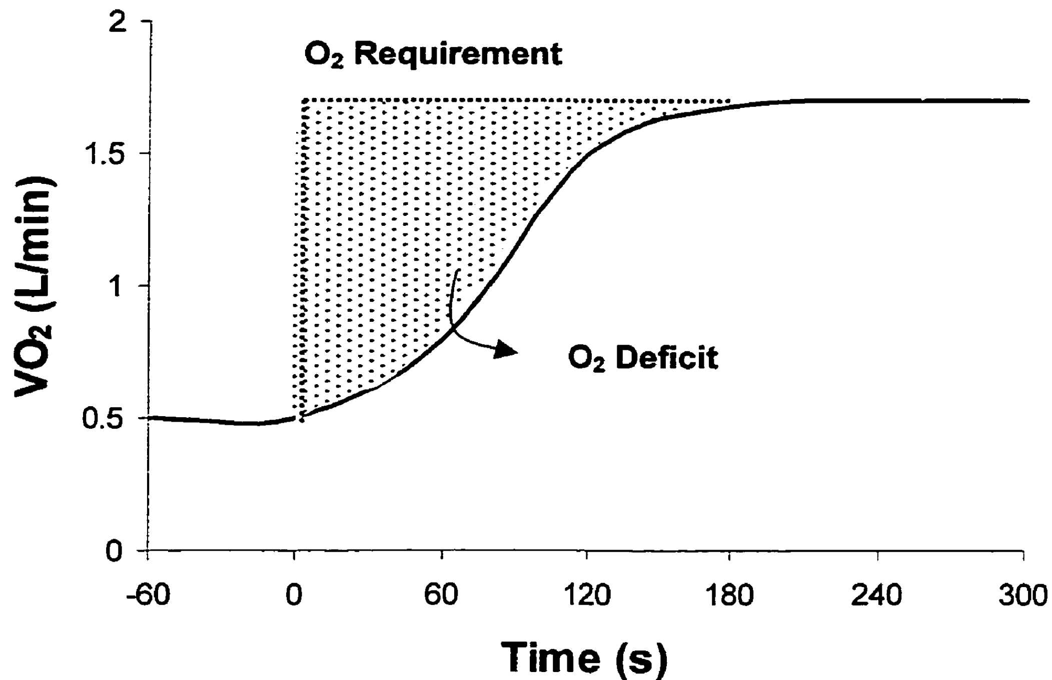


Figure 1. Schematic representation of oxygen uptake during submaximal exercise. O_2 requirement indicates the demand for oxidative ATP production corresponding to a step increase to a moderate power output. Shaded area represents the O_2 deficit (non-oxidative ATP production).

Despite the activation of substrate level phosphorylation and aerobic metabolism at exercise onset there is a small initial mismatch in ATP utilization

and ATP resynthesis resulting in the accumulation of free ADP, adenosine-5'-monophosphate (AMP), and P_i. While this mismatch does not result in significant decreases in ATP concentration from rest (~ 25-30 mmol/kg dry muscle) there are still significant increases in the concentrations of both free ADP and AMP, due to much smaller initial concentrations (~ 70 - 100 μmol/kg dm and < 1 μmol/kg dm, respectively). These metabolites are typically expressed as the ratio [ATP]/[ADP][P_i] and describe the phosphorylation potential or energy status of the cell. Phosphorylation potential is thought to be a key regulator of glycogenolysis and oxidative phosphorylation due to its role in the regulation of PHOS, PFK, and pyruvate dehydrogenase (PDH) (as reviewed by Erecinska and Wilson 1982).

As exercise intensity increases, the time it takes for aerobic metabolism to reach a metabolic steady state increases (40% VO_2max, $t_{1/2} = 23.25 \pm 5.7$ s; 50% VO_2max, $t_{1/2} = 32.4 \pm 3.2$ s; 60% VO_2max, $t_{1/2} = 38.1 \pm 2.1$ s; 70% VO_2max, $t_{1/2} = 41.8 \pm 1.6$ s where $t_{1/2}$ = the time to reach 50% of the maximal response) (Barstow *et al.* 1993, Barstow 1994, Hickson *et al.* 1978, Whipp and Wasserman 1972). Therefore, the greater the power output, the greater the mismatch between the rate of ATP utilization and the rate of oxidative ATP production and therefore, the increased reliance on non-oxidative ATP production. At very high exercise intensities, substrate level phosphorylation provides as much as 80% of the energy during the first 30 s of exercise (Bangsbo *et al.* 1990, Parolin *et al.* 1999). Reliance on PCr and anaerobic glycolysis decreases once steady state is reached. This occurs within a few minutes at power outputs up to 80 – 85%

VO_2max, depending on the fitness level of the individual. However, at exercise power outputs above 100% VO_2max, reliance on substrate level phosphorylation for ATP production remains significant, as oxidative ATP provision is unable to solely meet the ATP demand.

The monoexponential on-kinetics of oxidative phosphorylation has lead to an intense investigation of the potential factors limiting activation of the aerobic system. Research has targeted 2 main hypotheses, namely that a) the delay in oxidative phosphorylation is a result of a metabolic inertia including a lag in enzyme activation or substrate availability and b) the delay in oxidative phosphorylation is due to a lack of oxygen at the mitochondrial level in some muscle fibers at exercise onset. Both hypotheses have been well studied and evidence in support of both an O_2 transport limitation and a metabolic inertia have been documented with varied experimental conditions, the details of which will be presented herein.

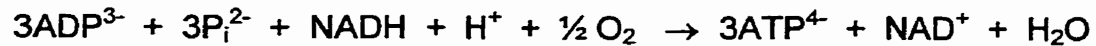
REVIEW OF LITERATURE

The aim of this review of literature is to provide a thorough yet concise overview of the literature regarding the cellular energetics and metabolism of skeletal muscle during the transition from rest to exercise. Emphasis will be given to studies investigating changes in muscle metabolite concentrations and VO_2 on-kinetics during exercise onset in response to altered substrate or O_2 availability, particularly in human subjects.

Regulation of Oxidative Phosphorylation

With oxidative phosphorylation being the main source of ATP production it is important to examine how this system is regulated and, in turn, target potential factors that may be responsible for limiting its activation at exercise onset. Oxidative phosphorylation describes the coupling of the oxidation of substrates, NADH and $FADH_2$, in the electron transport chain (ETC) with ATP synthesis via the F_1F_0 ATPase (ATP synthase) by an electrochemical gradient. The ETC is a series of four enzyme complexes located on the inner mitochondrial membrane. Complex I and II accept reducing equivalents from mitochondrial NADH and $FADH_2$, respectively, with the end result being reduction of O_2 to H_2O, as shown in Figure 2. This series of oxidation-reduction reactions is accompanied by the pumping of protons out across the mitochondrial inner membrane at complexes I, III, and IV, generating an electrochemical gradient (membrane potential and pH gradient). The energy of this electrochemical gradient is harnessed by the F_1F_0

ATPase for synthesis of ATP. The net reaction of oxidative phosphorylation is described as:



$$3ADP^{3-} + 3P_i^{2-} + NADH + H^+ + \frac{1}{2} O_2 \rightarrow 3ATP^{4-} + NAD^+ + H_2O$$

From this equation it is evident that the activation of oxidative phosphorylation requires a supply of ADP, P_i, NADH, and O_2.

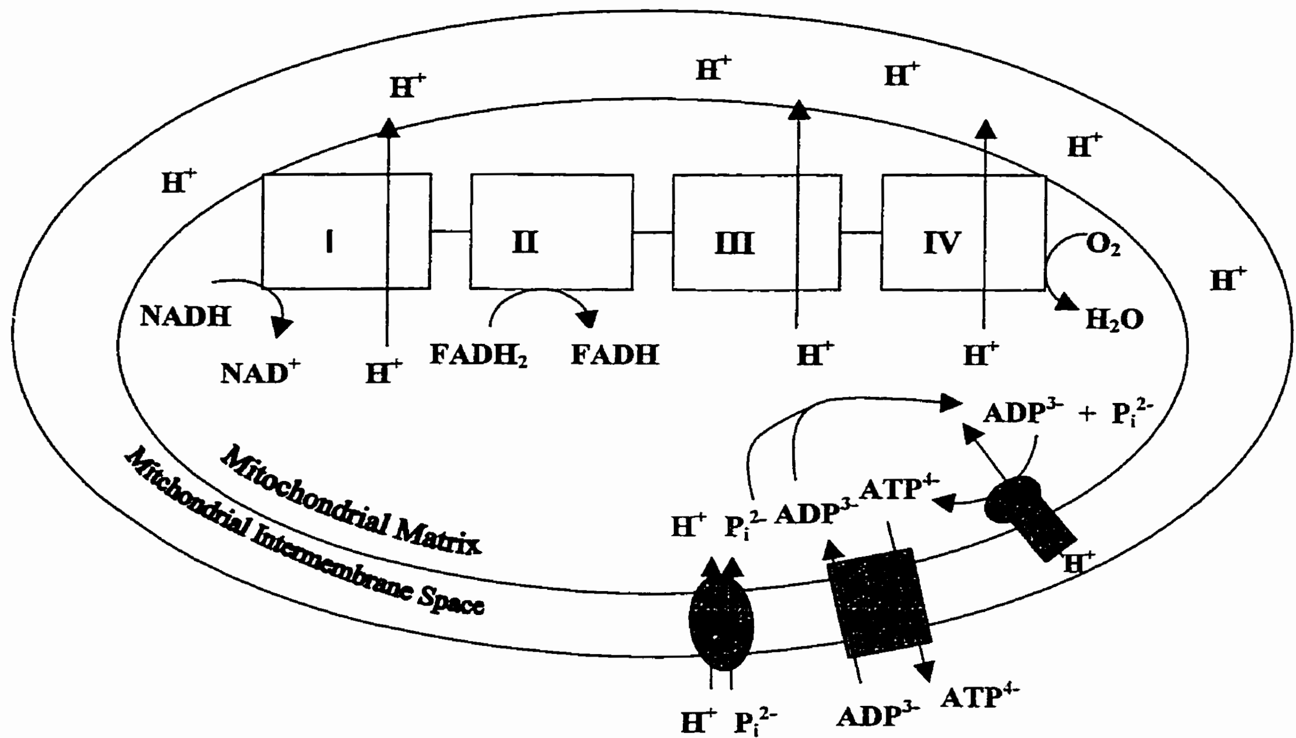


Figure 2. Schematic representation of mitochondrial oxidative phosphorylation. Transporters spanning the mitochondrial intermembrane space include the P_i transporter (oval) and the adenine nucleotide transporter (rectangle). Inner mitochondrial membrane transporters include the F_1F_0 ATPase and the electron transport chain, comprising complexes I, II, III, and IV.

Two hypotheses currently exist which attempt to explain the mechanisms by which these metabolic signals regulate the rate of oxidative phosphorylation: 1) the near-equilibrium hypothesis and 2) the adenine nucleotide translocase hypothesis. The near-equilibrium hypothesis suggests that the reactions of the ETC are near-equilibrium reactions (with the exception of cytochrome oxidase) and thus, the rate of oxidative phosphorylation is dependent on substrate (oxygen) availability and the ratio of substrate/product concentrations ([ATP]/[ADP][P_i], [NAD^+]/[NADH]) (as reviewed by Balaban 1990, Erecinska and Wilson 1982). The adenine nucleotide translocase hypothesis postulates that the exchange of cytosolic ADP and mitochondrial ATP across the inner mitochondrial membrane via the adenine nucleotide translocase is the rate limiting step and that extramitochondrial [ATP]/[ADP] determines the overall rate of respiration (Kunz *et al.* 1981). Much of the work examining these hypotheses has been conducted *in vitro*, with results being highly dependent on external conditions. This, coupled with the inability to measure mitochondrial redox state and free concentrations of ATP, ADP, and P_i *in vivo*, has made it difficult to elucidate the control mechanisms operating in skeletal muscle. However, due to the fact that oxidative phosphorylation is comprised of near-equilibrium reactions, this limitation does not impinge upon the fact that interplay between levels of ADP, P_i, NADH, and O_2 are important in setting or fine-tuning the rate of oxidative phosphorylation. A limitation of any of these factors at the onset of exercise, as reflected by the ratios [ATP]/[ADP][P_i], [NAD^+]/[NADH], and the mitochondrial O_2 concentration, could account for the delayed activation of oxidative

phosphorylation. Thus, provision of ADP, P_i and NADH (metabolic inertia) and O_2 (inertia of O_2 delivery) will be examined during exercise.

Regulation of ADP and P_i Provision

The occurrence of action potentials, preceding muscle contraction, results in the release of Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} release stimulates muscle contraction, through interaction with troponin and tropomyosin, and activates several key regulatory enzymes including PHOS, PDH, and TCA cycle dehydrogenases. Hence, Ca^{2+} is believed to be an early warning signal coordinating muscle contraction (ATP utilization) with the activation of anaerobic and aerobic metabolism (ATP resynthesis).

During the onset of exercise, the hydrolysis of ATP by ATPases results in the accumulation of ADP and P_i, due to the initial inability of oxidative ATP production to match ATP breakdown. Thus, there is an immediate decrease in the phosphorylation potential ([ATP]/[ADP][P_i]) which acts as an additional signal to fine-tune the rate of ATP provision by stimulation of glycogenolysis (PHOS and PFK) and oxidative phosphorylation (PDH and F_1F_0 ATPase). The ADP and P_i produced from ATP hydrolysis accumulate in the cytosol and thus, must be transported into the mitochondria for use by the F_1F_0 ATPase. P_i is transported electroneutrally in symport with a proton or antiport with a hydroxyl ion (as reviewed by Hansford 1980). ADP is transported by the adenine nucleotide translocase as mentioned above. The tight coupling observed between ATP

utilization and increased mitochondrial ATP synthesis indicates the importance of ATP, ADP, and P_i in the regulation of oxidative phosphorylation.

Regulation of NADH Provision

The oxidation of acetyl-CoA in the TCA cycle provides the majority of NADH for the ETC at the onset of exercise. Acetyl-CoA can be derived from both fat and carbohydrate sources. While the reactions of β-oxidation generate a great deal of NADH, reliance on fat as a fuel is minimal during the onset of exercise compared to carbohydrates. This is evidenced by the greater reliance on muscle glycogen during exercise onset. As such, this review will focus on NADH provision from TCA cycle activity with a brief discussion of the potential for increased NADH provision from enhanced fat oxidation.

An increase in TCA cycle flux is necessary at the onset of exercise to increase provision of substrate for oxidative phosphorylation and thus, counter the increase in ATP hydrolysis during exercise. The factors regulating TCA cycle flux and thus, NADH provision, include the activity of TCA cycle enzymes and possibly, the concentration of TCA cycle intermediates (TCAI). Other potentially important control sites upstream of the TCA cycle are the provision of acetyl-CoA, regulated by PDH and carnitine-acylcarnitine translocase (CPTI).

Regulation of the Tricarboxylic Acid Cycle

TCA cycle flux is believed to be regulated primarily by three non-equilibrium enzymes, citrate synthase (CS), isocitrate dehydrogenase (IDH), and

2-oxoglutarate dehydrogenase (2-OGDH) (as reviewed by Hansford 1980, Spriet and Howlett 1999, Williamson and Cooper 1980). Citrate synthase catalyzes the first reaction in the TCA cycle and is thought to be stimulated by its substrates, oxaloacetate and acetyl-CoA, activated by ADP, and inhibited by its product, citrate (as reviewed by Hansford 1980, Newsholme and Leech 1983). Large increases in flux through CS at exercise onset suggest that increases in substrate and ADP concentrations may be more important in regulating CS during exercise as the inhibitory effects of citrate appear to be overridden. Mitochondrial redox state has also been suggested to influence CS flux (Williamson and Cooper 1980) possibly due to its involvement in the malate dehydrogenase reaction, which produces oxaloacetate.

Greater weight has been attributed to the importance of IDH and 2-OGDH in the regulation of TCA cycle flux as these enzymes have lower maximal activities than the other TCA cycle enzymes and their activity appears to correlate with TCA cycle flux (Blomstrand *et al.* 1997) and flux through oxidative phosphorylation (Moreno-Sanchez *et al.* 1990). Both IDH and 2-OGDH are stimulated by Ca^{2+} (Denton and M^c^Cormack 1985, Williamson and Cooper 1980), ADP (as reviewed by Hansford 1980), and $[NAD^+]/[NADH]$ (as reviewed by Hansford 1980, Williamson and Cooper 1980). Thus, the increase in Ca^{2+} and ADP that occurs at the onset of muscle contraction favours the activation of IDH and 2-OGDH, which would stimulate TCA cycle flux and increase the provision of NADH for the ETC. While mitochondrial redox state is thought to play a role in

the regulation of IDH and 2-OGDH, the changes in mitochondrial redox state with exercise are less well understood.

It is well documented that NAD^+ acts as a coenzyme for IDH, 2-OGDH, and malate dehydrogenase in the TCA cycle. Thus, one would expect that an increase in NAD^+ at exercise onset would favour the activation of these enzymes, resulting in an increase in TCA cycle flux and NADH provision for the ETC. However, measurements of the changes in the mitochondrial redox state during exercise have been varied. Initial experiments using surface fluorometry showed that there was an increase in the mitochondrial $[NAD^+]/[NADH]$ ratio in contracting dog muscle (Jobsis and Stainsby 1968). Since then, some studies have supported an increase in the oxidation state of mitochondria (increased $[NAD^+]/[NADH]$) using near-infrared spectroscopy of cytochrome aa_3 in dog skeletal muscle (Stainsby *et al.* 1989) and the GDH equilibrium reaction in human skeletal muscle (Graham and Saltin 1989) as indicators of mitochondrial redox state. Conversely, other studies have shown a reduction in mitochondrial redox state using near-infrared spectroscopy of cytochrome aa_3 (Duhaylongsod *et al.* 1993) and the bioluminescent technique under conditions of ischemia (reduced oxygen content due to a reduced blood flow) (Sahlin 1983) and during moderate (75% VO_2max) (Sahlin *et al.* 1987) and intense (100% VO_2max) exercise (Sahlin 1985). The variations in results may be due to an inability of some techniques to accurately measure mitochondrial and not cytosolic NAD^+ and NADH concentrations. Despite these conflicting results, the fact that NAD^+ is a substrate for TCA cycle dehydrogenase enzymes and PDH, and NADH is a

substrate for the ETC, strongly suggests that the $[NAD^+]/[NADH]$ ratio is an important energy-linked regulatory factor of both the TCA cycle and the ETC.

An increase in TCAI pool size has also been suggested to be important in order to augment TCA cycle flux (Lee and Davis 1979, Sahlin *et al.* 1990, Williamson and Cooper 1980) and could represent a site of metabolic inertia. Studies in both electrically stimulated rodent muscle (Aragon and Lowenstein 1980) and humans (Gibala *et al.* 1997a, 1997b, 1998) have shown a net accumulation of TCAI during exercise intensities of at least 60% VO_2max. The increase in TCAI peaks within the first few minutes of exercise suggesting a rapid adaptation of TCAI pool size in response to an increased energy demand (Gibala *et al.* 1997a, 1997b). Further work examining the relationship of TCAI to energy demand showed that the sum of TCAI appear to be exponentially related to work intensity, based on changes in citrate, malate, and fumarate (Sahlin *et al.* 1987, 1990, 1995) and the rate of flux through the TCA cycle (Gibala *et al.* 1998).

Despite the evidence suggesting a role for TCAI pool size in the regulation of TCA cycle flux, it remains inconclusive as to whether the increase in TCAI pool size is necessary to support an increase in TCA cycle flux or is simply a consequence of the increase in flux. The mechanism proposed for the increased TCAI pool size is increased flux through the alanine aminotransferase reaction as a result of pyruvate production through glycolysis exceeding the rate of pyruvate oxidation (Gibala *et al.* 1997a, 1998, Sahlin *et al.* 1990). However, the increase in TCAI pool size (~ 3-4-fold higher) that occurs early in exercise seems inadequate to account for the up to ~100-fold increase in TCA cycle flux during

exercise. Further disparity exists due to the fact that changes in TCAI during exercise are not uniform and have been shown to vary in time course, magnitude, and direction of change (as reviewed by Graham and Gibala 1998). Early work in rat skeletal muscle focused on changes in malate, fumarate, and citrate at exercise onset (Aragon and Lowenstein 1980). These TCAI were shown to account for more than 60% of the total TCAI pool size in rodent (Aragon and Lowenstein 1980) and human skeletal muscle (Gibala *et al.* 1997b). Recent studies have found that the span 2 (left half of the TCA cycle) TCAI malate, fumarate, and succinate show the largest increases (up to ~ 600%) at exercise onset and account for ~ 80-90% of the TCAI pool size (Gibala *et al.* 1997a, 1998). The increases observed in citrate (50%), isocitrate (175%), and oxaloacetate (100%) are less significant and 2-oxoglutarate actually decreases during exercise (Gibala *et al.* 1997b). In addition, the large increase in TCAI, particularly succinate, malate, and fumarate are not maintained and gradually decline during prolonged exercise and at exhaustion (Gibala *et al.* 1997b, Sahlin *et al.* 1990). Variation also exists with respect to exercise intensity, as during low intensity exercise an increase in TCA cycle flux occurs independent of changes in TCAI (Sahlin *et al.* 1995). Many of the changes in TCAI observed with exercise may actually reflect changes occurring in the mitochondrial and cytosolic compartments. At least six TCAI and most TCA cycle enzymes are present in both the cytosolic and mitochondrial compartments (as reviewed by Gibala *et al.* 2000). The inability to isolate mitochondrial and cytosolic concentrations of TCAI contributes to the difficulty in determining the role of the

TCAI in regulating TCA cycle flux. Thus, the importance of TCAI on TCA cycle flux and oxidative energy metabolism remains unclear.

Regulation of Pyruvate Dehydrogenase

Pyruvate dehydrogenase is a multienzyme complex located on the inner mitochondrial membrane that catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA. The PDH complex is comprised of multiple copies of 3 catalytic subunits: E1 – pyruvate decarboxylase, E2 – dihydrolipoate acetyltransferase, and E3 – dihydrolipoyl dehydrogenase. These three enzymes act sequentially to convert pyruvate, CoASH, and NAD^+ to acetyl-CoA, NADH, H^+, and CO_2 (Behal *et al.* 1993). The E1-pyruvate decarboxylase subunit is considered to be the rate limiting step in the production of acetyl-CoA while the E2 and E3 subunits catalyze near-equilibrium reactions (Cooper *et al.* 1975). The activity of PDH is regulated by a phosphorylation-dephosphorylation cycle as was initially observed in beef heart, beef kidney, and pork liver mitochondria (Linn *et al.* 1969) and later in skeletal muscle (Taylor and Halperin 1973). Phosphorylation of 3 serine residues in the E1 subunit by PDH kinase causes inactivation of the enzyme (Yeaman *et al.* 1978). Conversely, dephosphorylation of these serine residues by PDH phosphatase causes activation of the enzyme. Thus, PDH exists in a non-active, phosphorylated "b" form and an active, non-phosphorylated "a" form, with the transformation of PDH and substrate entry into the TCA cycle being controlled by the relative activities of the kinase and phosphatase (Figure 3)(as reviewed by Weiland 1983).

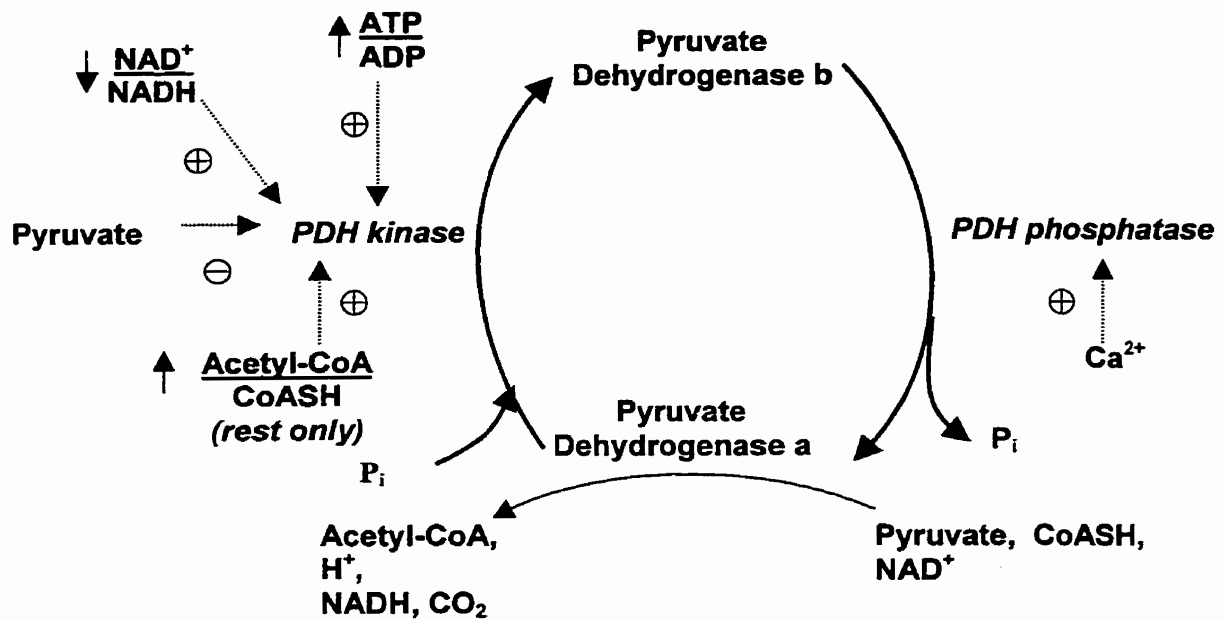


Figure 3. Schematic of the acute regulation of PDH (Spriet *et al.* 1999).

PDH kinase is acutely regulated by allosteric modulators (Figure 3). Increases in the ratios of [ATP]/[ADP] (Hansford 1976, Linn *et al.* 1969, Taylor and Halperin 1973) [NADH]/[NAD^+] (Cooper *et al.* 1975, Hansford 1976) and [Acetyl-CoA]/[CoASH] (Cooper *et al.* 1975, Garland and Randal 1964, Hansford 1976) act to stimulate PDH kinase while low ratios of these metabolites and high pyruvate concentrations cause inhibition of PDH kinase (Linn *et al.* 1969). Pyruvate, a substrate for PDH, also acts to increase flux through the reaction. PDH phosphatase is acutely regulated primarily by Ca^{2+} stimulation (Denton *et al.* 1972, Pettit *et al.* 1972, Taylor and Halperin 1973).

At rest, the high ratios of [ATP]/[ADP], low [CoASH]/[acetyl-CoA], and low pyruvate concentrations favour the activation of PDH kinase. The absence or

low level of muscle contraction causes low Ca^{2+} concentrations and results in a lack of stimulation of PDH phosphatase. Thus, PDH remains mainly in the non-active "b" form.

Transformation to PDHa during the transition from rest to exercise is primarily mediated by an increase in Ca^{2+}, a potent activator of PDH phosphatase. Increases in ADP and pyruvate concentrations also act to increase PDHa during exercise. The rise in ADP is due to ATP hydrolysis and the build up of pyruvate results from the rate of glycogenolysis exceeding the rate of pyruvate oxidation (and other pyruvate fates). These changes inhibit PDH kinase while increased Ca^{2+} concentrations stimulate PDH phosphatase, thus favouring transformation of PDH to the active form. Although the concentration of acetyl-CoA increases during exercise its effects on PDH kinase are overridden (Constantin-Teodosiu *et al.* 1991a, Howlett *et al.* 1998, Parolin *et al.* 1999, Putman *et al.* 1995). The role of the [NADH]/[NAD^+] ratio in PDH transformation has yet to be elucidated due to the difficulties in measuring mitochondrial redox state as previously discussed. However, it is interesting to note that PDH and the dehydrogenases of the TCA cycle (IDH and 2-OGDH) are all believed to be stimulated by increasing [ADP]/[ATP], [NAD^+]/[NADH], and Ca^{2+} thus, providing a concerted mechanism of control to increase aerobic metabolism in response to an altered energy state. The increase in PDHa observed during exercise is directly proportional to increasing power output (Constantin-Teodosiu *et al.* 1991a, Howlett *et al.* 1998). Since it is known that PDH activity closely matches flux through the enzyme (Howlett *et al.* 1998, Putman *et al.* 1995) in most normal

situations, the increase in PDHa with exercise intensity indicates a greater carbohydrate oxidation with increasing energy demands. The extent of PDH activation at increasing power outputs concurs with greater concentrations of ADP relative to ATP, higher accumulations of pyruvate, and increased release of Ca^{2+} at higher exercise intensities.

The role of PDH as a key regulator of acetyl-CoA provision for entry into the TCA cycle makes it a potential target as a limiting factor of aerobic metabolism. During the transition from rest to exercise the rate of glycogenolysis exceeds the rate of pyruvate oxidation by PDH. Consequently, pyruvate accumulates and is converted to lactate by lactate dehydrogenase (LDH) or to alanine by alanine aminotransferase, an anapleurotic process (replenishing of TCA cycle intermediates) in human skeletal muscle (Gibala *et al.* 1997a). Characterization of the time course of PDH activation suggests that PDH may be limiting during submaximal exercise. During exercise at 35% and 65% VO_2max PDH reaches the appropriate level of activity by ~ 1 min (Howlett *et al.* 1998) and during maximal sprinting exercise, 95% activation is achieved by 15 sec (Parolin *et al.* 1999). Evidence supporting a delay in PDH activation at the onset of exercise was shown with the use of dichloroacetate (DCA). DCA acts to increase the amount of PDH in the "a" form in resting skeletal muscle by inhibition of PDH kinase (Stacpoole 1989). This results in maximal flux of pyruvate through PDH at exercise onset as well as acetylation of CoA and carnitine pools (Timmons *et al.* 1997). Administration of DCA increased PDHa at rest and caused a decrease (compared to control) in lactate accumulation and

PCr and glycogen breakdown during muscle contractions in ischemic dog muscle (Timmons *et al.* 1996, 1997), ischemic human muscle (Timmons *et al.* 1998b), and human muscle with normal blood flow (Howlett *et al.* 1999a, Timmons *et al.* 1998a). Therefore, an increase in PDHa at rest was able to increase the rate of onset of oxidative phosphorylation and reduce the O_2 deficit, as evidenced by a decrease in substrate level phosphorylation. While the above results seem promising in attributing the lag in aerobic metabolism to substrate provision from PDH activation, they do not exclude the possibility that the elevated acetylcarnitine store was responsible for enhanced provision of acetyl-CoA.

<u>Acetylcarnitine</u>

The exercise induced increase in acetyl-CoA results in the subsequent accumulation of acetylcarnitine. The conversion of acetyl-CoA to acetylcarnitine is catalyzed by the near-equilibrium enzyme carnitine acetyltransferase (CAT) (Figure 4). The obligatory increase in acetylcarnitine during exercise is due to the function of carnitine in buffering the excess formation of acetyl groups generated from flux through PDH exceeding TCA cycle flux (Alkonyi *et al.* 1975). This is an important mechanism for preventing the depletion of the mitochondrial CoASH pool, which would otherwise inhibit flux through PDH and 2-OGDH and other mitochondrial reactions involving CoASH (Constantin-Teodosiu *et al.* 1991a, Putman *et al.* 1995).

As indicated above, DCA administration causes an increase in PDHa, acetyl-CoA, and acetylcarnitine at rest (Timmons *et al.* 1996, 1997, 1998a,

1998b, Howlett *et al.* 1999a). It is disputed whether the sparing of non-oxidative ATP production observed with DCA at the onset of exercise is from increased acetyl-CoA availability via PDHa or the acetylcarnitine store or both (Figure 4). Timmons and coworkers have shown a decrease in the acetylcarnitine store during the first few minutes of muscle contraction in ischemic dog muscle (Timmons *et al.* 1996, 1997) and ischemic human muscle (Timmons *et al.* 1998b) suggesting that PDH flux is not sufficient to meet the demand for flux through the TCA cycle. While the observed decrease in acetylcarnitine was small (1-2 mmol acetylcarnitine/kg dm in humans), the oxidation of 1 mmol/kg dm acetyl-CoA from 1 mmol/kg dm acetylcarnitine results in the production of 12 mmol ATP. Thus, it has been argued that increased acetyl-CoA availability from acetylcarnitine is responsible for the decreased O_2 deficit (Timmons *et al.* 1996, 1997). The proposed mechanism is that acetylcarnitine may provide a substrate store for the TCA cycle dehydrogenases to increase TCA cycle flux (Timmons *et al.* 1998b) based on previous work that dehydrogenase activity is a major limit to flux through oxidative phosphorylation (Moreno-Sanchez *et al.* 1990). However, previous results have shown no decrease in muscle acetylcarnitine during the onset of exercise at 65% VO_2max with DCA (Howlett *et al.* 1999a).

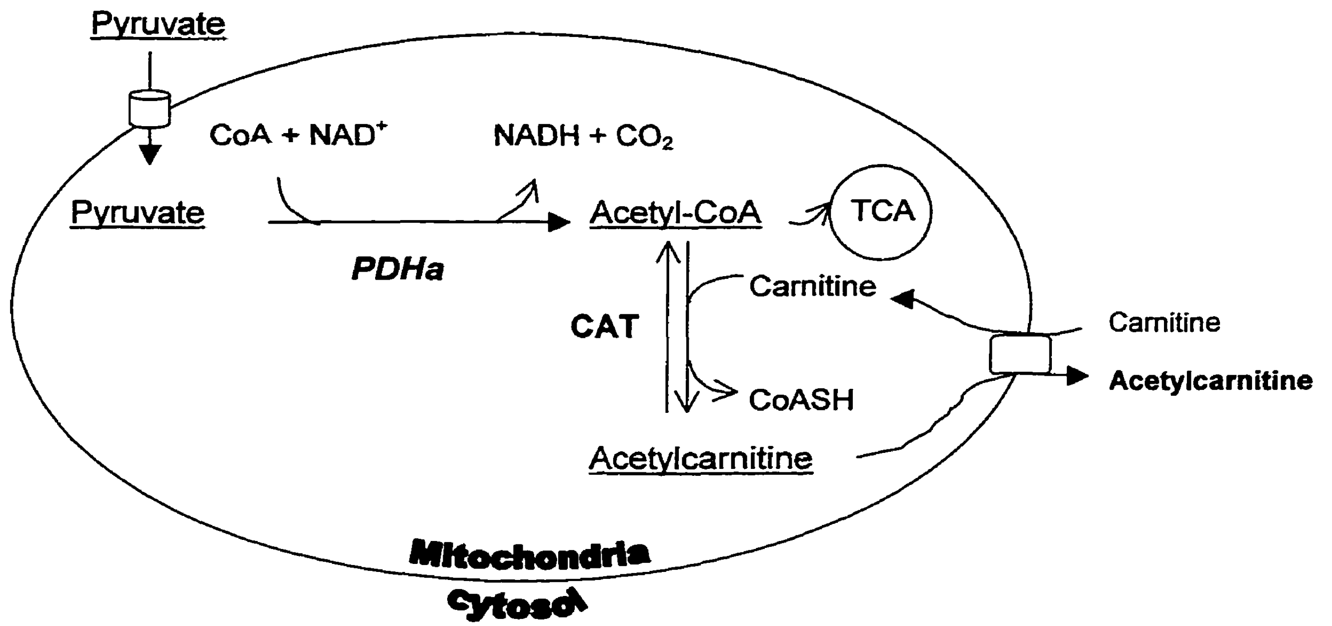


Figure 4. Schematic depicting the relationship between pyruvate, PDHa, acetyl-CoA, and acetylcarnitine. Acetylcarnitine and carnitine are exchanged between the mitochondria and cytosol by a mitochondrial transporter. ***CAT***, carnitine acetyltransferase.

A decrease in acetyl-CoA and acetylcarnitine accumulation has also been observed in humans during cycling at 75% VO_2max following 3 days of a low carbohydrate diet (Putman *et al.* 1993). The reliance on fat for fuel increased acetyl group accumulation at rest. Despite high resting concentrations, acetyl-CoA levels fell with exercise as the rate of oxidation of acetyl-CoA exceeded the rate of provision by β-oxidation. In addition, a significant decline in acetylcarnitine levels was observed at exhaustion but not at 16 minutes of exercise. Although no direct comparison can be made with respect to energy

provision, this study indicates that acetylcarnitine can provide a source of acetyl-CoA during exercise. However, because the changes in acetylcarnitine during exercise with a high carbohydrate diet are equivocal, it still remains to be determined whether acetylcarnitine is responsible for the sparing of substrate level phosphorylation observed during exercise with DCA or whether increased substrate provision was via enhanced flux through PDH. A dissociation of increased PDHa and acetylcarnitine stores at exercise onset would provide insight into the factor responsible for a faster onset of oxidative phosphorylation.

Carnitine-acylcarnitine translocase (CPTI)

Fat is an important energy providing fuel during most exercise situations. Fat is stored predominantly in adipose tissue but is also present in skeletal muscle, as muscle triacylglycerol. The most usable form of fat is free fatty acids (FFA). Skeletal muscle is unable to synthesize FFA de novo and thus, relies on the uptake of albumin bound FFA present in the blood and the store of muscle triacylglycerol. The regulation of adipose tissue lipolysis, FFA delivery, and uptake by the muscle will not be discussed. For a comprehensive review see Turcotte *et al.* (1995) and Vander Vusse and Reneman (1996).

Fatty acyl-CoA units are oxidized in the mitochondria by the process of β-oxidation. The β-oxidation pathway consists of 4 near-equilibrium reactions, which collectively generate a two-carbon acetyl-CoA molecule, one $FADH_2$, and one NADH. The reducing equivalents produced both from β-oxidation and the oxidation of acetyl-CoA in the TCA cycle from one long chain free fatty acid

(FFA) far exceeds that generated from glycogenolysis on a mole basis. The oxidation of an 18-carbon chain fatty acid produces 146 mmol ATP compared to 39 mmol ATP produced from glycogen breakdown. However, fat oxidation is limited in that the maximal rate of ATP production from fat can only sustain exercise up to 55-75% VO_2max, depending on the training state of the individual, whereas ATP production from carbohydrate can sustain exercise up to 100% VO_2max.

A major regulatory step in fat oxidation is on the outer mitochondrial membrane at CPTI. Upon delivery of FFA to the mitochondria, FFA are first activated by fatty acyl-CoA synthetase, producing fatty acyl-CoA. CPTI then catalyzes the conversion of fatty acyl-CoA to acyl-carnitine, a form which can be transported across the inner mitochondrial membrane by carnitine-acylcarnitine translocase (CAT). On the matrix side, CPTII catalyzes the conversion of acyl-carnitine back to fatty acyl-CoA for oxidation. Both CAT and CPTII are near-equilibrium enzymes and thus, CPTI controls the entry of fatty acyl-CoA units into the mitochondria for oxidation. Malonyl-CoA, the first committed intermediate in de novo fatty acid synthesis, acts to inhibit CPTI *in vitro* (Berthon *et al.* 1998, McGarry *et al.* 1983, Saggerson and Carpenter 1981). However, the physiological significance of malonyl-CoA during exercise has not been confirmed in human skeletal muscle (Odland *et al.* 1996).

An increase in the availability of FFA and thus, enhanced fat oxidation, by administration of Intralipid (Dyck *et al.* 1993, 1996) or caffeine (Chesley *et al.* 1998), has recently been shown to decrease the reliance on substrate level

phosphorylation during exercise, as evidenced by a reduction in PCr use and accumulation of ADP, AMP, and P_i. A fat-induced increase in mitochondrial NADH concentration is thought to be responsible for the improved phosphorylation state (Chesley *et al.* 1998), however, this mechanism has not been confirmed with direct measurements. It has been hypothesized that enhanced fat oxidation causes an increase in mitochondrial NADH, resulting in a decreased requirement of ADP and P_i to achieve the required rate of oxidative ATP production (Chesley *et al.* 1998). Since PHOS is regulated by P_i (substrate availability) and AMP (allosteric modulator), a decreased accumulation of AMP and P_i would decrease flux through PHOS causing less glycogen breakdown. The sparing of substrate level phosphorylation by enhanced FFA oxidation provides support for the importance of NADH provision in activating oxidative phosphorylation at the onset of exercise.

Regulation of Oxygen Delivery

An adequate supply of oxygen is essential for the normal functioning of all cells. As oxygen is a substrate for oxidative phosphorylation the demand for oxygen in skeletal muscle can vary over a large degree from rest to intense exercise. In order to accommodate the need for O_2 at the level of oxidative phosphorylation, O_2 in the air must be inspired into the lungs, diffuse through the lung tissue into the blood and be transported to skeletal muscle via the central and peripheral circulations for diffusion into the muscle cell and mitochondria. The extensiveness of this pathway outlines the many potential barriers for

transporting O_2 from the air to the muscle mitochondria. However, this alone does not prove that O_2 is limiting at the onset of exercise.

It is interesting that the concentrations of ADP, P_i, and NADH (the other three substrates for oxidative phosphorylation) increase at the onset of exercise while the mitochondrial oxygen concentration is believed to decrease. However, it is unknown whether the fall in O_2 is severe enough to limit the rate of oxidative phosphorylation and therefore, be responsible for the lag in aerobic metabolism at the onset of exercise. Thus, great debate exists regarding whether or not mitochondrial oxygen content is sufficient, or adjusts rapidly enough, to support the large increase in the rate of oxidative phosphorylation during exercise. This is largely due to the difficulty in accurately measuring mitochondrial partial pressure of O_2 (P_mO_2) *in vivo* during exercise. Investigations into the possibility of an O_2 supply limitation have been conducted by increasing or decreasing hemoglobin (Hb) content and blood flow (convective O_2 delivery), increasing hemoglobin-oxygen ($Hb\text{-}O_2$) dissociation (diffusive O_2 delivery), increasing or decreasing the fraction of inspired air (F_IO_2) (convective and diffusive O_2 delivery), and estimating P_mO_2 *in vivo*. Less attention has been given to the pulmonary system as a limiting factor as the arterial O_2 saturation does not decrease during exercise, except in highly trained endurance athletes during maximal exercise (as reviewed by Dempsey and Wagner 1999). Since a focus of this thesis is examining the effects of hyperoxia ($F_IO_2 > 0.21$) on O_2 deficit, emphasis is given to literature investigating VO_2 on-kinetics and muscle metabolite concentrations during the onset of exercise in hyperoxic conditions.

Convective Oxygen Delivery

The delivery of oxygen to skeletal muscle is dependent on the convective and diffusive components of the circulatory transport system. Convective O_2 delivery is the transport of oxygen by the bulk flow of blood. The amount of O_2 delivered to the muscle is determined by arterial O_2 content (CaO_2) and blood flow (Q). CaO_2 includes both the oxygen bound to Hb and the oxygen dissolved in the plasma. The majority (~ 98%) of O_2 is reversibly bound to Hb within the red blood cell while ~ 2% is dissolved in the plasma and the red blood cell cytoplasm. It is the dissolved O_2 which determines the PO_2. The O_2 content of the blood can be influenced by F_IO_2, pulmonary perfusion and exchange, Hb content, and the O_2 saturation of Hb. Variations in these parameters have been used to observe the effects of increasing O_2 delivery to the working muscle during exercise. As hyperoxic conditions only alter F_IO_2 and O_2 saturation of Hb, only these components of CaO_2 will be discussed in addition to the effects of blood flow.

A controlled approach of increasing convective O_2 delivery is to increase blood flow to the exercising muscle. As CaO_2 is unaffected by blood flow changes, O_2 delivery to the muscle directly mirrors changes in muscle blood flow. In electrically stimulated dog muscle, a transition from rest to 60-70% VO_2 peak under conditions of elevated blood flow at exercise onset did not affect muscle VO_2 kinetics. Thus, despite significantly enhanced O_2 delivery, oxygen uptake during the transition state remained the same due to a reduced arteriovenous O_2 content difference (Grassi *et al.* 1998a). While the high blood flow rates may be

the cause of a reduced O_2 extraction, Richardson et al. (1993) found no difference in maximal O_2 extraction in humans between normal cycling and maximal knee extensor exercise (high leg blood flow). However, the applicability of this finding to dogs may be limited due to fiber type differences and much higher induced blood flows. Investigations into changes in VO_2 on-kinetics with increased blood flow in humans is limited by difficulties in measuring non-steady state blood flow due to the lack of time resolution with present techniques.

The lack of effect of increased blood flow in dogs concurs with the finding that the monoexponential increase in blood flow at the onset of muscle contraction occurs more rapidly than muscle VO_2 on-kinetics (Grassi *et al.* 1998a). This has also been observed in humans (Grassi *et al.* 1996). This suggests that VO_2 on-kinetics are not limited by convective O_2 delivery but by an intrinsic metabolic inertia or diffusive O_2 delivery. In addition, the observation of a spatial and temporal heterogeneity of blood flow in contracting dog muscle (Marconi *et al.* 1988) may suggest that the inability to extract extra oxygen in the initial phase of exercise is due to inefficient blood flow distribution (Bangsbo 2000). In humans, ischemic conditions induced by supine exercise without lower body negative pressure showed slower VO_2 on-kinetics than supine exercise with lower body negative pressure (Hughson *et al.* 1993). Similarly, slowed blood flow kinetics in arm exercise above vs. below the heart resulted in slower muscle VO_2 kinetics and mean response time (Hughson *et al.* 1996). These results suggest a dependence of muscle O_2 uptake at exercise onset on O_2 delivery. However, the fact that reduced O_2 delivery slows down VO_2 on-kinetics does not

demonstrate an O_2 limitation in normal conditions. Thus, increasing blood flow and O_2 delivery above that normally observed during exercise in isolated dog muscle does not appear to enhance muscle VO_2 on-kinetics during the transition from rest to exercise.

Hyperoxic air has a higher percentage of oxygen (up to F_IO_2=1.0) compared to normal air (F_IO_2 = 0.21). While this increases the inspired PO_2 by almost fivefold, it only increases CaO_2 by ~ 8-10% due to the almost complete saturation of Hb with O_2 at normoxic PO_2 (Figure 5) (as reviewed by Wagner 1996). Some researchers have found that during submaximal cycling exercise (55-70 % VO_2max) the 10% increase in CaO_2 from breathing 100% O_2 is counter balanced by a 7-11% decrease in steady state leg blood flow such that O_2 delivery remained unchanged from normoxia (Welch *et al.* 1977). Leg blood flow measurements during the transition from rest to knee-extensor exercise below ventilatory threshold showed a slight but insignificant decrease in leg blood flow with hyperoxia such that O_2 delivery was not different between hyperoxia and normoxia (MacDonald *et al.* 2000). Others have reported no change in leg blood flow with hyperoxia (F_IO_2 = 1.0) during incremental cycling exercise to 100% VO_2max (Knight *et al.* 1993) and maximal knee extensor exercise (Richardson *et al.* 1999) with significant increases in O_2 delivery. In separate subject pools, Cardus et al. (1998) found no difference in blood flow during maximal exercise between normoxia (F_IO_2 = 0.21) vs. hypoxia (reduced O_2 content due to a reduced F_IO_2) (F_IO_2 = 0.15 and 0.12) and between hyperoxia (F_IO_2 = 1.0) vs. hypoxia suggesting no difference between hyperoxia and normoxia, although no

direct comparison was made. It remains inconclusive whether hyperoxic conditions actually increase convective O_2 delivery because of discrepant results in changes in leg blood flow. Although O_2 uptake would typically be a good indicator of whether O_2 delivery was increased, the fact that hyperoxia potentially alters both convective and diffusive O_2 delivery makes it impossible to differentiate which factor is responsible for the increased O_2 uptake. Thus, changes in oxygen uptake with hyperoxia will be discussed later incorporating both factors.

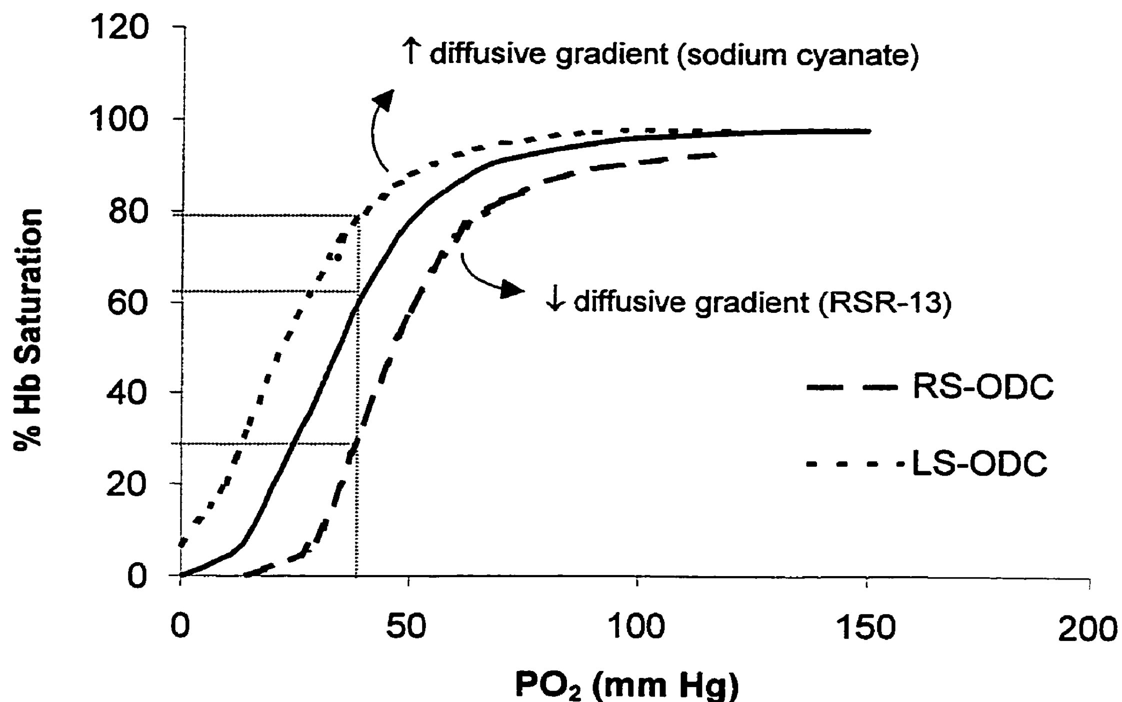


Figure 5. Schematic of the Hb-O_2 dissociation curve (ODC) during normal conditions (solid bold line). Right-shifted oxygen dissociation curve (RS-ODC) and left-shifted oxygen dissociation curve (LS-ODC).

Diffusive Oxygen Delivery

The diffusive movement of oxygen describes the movement of oxygen from Hb in the red blood cell to the muscle fiber mitochondria. This includes the dissociation of O_2 from Hb, diffusion out of the red blood cell and through the plasma in the capillary, across the capillary wall and interstitial space, and then into the muscle fiber for diffusion into the mitochondria. The diffusive component is determined by the magnitude of the PO_2 gradient from the blood to the O_2-consuming mitochondria and the physical conductance for O_2 along this pathway. Thus, $VO_2 = DO_2(P_cO_2 - P_mO_2)$ where DO_2 is an O_2 conductance coefficient, P_cO_2 is the PO_2 in the capillary, and P_mO_2 is the PO_2 in the mitochondria. The normal capillary PO_2 in humans is ~ 100 Torr while the PO_2 in active muscle is thought to be < 10 Torr and the P_mO_2 is estimated to be near zero during exercise (as reviewed by Conley *et al.* 2000, Wagner 1996). Hyperoxic conditions increase P_cO_2 to ~ 600 Torr and therefore, greatly increase the diffusion gradient from blood capillary to muscle fiber. Thus, some of the metabolic changes observed during hyperoxia may be due to greater diffusive oxygen delivery.

The factors which influence the diffusion process include the Hb-O_2 and myoglobin (Mb)-O_2 dissociation reactions, the effective diffusive capacity of the tissues, the thickness of the plasma layer around the red blood cell, the distance for diffusion, and the difference in O_2 tension between the capillary and the mitochondria. Due to the large diffusion distance between the red blood cell and the muscle cell, and the residual PO_2 in femoral venous blood, some researchers

strongly suggest the existence of a diffusion limitation during exercise (Cardus *et al.* 1998). Studies in which increased O_2 delivery (via hyperoxic conditions or increased blood flow) failed to enhance muscle oxygen uptake is believed by some to be evidence of a diffusion limitation, supported by decreases in muscle O_2 extraction and O_2 conductance with increasing F_IO_2 (Cardus *et al.* 1998, Welch *et al.* 1977). However, a recent study using the knee extensor model showed that percent O_2 extraction and O_2 conductance did not vary with F_IO_2 such that increased O_2 delivery with hyperoxia (F_IO_2 = 1.0) caused an increase in muscle VO_2 (Richardson *et al.* 1999). It is well documented that hyperoxia increases the PO_2 of arterial blood by ~ 6-fold causing a large increase in the O_2 gradient from capillary to mitochondria. The impact of this increase in diffusive O_2 delivery will be considered in the discussion of VO_2 on-kinetics, VO_2max, and performance.

While many of the factors directly affecting diffusive O_2 transport cannot be manipulated, there has been some investigation into the effects of altering Hb-O_2 binding affinity on VO_2 to examine whether a diffusion limitation exists (Hogan *et al.* 1991, Richardson *et al.* 1998). A change in Hb-O_2 dissociation directly affects the PO_2 of the blood which results in changes in the diffusion gradient between the capillary and the mitochondria. A right-shifted Hb-O_2 dissociation curve (ODC) causes a decreased Hb affinity and an increase in the capillary-to-tissue PO_2 gradient while a left-shifted ODC results in an increased Hb affinity and a decrease in the diffusion gradient (Figure 5). In dogs, two studies have been conducted in which a right-shifted ODC was induced with the drug RSR-13

under hyperoxic conditions causing elevated arterial PO_2 and normal Hb saturation. During maximal contractions, these conditions significantly increased O_2 extraction and muscle VO_2max (Richardson *et al.* 1998). However, during the transition to 60-70% VO_2 peak, these conditions failed to alter VO_2 on-kinetics (Grassi *et al.* 1998b). This may suggest an ability to increase O_2 extraction during steady state exercise but not during the transition state. The discrepancy in results may also be explained by observations of the presence of responders and non-responders. Upon close examination, Richardson et al. (1998) found that animals with initially poor extraction greatly increased O_2 extraction with hyperoxia and RSR-13 whereas initially high extractors showed little change. This phenomenon has not been examined in humans. A left-shifted ODC from the administration of sodium cyanate with constant muscle blood flow and arterial O_2 saturation caused a 17% decrease in O_2 extraction and VO_2max in dogs (Hogan *et al.* 1991). Thus, while increased PO_2 with a right-shifted ODC appears to increase O_2 extraction, the increased P_mO_2 only seems to be beneficial during prolonged steady state exercise and not during exercise onset.

<u>Hyperoxia</u>

The breathing of hyperoxic air affects both the convective and diffusive components of O_2 delivery. Therefore, observed changes resulting from hyperoxic conditions could result from either of these two factors. The components of convective O_2 delivery to the muscle are commonly measured. However, only estimates of muscle O_2 conductance (from intracellular PO_2) can

be determined and are not often reported. As the main thrust of this thesis is changes during the transition from rest to exercise, emphasis will be given to the effects of hyperoxia on oxygen uptake kinetics. In addition, implications for O_2 as a potential modulator of mitochondrial respiratory rate during steady state exercise are briefly discussed by examining the effects of hyperoxia on VO_2max and performance.

Oxygen Uptake Kinetics

Measurements of oxygen uptake via breath by breath analysis show that VO_2 follows a monoexponential function during the transition from rest to exercise (Figure 1). This monoexponential increase in VO_2 indicates a lag in oxygen uptake compared to the step increase in power output. As the increase in VO_2 in response to exercise is a result of the combined responses of O_2 transport, O_2 extraction, and O_2 utilization, delayed or insufficient changes in any of these factors could be responsible for the lag in VO_2. The VO_2 on-kinetics are typically characterized by $t_{1/2}$, which describes the time to reach 50% of the amplitude of the response, or the time constant τ, which is the time to reach 63% of the response. These measures can be used to determine potential factors limiting the rate of change in VO_2 and have previously been shown to change with exercise intensity (Barstow *et al.* 1993), aerobic training (Hickson *et al.* 1978), previous exercise (Macdonald *et al.* 1997), and hyperoxic conditions (Linnarsson *et al.* 1974, Macdonald *et al.* 1997, Pederson 1983).

VO_2 measured at the mouth is thought to be a good indicator of oxygen uptake by the exercising muscles (Grassi *et al.* 1996). Much of the initial research investigating the possibility of an oxygen limitation during exercise relied on the measurement of VO_2 on-kinetics. This is an indirect method of determining O_2 deficit. An increase in the VO_2 on-kinetics while breathing hyperoxic air suggests an O_2 supply limitation at exercise onset. Breathing hyperoxic air ($F_IO_2 > 0.21$) causes an increase in CaO_2 with the potential for increased bulk O_2 delivery to the muscle. Varied initial results of VO_2 on-kinetics were due to inaccuracies in measuring VO_2 during hyperoxic conditions, particularly using the conventional Douglas bag technique. This technique was found to give erroneously high values as a result of contamination with ambient air (Welch and Pedersen 1981). The use of a mixing chamber has been shown to give more accurate values (Prieur *et al.* 1998, Welch and Pedersen 1981). However, current work has favoured the use of an automated breath by breath gas system or the measurement of blood flow and blood gas concentrations.

During submaximal exercise (138W, below ventilatory threshold) with hyperoxia ($F_IO_2 = 0.7$), Hughson and Kowalchuk (1995) reported no change in VO_2 on-kinetics and suggested that VO_2 is set by metabolic demand rather than O_2 availability. In support of these findings, a recent study showed that hyperoxia did not enhance muscle VO_2 on-kinetics during submaximal (below ventilatory threshold) knee-extensor exercise (MacDonald *et al.* 2000). They observed that small decreases in leg blood flow with hyperoxia compensated for the increase in CaO_2 at exercise onset. Further studies during heavy

submaximal exercise (70-80% VO_2max) showed that hyperoxia (F_IO_2 = 0.6 and 0.7, respectively) did result in faster VO_2 on-kinetics (Macdonald *et al.* 1997, Pederson 1983).

Only two studies have made direct measurements of O_2 deficit through the measurement of ATP, PCr, and lactate concentrations in muscle biopsies. Linnarson et al. (1974) were the first to investigate the effects of hyperoxic conditions on skeletal muscle metabolism. They found that hyperoxic conditions (P_iO_2=212 mmHg) decreased the O_2 deficit, as shown by less PCr degradation and lactate accumulation, after 4 minutes of submaximal (50-60% VO_2max) exercise. This relationship was not observed during intense exercise (Linnarsson *et al.* 1974). Recent work by Linossier and coworkers seems to contradict these findings. During approximately 5 minutes of maximal cycling exercise (287 ± 12 W), hyperoxic conditions (F_IO_2 = 0.6) induced a strong trend for less PCr degradation (52.7% degradation of PCr compared to 76.4% in control) and a 20% decrease in lactate accumulation with 5 subjects (Linossier *et al.* 2000). They also observed a higher glycogenolytic rate with hyperoxia despite lower lactate accumulation and unchanged pyruvate accumulation suggesting that hyperoxic conditions enhanced the activation of aerobic metabolism. An important difference between these two studies that may account for the differing results is that the study by Linnarson et al. (1974) was carried out under varied atmospheric pressures and inspired oxygen partial pressures. Therefore, these results must be viewed with caution as effects of

absolute pressure can affect performance independent of changes in O_2 pressure (as reviewed by Welch 1982).

The above results support the belief by some researchers that hyperoxic conditions only improve VO_2 on-kinetics during severe exercise (Welch and Pedersen 1981). MacDonald et al. (2000) suggests that during submaximal exercise, O_2 delivery may not be a significant factor in determining the rate of increase in O_2 uptake, possibly due to a lower metabolic demand. Thus, particular attention must be given to the percentage of VO_2max utilized during the exercise protocol with hyperoxia. In addition, responses to hyperoxia have been shown to vary depending on the training status of the individual. Evidence suggests that greater fitness is correlated with greater vulnerability to limited O_2 supply (Roca *et al.* 1992).

VO_2max and Performance

Hyperoxic conditions have had positive effects on both VO_2max and performance. Hyperoxic conditions have resulted in up to a 22% increase in VO_2max (as reviewed by Welch 1982) based on VO_2 measurements at the mouth. More reliable measurements of VO_2max using the Fick equation ($VO_2max = Qmax(CaO_2 - CvO_2)$; where Q = blood flow and $CaO_2 - CvO_2$ = arteriovenous O_2 content difference) have shown less dramatic yet significant increases with a 12.6% increase in whole body VO_2max with $F_IO_2 = 0.5$ (Ekblom *et al.* 1975) and an 8.1% increase in leg VO_2max (Knight et al. 1993).

In agreement with the increases in VO_2max, hyperoxia increased performance time to exhaustion during both submaximal (50% VO_2max) (Linnarsson *et al.* 1974) and maximal (Linossier *et al.* 2000) exercise by up to 45%. This result was also observed during incremental plantar flexion exercise such that exhaustion during hyperoxia occurred at a higher power output compared to normoxia ($F_IO_2 = 0.21$) (Hogan *et al.* 1999). In addition, PCr degradation during steady state exercise was greater in hypoxia and less in hyperoxia (Haseler *et al.* 1999, Hogan *et al.* 1999). Hypoxic conditions (F_IO_2 = 0.10 and 0.07) in dogs showed a significant decrease in muscle VO_2 during steady state electrical stimulation (Hogan *et al.* 1993), providing further support for the influence of O_2 levels. The decrease in muscle VO_2 observed was significantly correlated with changes in [PCr], [ADP], and the ratio [ATP]/[ADP][P_i] such that greater PCr degradation and ADP accumulation were required during hypoxic conditions. These findings suggest that the extent of tissue oxygenation is not only important at levels thought to be rate limiting but may also be important in modulating the level of metabolic controllers to maintain the rate of oxidative phosphorylation.

Estimation of mitochondrial PO_2 (P_mO_2)

The P_mO_2 is determined by the capillary O_2 driving pressure and the local diffusion capacity as well as the rate of O_2 utilization. Investigations into the level of P_mO_2 at which oxidative phosphorylation becomes limited (critical PO_2) have reported PO_2 values of < 1 mmHg (Jobsis 1977, Wilson *et al.* 1979). This result

was confirmed in isolated cardiac myocytes and cell suspensions, which was also consistent with the km for cytochrome oxidase (Richmond *et al.* 1997). Therefore, < 1 mmHg is typically assumed to be the critical PO_2 for skeletal muscle *in vivo*. However, it is uncertain whether values obtained from isolated mitochondria or isolated cells accurately reflect the *in vivo* situation, due to the absence of the normal regulatory mechanisms.

Many of the *in vivo* estimations of P_mO_2 are based on measurements of myoglobin saturation, determined by proton magnetic resonance imaging, which is believed to be a good indicator of the PO_2 to which cytochrome oxidase is exposed (Gayeski *et al.* 1985). Measurements of Mb saturation *in situ* in quick frozen dog muscle found that the km for cytochrome aa_3 was not different than in isolated mitochondria and that maximal oxygen consumption could be maintained at a P_mO_2 of > 0.5 mmHg (Gayeski *et al.* 1987). While this indicates that a very low critical PO_2 is required to maintain mitochondrial respiration, the changes in P_mO_2 in response to exercise have yet to be thoroughly examined. Electrical stimulation of dog muscle (Gayeski *et al.* 1985) and submaximal exercise in humans (Richardson *et al.* 1995) was shown to cause a rapid desaturation of Mb (up to 50% of maximum) within 20 seconds of exercise onset, remaining unchanged despite continued exercise. The finding that cytochrome oxidase does not become O_2 limited until Mb is almost fully desaturated (Connett *et al.* 1985) suggests that O_2 is not limiting during the onset of submaximal exercise. However, these results do support the idea that respiratory rate can be maintained at a reduced P_mO_2 through changes in phosphorylation potential and

mitochondrial redox state. In resting rat skeletal muscle, Richmond et al. (1999) found that an abrupt fall in PO_2 (with reduced blood flow) coincided with a significant rise in NADH fluorescence (corresponding with a critical PO_2 of 2.1mmHg). Therefore, an increase in the $[NADH]/[NAD^+]$ ratio, and likely a reduced phosphorylation potential (decreased $[ATP]/[ADP][P_i]$) can compensate for a reduced P_mO_2 in order to maintain the rate of oxidative phosphorylation (Hogan *et al.* 1992, Wilson *et al.* 1979, 1988). Typically, critical PO_2 values correspond to a PO_2 at which changes in redox state or phosphorylation potential are no longer able to prevent a drop in maximal mitochondrial respiration. However, as PO_2, phosphorylation potential, and mitochondrial redox state all interact to modulate the rate of oxidative phosphorylation, critical PO_2 values observed may be dependent on experimental conditions and moreover, may vary significantly with physiological conditions.

STATEMENT OF THE PROBLEM AND RATIONALE FOR THIS STUDY

The purpose of this study was twofold. Firstly, to determine whether an increased muscle acetylcarnitine store (acetate infusion) would enhance the provision of acetyl-CoA for the TCA cycle at the onset of submaximal exercise and cause a faster activation of oxidative phosphorylation and thus, a decreased reliance on substrate level phosphorylation. Secondly, to determine whether breathing 100% oxygen would enhance the activation of oxidative phosphorylation at the onset of submaximal exercise. Both of these experimental conditions were conducted to target the question of what is limiting aerobic metabolism at the onset of exercise. Muscle biopsies were taken at rest, 30 s, and 120 s for measurement of ATP, PCr, and lactate, which was a direct measurement of the O_2 deficit.

Work to date has shown a decrease in the reliance on substrate level phosphorylation (PCr degradation and anaerobic glycolysis) during exercise onset with DCA. DCA increases the activation of PDH, which leads to an accumulation of acetyl-CoA and acetylcarnitine. Thus, it is not clear whether the sparing of substrate level phosphorylation at the onset of exercise was due to enhanced provision of acetyl-CoA from acetylcarnitine or PDHa. Acetate infusion elevated muscle acetyl-CoA and acetylcarnitine without affecting the activation of PDH. This allowed us to isolate the source of enhanced acetyl-CoA provision at exercise onset and determine a site of metabolic inertia.

There is debate regarding whether O_2 is limiting at the onset of exercise. Hyperoxic conditions have been shown to enhance the VO_2 on-kinetics during intense (> 70% VO_2max) submaximal exercise but not during moderate (≤ 65% VO_2max) submaximal exercise. Previous work from our laboratories has shown a 35% reduction in the reliance on substrate level phosphorylation during the onset of exercise at 65% VO_2max with DCA. Thus, enhanced substrate availability was not able to completely eliminate the reliance on substrate level phosphorylation suggesting the potential that oxygen is also limiting at exercise onset. To further investigate this possibility, this study was also conducted at an exercise intensity of 65% VO_2max.

HYPOTHESES

1. Acetate infusion would not affect the reliance on substrate level phosphorylation (PCr degradation and lactate accumulation) compared to control during the onset of submaximal (65% VO_2max) cycling exercise.
2. Breathing 100% oxygen (hyperoxic conditions) would not affect PCr degradation or lactate accumulation compared to control at the onset of submaximal (65% VO_2max) cycling exercise.

METHODS

Subjects: Eight healthy, moderately active male subjects volunteered to participate in this study. Their mean (± SE) age, height, weight, and VO_2max were 22.9 ± 1.0 yrs, 180.0 ± 2.7 cm, 77.0 ± 2.8 kg, and 54.7 ± 3.0 $ml \cdot kg^{-1} \cdot min^{-1}$, respectively. Written informed consent was obtained from each subject subsequent to a thorough explanation of the study protocol and the associated risks. The study was approved by the ethics committee of the University of Guelph and McMaster University.

Experimental Infusions: Sodium acetate (4M) was obtained from the McMaster University Medical Center and administered to the subjects intravenously in a 500 ml saline solution at a dose of 4 mmol/kg body weight. The acetate solution was infused over 1 hr immediately prior to exercise. For control and hyperoxic trials, 500 ml of saline was infused over the same time course.

Pre-experimental protocol: Subjects underwent a continuous incremental exercise test on a bicycle ergometer to determine VO_2max. This was determined by analyzing expired breaths (every 20 seconds) for gas concentrations (O_2 and CO_2) and volume using a metabolic cart (Quinton Q-Plex 1, Quinton Instruments, Seattle, WA). From this test, power outputs eliciting 65% of the measured VO_2max were calculated. On an ensuing day, subjects returned for a 5 min practice ride to validate the power output eliciting 65% of their VO_2max. Before cycling, subjects were asked to breathe room air through a mouthpiece for 20 min to simulate testing conditions. Subjects pedaled between 90-100 rpm during

the VO_2max test and practice ride and maintained this cadence during all three experimental trials. The mean (± S.E.) absolute power output for the trials was 186.3 ± 8.7 W.

Experimental protocol: On three separate experimental days (each separated by one week), subjects arrived at the laboratory at the same time of day having eaten a carbohydrate rich meal 2 – 4 hrs before the trial. Subjects were asked to replicate their diet each week for the three meals consumed prior to testing and to refrain from prolonged or intense physical activity for 24 hrs before each trial. Caffeine consumption was maintained at each subjects' normal daily intake.

The three experimental conditions were control (breathing room air and saline infusion), acetate (breathing room air and acetate infusion), and hyperoxia (breathing 100% oxygen and saline infusion). The order of the trials was randomly assigned and the subjects were blind to which treatment they received. On each test day, a catheter was inserted into a forearm vein of the subject and 500 ml of either an acetate or saline solution was infused over the course of 1 hr. During this hour the subjects rested quietly on a bed. At 30 min into the infusion, one leg was prepared for needle biopsy (Bergstrom 1975) with three incisions made through the skin superficial to the vastus lateralis muscle under local anesthesia (2% lidocaine without epinephrine). The contralateral leg was prepared for biopsy in trial 2 and the initial biopsied leg repeated in trial 3. During the final 20 min of this hour, the subjects breathed either room air (21% oxygen) or hyperoxic air (100% oxygen). At the completion of 1 hr, the catheter was

removed and a resting biopsy was taken. Subjects then moved to an electrically braked cycle ergometer (Excalibur, Quinton Instruments, Seattle, WA) while continuing to breath the specified gas mixture and began pedaling at the prescribed power output for 120 s. Exercise biopsies were taken at 30 s and 120 s while the subject remained on the cycle ergometer. The interval time between stopping at 30 s and restarting cycling for the final 90 s was 25 – 35 s in all trials. Muscle biopsies were immediately frozen in liquid N_2 (3-5 s after needle insertion) and removed from the needle while frozen. Muscle samples were stored in liquid N_2 until analysis.

Analysis: The muscle sample remained submerged in liquid N_2 while a small piece of frozen wet muscle (8-15 mg) was removed for the determination of PDH activity (PDHa), as described by Constantin-Teodosiu *et al.* (1991b) and modified by Putman *et al.* (1993). Total creatine concentrations were measured for each muscle homogenate and PDHa values were corrected to the highest total creatine value among all of the biopsies within each subject. The remainder of the biopsy sample was freeze-dried, dissected of all visible blood, connective tissue, and fat, and powdered for subsequent analysis.

One aliquot of freeze-dried muscle (8-10 mg) was extracted with 0.5 M perchloric acid (PCA) (containing 1 mM EDTA) and neutralized with 2.2 M $KHCO_3$. This extract was used for the determination of adenosine-5'-triphosphate (ATP), phosphocreatine (PCr), creatine, lactate, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), glycerol-3-phosphate (G-3-P), glucose-1-phosphate (G-1-P), and glucose by enzymatic spectrophotometric

assays (Bergmeyer 1974, Harris *et al.* 1974). Pyruvate was analyzed on this extract using a fluorometric assay (Passoneau and Lowry 1993). Acetyl-CoA and acetylcarnitine concentrations were determined by radiometric measures (Cederblad *et al.* 1990). Muscle glycogen content was measured in duplicate on a second aliquot of freeze-dried muscle (4 – 6 mg) from resting samples (Harris *et al.* 1974). Metabolite concentrations were also corrected for the highest total creatine measured in the 9 biopsies from each subject.

Calculations: Free ADP and AMP concentrations were calculated by assuming equilibrium of the creatine kinase and adenylate kinase reactions as previously described (Dudley *et al.* 1987). Free ADP was calculated using the measured ATP, Cr, and PCr values, an estimated H^+ concentration, and the creatine kinase equilibrium constant of 1.66×10^9. The H^+ concentration was estimated from the measured lactate and pyruvate content using the regression equation described by Sahlin *et al.* (Sahlin *et al.* 1976). Free AMP was calculated from the estimated free ADP and measured ATP content using the adenylate kinase equilibrium constant of 1.05. Free P_i was calculated by adding the estimated resting free phosphate of 10.8 mmol/kg dry weight (Dudley *et al.* 1987) to the difference in PCr content (Δ PCr) minus the accumulation of the glycolytic intermediates G-6-P and G-3-P between rest and each exercise time point.

Anaerobic energy yield (mmol ATP/kg dry muscle) was determined for each treatment at 30 s and 120 s by adding the PCr utilization plus 1.5 times the lactate accumulation.

Statistics: All data are presented as means ± S.E. For all metabolite contents, except glycogen, a 2-way ANOVA (time x trial) with repeated measures was used to test for significance. Glycogen and anaerobic energy yield were analyzed using a 1-way ANOVA with repeated measures. Results were considered significant at $p < 0.05$ and a Tukey post hoc test was used to determine where the significant differences occurred.

RESULTS

Power Output and VO_2. During each trial subjects rode at a specified power output ranging from 155 – 220 W, corresponding to an average VO_2 of 36.5 ± 1.6 ml· kg^{-1}· min^{-1} or $64.8 \pm 0.9\%$ VO_2max. Measurements of VO_2 during the practice ride showed that all subjects reached a steady state VO_2 by 100 - 120 s of exercise.

PDHa. There was no significant difference in PDHa between CON, ACE, and HYP at any time point (Figure 6). All treatments showed a significant increase in PDHa over time with values at 120 s ranging from $2.14 \pm 0.21 - 2.50 \pm 0.19$ mmol/kg wet weight/min.

Resting muscle metabolites. There was no significant difference in muscle ATP, PCr, lactate, and glycogen contents between CON, ACE, and HYP trials at rest (Table 1). However, ACE resulted in more than a two-fold increase in acetyl-CoA and a three-fold increase in acetylcarnitine compared to CON and HYP (Table 1).

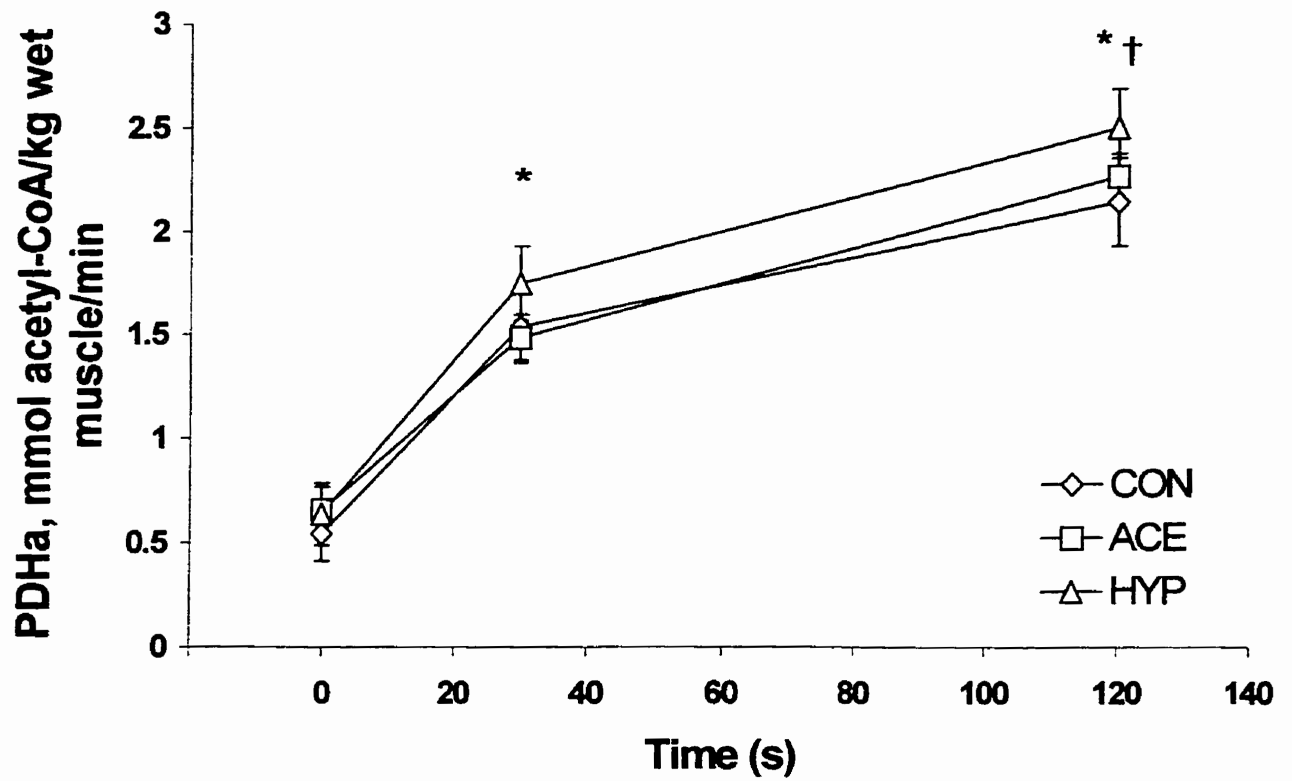


Figure 6. Pyruvate dehydrogenase activation during control (CON), acetate (ACE), and hyperoxia (HYP) trials. * Significantly different than rest for all trials. † Significantly different than 30 s for all trials.

Table 1. Resting muscle metabolite concentrations. All values are mean ± SE. Units are mmol/kg dry muscle except for acetyl-CoA (μmol/kg dry muscle).

Metabolite	CON	ACE	HYP
ATP	23.2 ± 1.0	23.6 ± 0.7	25.0 ± 0.7
PCr	81.0 ± 2.2	83.0 ± 2.9	81.8 ± 2.4
Lactate	3.91 ± 0.66	6.61 ± 1.44	2.47 ± 0.40
Glycogen	404.5 ± 21.8	466.4 ± 55.3	404.3 ± 16.0
Acetyl-CoA	8.89 ± 2.37	22.88 ± 2.75 +	10.47 ± 1.82
Acetylcarnitine	3.45 ± 1.31	11.03 ± 1.15 +	3.99 ± 1.19

+ Significantly different than CON and HYP.

Muscle metabolites during exercise. There was no significant difference in ATP content between trials or during exercise (Table 2). Levels of free ADP, free AMP, and free P_i were not significantly different between trials, however, all trials showed a significant increase over time (Table 2).

PCr content significantly decreased by 30 s of exercise in all trials with no difference between trials (Figure 7). A further decrease in PCr at 120 s occurred in both CON and ACE trials but not in HYP such that PCr degradation in HYP was less than ACE (p=0.05). There was a significant increase in lactate accumulation by 30 s in all trials with no significant difference between trials (Figure 8). A further increase in lactate occurred at 120 s in both CON and ACE but not in HYP however, the difference between trials at 120 s was not significant ($p > 0.07$). The lack of significant differences in PCr and lactate between trials resulted in no difference in anaerobic ATP provision between trials during 30 s of exercise (Figure 9) or from 30-120 s of exercise (CON: 40.27 ± 9.79; ACE: 45.92 ± 11.78; HYP: 24.26 ± 7.38 mmol/kg dry muscle).

Pyruvate content significantly increased during exercise in all trials (Figure 10). By 120 s, pyruvate was significantly greater in ACE than HYP. Glucose and F-6-P showed no changes with exercise or between trials and G-6-P increased over time in all trials (Table 2).

Acetyl-CoA content remained significantly elevated in ACE at 30 s compared to CON and HYP and ACE showed no change in acetyl-CoA content over time (Figure 11A). However, both CON and HYP showed a significant increase in acetyl-CoA from 30 s to 120 s such that there was no difference

between trials at 120 s. Acetylcarnitine concentration was significantly elevated in ACE at all time points compared to CON and HYP and showed no change over time (Figure11B). Acetylcarnitine content increased in CON and HYP over time but remained significantly less than ACE.

Table 2. Muscle metabolite concentrations at rest and at 30 and 120 s of cycling during CON, ACE, and HYP. All values are mean ± SE. Units are mmol/kg dry muscle except for free ADP and AMP (μmol/kg dry muscle).

Metabolite	Time (s)	CON	ACE	HYP
ATP	0	23.2 ± 1.0	23.6 ± 0.7	25.0 ± 0.7
	30	24.4 ± 1.1	23.4 ± 0.9	25.7 ±1.1
	120	23.0 ± 1.4	23.6 ± 0.8	26.5 ± 0.9
free ADP	0	77.9 ± 5.0	72.2 ± 5.3	84.4 ± 6.2
	30	148.4 ± 13.2	150.2 ± 18.0	182.5 ± 14.5*
	120	234.6 ± 60.6 *	259.3 ± 42.2 * †	240.4 ± 44.4*
free AMP	0	0.25 ± 0.02	0.21 ± 0.03	0.28 ± 0.04
	30	0.89 ± 0.15	1.01 ± 0.26	1.30 ± 0.20
	120	3.10 ± 1.71 *	3.35 ± 1.12 *	2.41 ± 0.88
free P_i	30	28.1 ± 2.5	34.0 ± 4.5	34.5 ± 3.8
	120	42.1 ± 6.3 †	51.4 ± 6.1 †	41.8 ± 5.1
Glucose	0	2.21 ± 0.33	3.40 ± 1.05	2.67 ± 0.73
	30	4.90 ± 1.05	5.71 ± 1.60	4.94 ± 1.53
	120	5.20 ± 0.86	6.00 ± 1.23	5.19 ± 0.53
G-6-P	0	0.19 ± 0.04	0.58 ± 0.10	0.35 ± 0.17
	30	1.39 ± 0.47 *	1.60 ± 0.66 *	1.27 ± 0.18
	120	1.73 ± 0.44 *	2.04 ± 0.43 *	1.49 ± 0.27 *
F-6-P	0	0.03 ± 0.02	0.20 ± 0.10	0.10 ± 0.04
	30	0.19 ± 0.08	0.32 ± 0.09	0.22 ± 0.10
	120	0.27 ± 0.07 *	0.27 ± 0.07	0.24 ± 0.04

* Significantly different than rest for the same trial. † Significantly different than 30 s for the same trial.

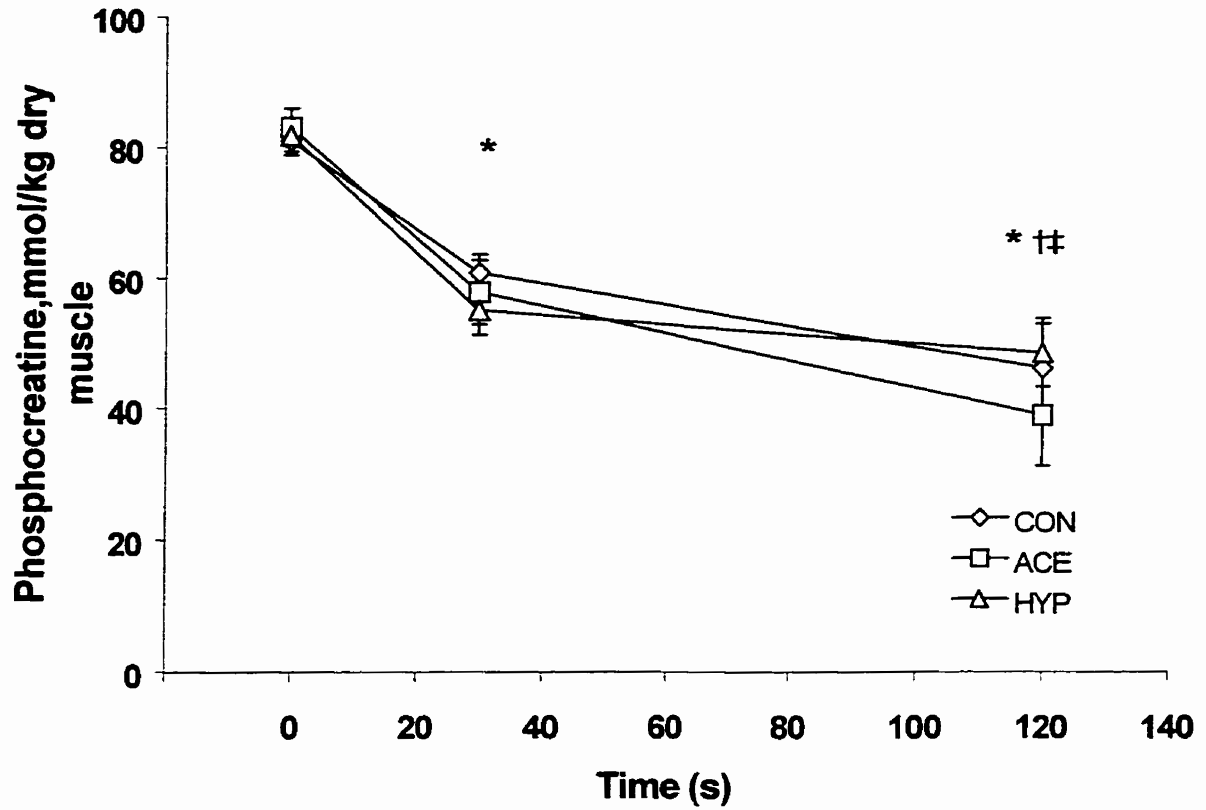


Figure 7. Phosphocreatine degradation during control (CON), acetate (ACE), and hyperoxia (HYP) trials. * Significantly different than rest for all trials. † Significantly different than 30 s for CON and ACE trials. ‡ HYP significantly different than ACE.

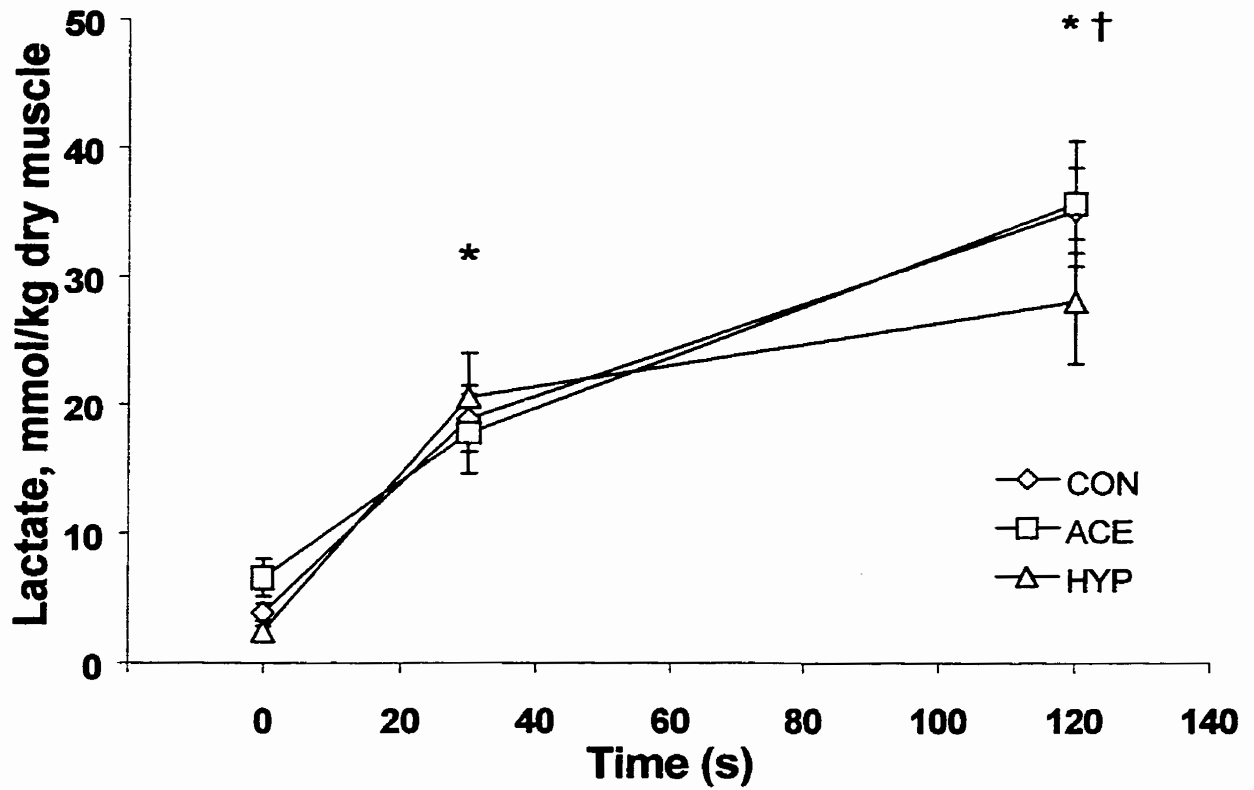


Figure 8. Lactate accumulation during control (CON), acetate (ACE), and hyperoxia (HYP) trials. * Significantly different than rest for all trials. † Significantly different than 30 s for CON and ACE.

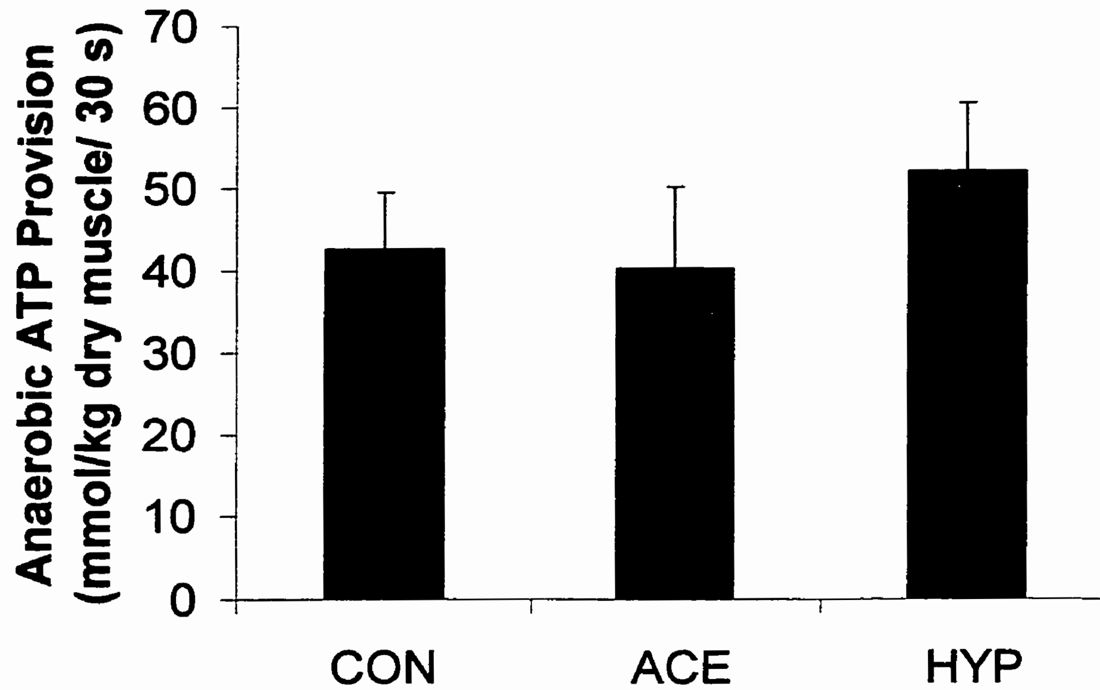


Figure 9. Calculated anaerobic ATP provision (from PCr degradation and anaerobic glycolysis) during 30 s of exercise in control (CON), acetate (ACE), and hyperoxia (HYP) trials. There was no significant difference between trials.

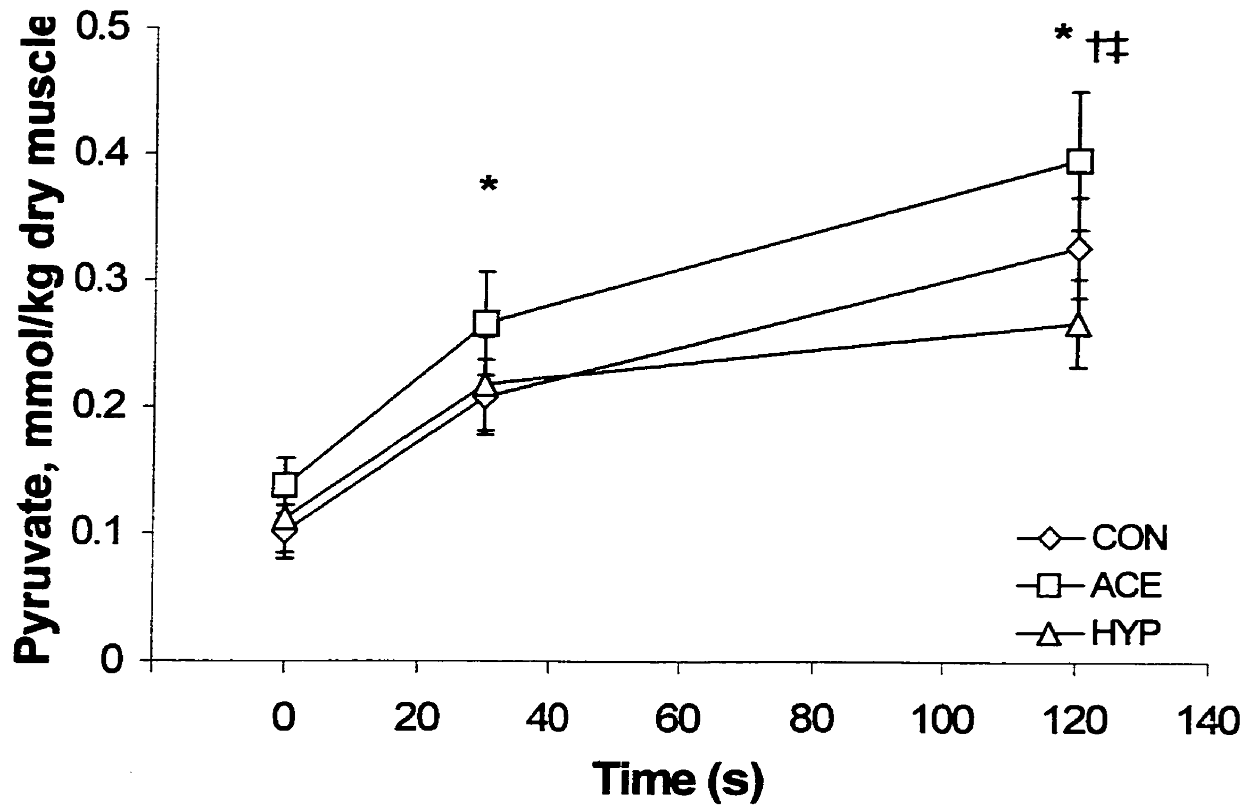


Figure 10. Pyruvate accumulation during control (CON), acetate (ACE), and hyperoxia (HYP) trials. * Significantly different than rest for all trials. † Significantly different than 30 s for CON and ACE. ‡ ACE significantly greater than HYP.

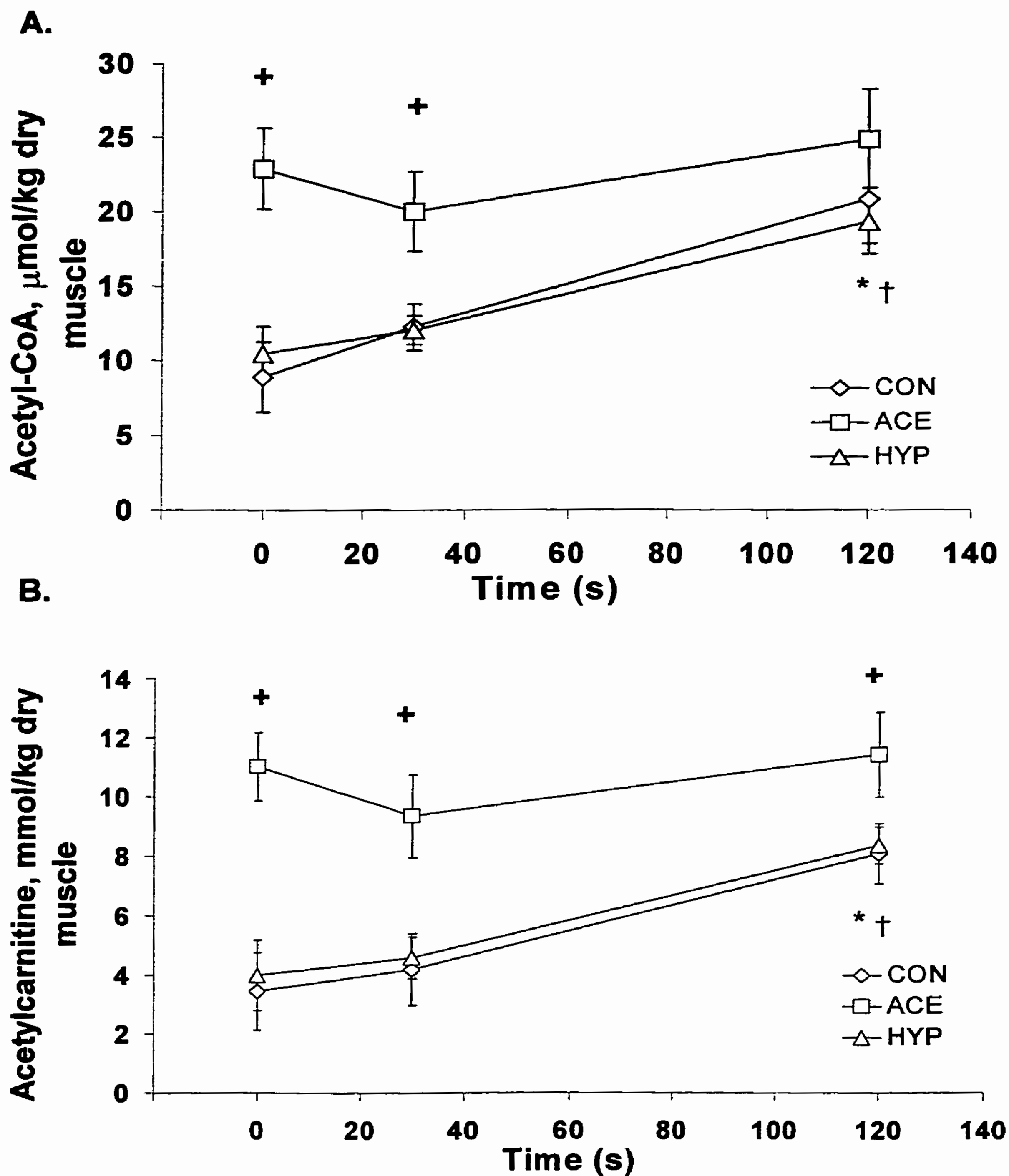


Figure 11. A) Acetyl-CoA accumulation and B) acetylcarnitine accumulation during control (CON), acetate (ACE), and hyperoxia (HYP) trials. + Significantly different than CON and HYP. * Significantly different than rest for CON and HYP. † Significantly different than 30 s for CON and HYP.

Discussion

It is well known that during the transition from rest to exercise there is a lag in the activation of oxidative phosphorylation, such that ATP production is partially supplemented by PCr hydrolysis and anaerobic glycolysis (substrate level phosphorylation). The present study examined whether increased substrate provision of acetyl-CoA from acetylcarnitine or increased inspired oxygen (100% O_2) would enhance the activation of oxidative phosphorylation and thus, reduce the reliance on substrate level phosphorylation during the transition from rest to 65% VO_2max.

Acetate

Acetate infusion (ACE) significantly elevated muscle acetyl-CoA and acetylcarnitine levels at rest compared to control as previously observed (Howlett *et al.* 1999b, Putman *et al.* 1995) and thus, achieved the aim of setting up the potential for enhanced substrate provision at exercise onset. This is consistent with the fact that acetate readily diffuses across the muscle and mitochondrial membranes and is directly converted to acetyl-CoA via acetyl-CoA synthetase (Vinay *et al.* 1987). Acetyl-CoA is either oxidized or converted to acetylcarnitine by the near-equilibrium reaction catalyzed by carnitine acetyltransferase. Muscle acetyl-CoA and acetylcarnitine levels increased as a function of time in the CON trial but remained elevated in the ACE trial. Despite high levels of acetyl-CoA at rest in ACE there was no difference in resting PDH activity between CON and ACE. While previous results have reported an inhibition of PDH at rest due to an

elevated [acetyl-CoA]/[CoASH] ratio (Putman *et al.* 1995) the results have been equivocal (Howlett *et al.* 1999a).

The lack of acetyl group accumulation observed in ACE compared to CON and the similar activities of PDH between conditions suggests that a greater amount of acetyl-CoA could have been oxidized in the TCA cycle in ACE. However, despite the potential for enhanced provision of substrate for the TCA cycle at exercise onset in ACE there was no significant difference in PCr degradation or lactate accumulation compared to CON at 30 or 120 s. Consequently, there was no difference in the anaerobic ATP provision between ACE and CON at any time point. These findings are consistent with previous work which showed no difference in cellular energetic state with acetate infusion compared to control following 5 minutes of cycling at 40% VO_2max and 15 minutes at 80% VO_2max (Putman *et al.* 1995). The results suggest that acetylcarnitine does not provide a significant source of acetyl-CoA during the onset of submaximal exercise for enhanced activation of oxidative phosphorylation.

This is in contrast to work by Timmons and coworkers who argue that the muscle acetylcarnitine store is important for increased oxidative substrate availability at exercise onset. This was supported by observed decreases in acetylcarnitine of 2-5 mmol/kg dry muscle in contracting ischemic dog muscle (Timmons *et al.* 1996, 1997) and 1 mmol/kg dry muscle during submaximal ischemic human knee extensor exercise (Timmons *et al.* 1998b) from elevated levels with DCA administration. The decreases in acetylcarnitine content were

accompanied by significantly less PCr degradation and lactate accumulation compared to CON (Timmons *et al.* 1996, 1997, 1998b). In addition, the aerobic ATP yield from the decrease in acetylcarnitine in ischemic dog muscle corresponded with the amount of ATP spared from substrate level phosphorylation (Timmons *et al.* 1997). Elevated levels of acetyl-CoA with ACE or DCA are too small to account for the sparing of non-oxidative ATP production. However, utilization of 1 mmol acetylcarnitine, via conversion to acetyl-CoA, can provide 12 mmol of ATP via oxidative metabolism. As 3 mmol of ATP is produced by anaerobic glycolysis from glycogen this would result in 4 mmol of glycogen spared and an 8 mmol decrease in lactate production. Thus, it is conceivable that small decreases in acetylcarnitine during exercise in ischemic dog and ischemic human muscle significantly contributed to the provision of acetyl-CoA and oxidative ATP production. It is arguable whether the assay techniques employed in the present study are sensitive enough to pick up small changes (1 mmol/ kg dry muscle) in acetylcarnitine. Regardless of this, the lack of sparing of substrate level phosphorylation with ACE indicates that acetylcarnitine was not a significant source of acetyl-CoA during exercise onset in this study.

Pyruvate dehydrogenase (PDH) activity controls the amount of carbohydrate derived substrate that can be oxidized during the transition from rest to exercise. Both muscle pyruvate and Ca^{2+} content are important regulators of PDH activation. Therefore, an important methodological difference between work by Timmons and coworkers and the present study is the use of DCA rather

than acetate to elevate acetyl-CoA and acetylcarnitine levels. DCA increases the activation of PDH in resting skeletal muscle (Stacpoole 1989). This results in the potential for maximal flux of pyruvate through PDH at exercise onset as well as acetylation of CoA and carnitine pools at rest (Timmons *et al.* 1997). Thus, DCA administration causes maximal activation of PDH whereas ACE does not influence the activation of PDH, as observed in this study. This suggests the possibility that the sparing of substrate level phosphorylation observed with DCA administration could also be a result of enhanced substrate provision from elevated PDHa at exercise onset.

DCA administration during submaximal exercise (45% and 65% VO_2max) with normal blood flow has been shown to cause a significant decrease in the reliance on substrate level phosphorylation at exercise onset (Howlett *et al.* 1999a, Timmons *et al.* 1998a). Enhanced activation of PDH and increased levels of acetyl-CoA and acetylcarnitine decreased PCr degradation during the initial 2 minutes of exercise resulting in less P_i accumulation and thus, a decrease in glycogenolysis (Howlett *et al.* 1999a). This, coupled with the decrease in lactate production, indicated that there was a better match between the rate of pyruvate production and the rate of pyruvate oxidation. It was inferred by the lack of observed decrease in acetylcarnitine that elevated PDHa was the primary source of the extra acetyl-CoA in the TCA cycle during exercise onset. A study by Timmons et al. (1998a) involving 8 minutes of knee extensor exercise with normal blood flow and DCA also showed no decrease in muscle acetylcarnitine levels despite enhanced activation of oxidative phosphorylation.

The results from Howlett et al. (1999a) showed that by pre-loading the muscle with acetyl-CoA and acetylcarnitine at rest from DCA administration, a greater percentage of flux through PDH was directed towards oxidative phosphorylation compared to CON, in which much of the PDH flux was directed towards increasing the acetylcarnitine store. Despite elevated acetyl-CoA and acetylcarnitine stores with ACE, our results showed no difference in the amount of acetyl-CoA diverted to oxidative phosphorylation. The insignificant decrease in acetylcarnitine (~ 1.65 mmol/kg dry muscle) observed in this study following 30 s of exercise corresponds with values reported in humans by Timmons et al. (1998a). This indicates that the slight drop in muscle acetylcarnitine content was likely not responsible for the decreased reliance on substrate level phosphorylation. In addition, the similar rates of PDH activation between ACE and CON in our study compared to enhanced activation of PDH with DCA suggests that PDH is an important regulatory site for controlling the amount of acetyl-CoA directed to oxidative phosphorylation at the onset of exercise.

However, it is difficult to determine the importance of acetylcarnitine in substrate provision due to the inability to isolate mitochondrial and cytosolic concentrations of acetylcarnitine. The accumulation of acetylcarnitine is a result of the important role of carnitine in buffering the excess production of acetyl groups during exercise onset in order to preserve the mitochondrial pool of CoASH. This is crucial for maintaining the catalytic activity of PDH and 2-oxoglutarate dehydrogenase, and other mitochondrial reactions using CoASH. As a result, acetylcarnitine is transported out of the mitochondria by a

mitochondrial transporter but the extent of acetylcarnitine efflux and thus, the mitochondrial concentration of acetylcarnitine remains unknown. The mitochondrial acetylcarnitine concentration would have a great impact on the near-equilibrium reaction, carnitine acetyltransferase. A large efflux of acetylcarnitine out of the mitochondria may cause the carnitine acetyltransferase reaction to more successfully compete for acetyl-CoA, which would explain the obligatory increase in acetylcarnitine during the onset of exercise.

A comparison of the observed changes with DCA during the exercise transition in ischemia vs. normal blood flow seems to indicate that the reliance on acetylcarnitine for acetyl-CoA provision at exercise onset is only observed during ischemic exercise (Timmons *et al.* 1996, 1997, 1998b). However, recent work showed no change in acetylcarnitine content with DCA during the onset of 15 minutes of submaximal exercise (55% VO_2max) under hypoxic conditions, despite a significant decrease in the reliance on substrate level phosphorylation (Parolin *et al.* 2000b). It has been previously shown that hypoxic conditions are associated with slowed VO_2 on-kinetics (Hughson and Kowalchuk 1995) and increased lactate production (Katz & Sahlin, 1990). In addition, hypoxic conditions have been shown to delay the activation of PDH at exercise onset (Parolin *et al.* 2000a). While increased PDH activation with DCA contributes to the reduced reliance on substrate level phosphorylation at the onset of hypoxic exercise it is possible that extra substrate provision from acetylcarnitine is necessary to drive oxidative phosphorylation in the face of an O_2 limitation. An increase in the oxidation of acetyl-CoA in the TCA cycle would increase the

provision of NADH for the electron transport chain and reduce the requirement for ADP and Pi to drive oxidative phosphorylation at the same rate as that in the CON condition. This mechanism has also been proposed for the decreased PCr degradation and lactate accumulation at exercise onset during the situation of enhanced FFA availability induced by Intralipid infusion (Dyck *et al.* 1993, 1996) and caffeine (Chesley *et al.* 1998).

Based on the findings in this study, coupled with the results from DCA administration during submaximal exercise with normal blood flow (Howlett *et al.* 1999a, Timmons *et al.* 1998a), it seems clear that substrate availability from PDH is a site of metabolic inertia at the onset of submaximal exercise. The fact that DCA administration did not totally alleviate reliance on substrate level phosphorylation suggests that PDH activation and thus, substrate provision, is not the sole factor limiting the activation of oxidative phosphorylation. Both the lack of a significant decrease in elevated acetyl-CoA levels in ACE and the accumulation of acetyl-CoA in CON suggest that there is an inability to use the available store of acetyl-CoA at exercise onset. This could be due to a metabolic inertia of the TCA cycle or an O_2 limitation at the mitochondria.

Hyperoxia

The results show that breathing 100% oxygen (HYP) prior to and during 120 s of submaximal (65% VO_2max) exercise did not enhance activation of oxidative phosphorylation, as indicated by comparable PCr degradation and anaerobic glycolysis in HYP and CON. This was further supported by an inability to better utilize the available acetylated compounds, with similar accumulation of

acetyl-CoA and acetylcarnitine in HYP and CON. The amount of oxygen delivered to the mitochondria is determined by convective O_2 delivery, including arterial oxygen content (CaO_2) and blood flow, and diffusive O_2 delivery, influenced by the PO_2 gradient from the red blood cell to the mitochondria. Breathing 100% oxygen increases CaO_2 by approximately 8-10% and increases the PO_2 of arterial blood by approximately 6-fold (as reviewed by Wagner 1996). There is debate regarding whether hyperoxic conditions actually increase the convective delivery of oxygen to the muscle. Some researchers argue that decreases in blood flow accompanying hyperoxic breathing offset the 8-10% increase in CaO_2 such that convective O_2 delivery in HYP is not different than normoxic conditions (21% O_2) (Welch *et al.* 1977). As arterial blood gases and blood flow were not measured in the present study it is unknown whether convective O_2 delivery was increased during the onset of exercise. However, it is widely accepted that hyperoxic conditions increase the arterial PO_2 to approximately 600 Torr compared to 100 Torr in normoxia (Cardus *et al.* 1998, Knight *et al.* 1993) suggesting that a large increase in the diffusive gradient was achieved during this study.

The lack of sparing of substrate level phosphorylation during submaximal (65% VO_2max) exercise with HYP in this study concurs with previous indirect measurements of O_2 deficit (VO_2 on-kinetics) during moderate submaximal exercise (Hughson and Kowalchuk 1995). During more intense submaximal exercise (70-80% VO_2 max), hyperoxia did cause enhanced activation of oxidative phosphorylation as shown by faster VO_2 on-kinetics (Macdonald *et al.*

1997, Pederson 1983). This was supported by a recent study with hyperoxic conditions (F_IO_2 = 0.6) during 5 minutes of maximal cycling which showed a trend for less PCr degradation (52.7% degradation of PCr compared to 76.4% in control) and a 20% decrease in lactate accumulation (Linossier *et al.* 2000). The small number of subjects (n=5) in this study may have limited the attainment of a significant decrease in PCr degradation with hyperoxia. In addition, it is possible that significant changes in PCr content occurred during the initial 30-60 s of exercise and were less pronounced at 5 minutes of exercise. Consistent with these results, some researchers believe that hyperoxic conditions only improve VO_2 on-kinetics during more intense exercise (Welch and Pedersen 1981).

However, our results showed a trend for less PCr degradation and lactate accumulation as well as less pyruvate accumulation following 120 s of cycling. This may suggest a slight increase in the rate of oxidative phosphorylation by 120 s of exercise compared to control as a result of enhanced O_2 availability. Measurements taken beyond 120 s of exercise at 65% VO_2max may show an improved energetic state with hyperoxia. Thus, it is possible that a metabolic inertia initially limits the activation of oxidative-phosphorylation at exercise onset such that enhanced O_2 availability is only beneficial once the metabolic inertia has been eliminated.

The difficulties in measuring the mitochondrial PO_2 and diffusive O_2 conductance make it difficult to elucidate the mechanisms for increased VO_2 on-kinetics during exercise at 70-80% VO_2max but not at exercise intensities <65% VO_2max with HYP. In dogs, studies directly altering the hemoglobin-oxygen

dissociation reaction to favour an increased diffusive O_2 gradient showed no change in the VO_2 on-kinetics suggesting that a diffusion limitation exists at exercise onset in dogs in spite of an increased O_2 gradient from capillary to mitochondria (Grassi *et al.* 1998b). This is in contrast to exercise during steady state conditions in which hyperoxia does increase O_2 delivery and O_2 uptake, either by enhanced convective or diffusive O_2 transport, as indicated by increases in VO_2max (Ekblom *et al.* 1975, Knight *et al.* 1993) and performance (Hogan *et al.* 1999, Linnarsson *et al.* 1974, Linossier *et al.* 2000). This may indicate that the amount of oxygen delivered during the onset of submaximal exercise does not influence the activation of oxidative phosphorylation because of the existence of a metabolic inertia. This would support our finding that HYP was not able to blunt the increase in acetyl-CoA and acetylcarnitine at exercise onset and enhance the activation of oxidative phosphorylation. As previously discussed, our results in combination with the effects of DCA (Howlett *et al.* 1999a,Timmons *et al.* 1998a) indicate that substrate availability from PDHa significantly limits the activation of oxidative phosphorylation.

A lag in the increase in TCA cycle flux at exercise onset may be another potential site of metabolic inertia. While citrate synthase is not considered to be a near-equilibrium enzyme because of allosteric regulation by ADP, flux through citrate synthase was shown to be highly correlated with the concentration of its substrate oxaloacetate (Gibala *et al.* 1997b). Resting concentrations of oxaloacetate are very low and increase rapidly (up to 100%) at the onset of exercise (Gibala *et al.* 1997b). Due to the limitations of compartmentalization it is

uncertain how much of the increase in oxaloacetate concentration is mitochondrial. Oxaloacetate also participates in the transport of reducing equivalents from the cytosol to the mitochondria in the malate/aspartate shuttle, a process which would also increase during exercise.

Conclusion:

Elevated levels of acetylcarnitine at the onset of exercise at 65% VO_2max did not enhance the provision of acetyl-CoA for the TCA cycle for faster activation of oxidative phosphorylation. This confirms previous results that substrate provision from flux through PDH, and not acetylcarnitine, is a site of metabolic inertia, partially accounting for the lag in oxidative phosphorylation (Howlett *et al.* 1999a).

Hyperoxic conditions (100% oxygen) did not enhance the activation of oxidative phosphorylation at the onset of exercise at 65% VO_2max suggesting that oxygen availability is not limiting at the onset of submaximal exercise.

Future Directions:

Our results, in combination with previous results with DCA (Howlett *et al.* 1999a, Timmons *et al.* 1998b) indicate that substrate provision from flux through PDH limits the activation of oxidative phosphorylation during the onset of exercise with normal blood flow. During ischemic conditions with DCA, significant decreases in acetylcarnitine have been observed in both contracting dog muscle (Timmons *et al.* 1996, 1997) and human skeletal muscle (Timmons

et al. 1998a), implying that the provision of acetyl-CoA from acetylcarnitine was the cause of the decreased reliance on substrate level phosphorylation. Thus, a future study to clarify whether the decreased reliance on substrate level phosphorylation in ischemic conditions was due to elevated PDHa or acetylcarnitine would be to infuse acetate during exercise with ischemic conditions in order to enhance acetylcarnitine stores without activating PDH.

While the hyperoxic literature appears to indicate that oxygen is limiting only at the onset of more intense exercise ($\geq$ 70% VO_2max) and not at moderate submaximal intensities ($\leq$ 65% VO_2max), relatively few studies have been done and have largely relied on measurements of VO_2 on-kinetics (no muscle measurements). In addition, there have been suggestions that the effect of hyperoxia or hypoxia may be dependent on the training level of the individual (Roca *et al.* 1992). Thus, an exercise study directly measuring the O_2 deficit (muscle ATP, PCr, and lactate concentrations) during the onset of exercise at 65% and 100% VO_2max both before and after training would directly test both of these hypotheses.

The delayed activation of PDH at the onset of exercise and thus, a delayed provision of NADH for oxidative phosphorylation, may limit the beneficial effect of enhanced oxygen availability at exercise onset. Therefore, hyperoxic conditions may only increase the rate of activation of oxidative phosphorylation once the metabolic inertia is eliminated. A future study to test this hypothesis would be to conduct an exercise trial with the conditions of hyperoxia and DCA.

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APPENDICES

ATP

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	23.06	24.60	23.05	25.34	24.77	24.66	25.13	23.55	23.81
DK	25.99	24.79	25.10	24.37	25.25	21.57	29.06	24.92	25.10
MR	23.97	24.05	28.48	25.81	26.95	26.96	25.53	26.58	25.51
LF	21.41	26.4	22.96	24.50	21.36	23.44	23.84	27.98	26.54
SP	23.19	24.85	25.48	23.65	19.12	19.63	25.40	24.01	26.53
GB	23.78	24.17	21.69	23.34	22.39	24.60	23.39	22.63	25.66
GS	17.70	18.55	16.58	22.12	23.20	23.53	24.13	31.46	27.81
DB	26.40	27.76	20.34	20.13	23.92	24.44	23.30	24.28	31.27
Mean	23.19	24.41	22.96	23.66	23.37	23.60	24.97	25.68	26.53
S.E.	1.03	1.01	1.36	0.69	0.92	0.83	0.71	1.09	0.85

PCr

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	79.21	57.53	41.88	84.49	72.22	30.64	75.65	43.52	30.65
DK	75.78	50.07	16.87	76.43	51.53	19.64	72.77	58.03	50.53
MR	79.37	70.79	75.71	85.19	76.51	76.29	84.04	73.99	66.96
LF	76.90	62.24	45.97	73.95	60.80	38.54	83.34	47.00	43.86
SP	80.98	51.29	54.51	83.01	47.58	39.37	83.01	59.31	49.25
GB	80.88	67.46	37.43	76.89	39.66	15.35	77.50	50.69	54.19
GS	94.71	65.12	61.44	98.21	65.65	59.47	93.56	60.39	63.18
DB	80.01	61.87	33.95	85.91	48.01	30.75	84.23	46.83	28.14
Mean	80.98	60.80	45.97	83.01	57.74	38.75	81.76	54.97	48.35
S.E.	2.21	2.79	6.81	2.89	4.94	7.66	2.43	3.77	5.24

Cr

Subjects	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	43.86	77.75	81.19	38.57	50.84	92.43	47.41	79.54	92.41
DK	34.93	60.64	93.84	34.28	59.45	91.06	37.94	52.67	58.52
MR	47.31	55.89	50.97	41.49	50.17	50.40	42.64	52.69	59.72
LF	38.64	53.30	77.24	41.58	54.73	76.99	32.20	68.54	71.68
SP	40.52	76.25	73.02	38.48	50.65	88.17	44.52	68.23	78.28
GB	40.53	53.95	83.98	44.52	81.74	106.06	43.91	70.71	66.57
GS	39.55	69.14	72.81	36.04	68.61	74.79	40.69	73.86	75.99
DB	38.81	56.96	84.87	32.91	70.81	88.07	34.59	71.99	90.68
Mean	40.52	62.99	77.24	38.48	60.88	83.50	40.49	67.28	74.23
S.E.	1.39	3.77	4.78	1.49	4.41	6.23	1.97	3.65	4.83

PDHa

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
DB	1.14	1.78	2.81	0.98	1.92	2.68	0.83	1.39	2.56
LF	0.81	1.10	1.45	0.79	1.45	1.95	0.27	2.12	2.67
PW	0.26	1.61	1.87	0.43	1.18	1.99	1.38	2.15	2.27
GB	0.81	1.36	2.75	1.14	1.80	2.44	0.51	1.44	2.74
GS	0.22	1.37	2.61	0.32	1.56	1.98	0.15	1.15	1.62
DK	0.38	0.96	1.92	0.48	1.23	2.65	0.57	1.72	2.67
SP	0.54	1.68	1.61	0.68	1.25	2.18	0.48	1.46	2.19
MR	0.17	2.44	2.15	0.40	0.69	2.27	0.87	2.55	3.33
Mean	0.54	1.54	2.15	0.65	1.49	2.27	0.63	1.75	2.51
SE	0.13	1.74	2.14	0.11	0.11	0.11	0.15	0.18	0.19

ADP_{free}

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	85.13	194.78	210.21	77.66	106.76	319.03	105.15	228.73	332.82
DK	82.37	167.31	609.82	66.57	174.86	477.65	103.31	151.16	179.69
MR	93.18	120.38	111.11	79.72	110.54	110.45	86.28	114.01	136.50
LF	70.49	134.58	191.08	89.39	119.40	230.96	62.78	219.23	230.60
SP	77.32	192.51	168.74	70.02	127.95	218.39	92.95	160.52	186.19
GB	78.87	109.75	238.55	87.34	244.39	259.32	88.77	185.55	171.67
GS	49.51	108.96	103.99	54.73	133.22	160.83	71.26	194.94	193.14
DB	86.23	159.22	243.52	52.20	184.10	297.93	64.77	206.24	492.71
Mean	77.89	148.44	234.63	72.20	150.15	259.32	84.41	182.55	240.42
S.E.	5.02	13.24	60.61	5.25	17.96	42.15	6.24	14.54	44.41

AMP_{free}

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	0.29	1.46	1.82	0.22	0.43	3.93	0.41	2.11	4.43
DK	0.24	1.07	14.10	0.17	1.15	10.07	0.34	0.87	1.22
MR	0.34	0.57	0.41	0.23	0.43	0.43	0.27	0.46	0.69
LF	0.22	0.65	1.51	0.31	0.63	2.16	0.15	1.63	1.90
SP	0.24	1.42	1.06	0.19	0.81	2.31	0.32	1.02	1.24
GB	0.24	0.47	2.49	0.31	2.54	3.34	0.32	1.44	1.09
GS	0.13	0.60	0.62	0.12	0.72	1.04	0.20	1.15	1.27
DB	0.26	0.86	2.77	0.12	1.34	3.45	0.17	1.66	7.39
Mean	0.25	0.89	3.10	0.21	1.01	3.34	0.27	1.29	2.40
S.E.	0.02	0.14	1.71	0.02	0.26	1.12	0.03	0.19	0.87

Free Pi

Subject	CON 30	CON 120	ACE 30	ACE 120	HYP 30	HYP 120
PW	30.87	45.51	20.21	62.81	37.76	52.03
DK	34.75	65.82	33.40	64.45	23.83	30.27
MR	17.12	12.43	20.11	20.00	18.01	24.61
LF	25.51	40.45	22.33	41.39	43.36	47.32
SP	31.96	26.83	47.21	48.23	32.01	42.22
GB	20.92	52.88	45.55	67.67	34.17	33.17
GS	35.68	40.25	35.29	45.02	38.40	38.43
DB	27.59	52.83	47.53	61.62	48.47	66.58
Mean	28.05	42.12	33.95	51.40	34.50	41.83
SE	2.49	6.27	4.55	6.07	3.76	5.08

Lactate

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	3.89	17.50	40.54	3.15	12.81	50.23	3.72	27.69	42.04
DK	0.63	22.73	48.33	13.37	14.90	38.92	1.48	3.61	11.83
MR	6.19	9.19	18.61	9.12	10.58	11.47	3.95	14.54	14.92
LF	5.72	16.12	35.12	6.71	11.34	35.55	1.53	26.55	27.66
SP	3.93	29.80	35.35	8.43	10.04	34.78	1.45	18.30	47.17
GB	4.66	20.78	36.21	7.15	28.06	37.83	3.38	17.13	25.12
GS	3.41	23.51	28.16	2.66	24.19	25.33	1.94	32.72	18.99
DB	2.84	11.03	38.62	2.23	29.62	51.05	2.28	23.62	36.43
Mean	3.91	18.83	35.12	6.60	17.69	35.64	2.47	20.52	28.02
S.E.	0.65	2.57	3.31	1.44	3.10	4.86	0.39	3.45	4.86

Pyruvate

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	0.03	0.19	0.40	0.21	0.33	0.40	0.24	0.42	0.39
DK	0.16	0.22	0.25	0.11	0.20	0.26	0.12	0.12	0.26
MR	0.04	0.09	0.19	0.12	0.13	0.18	0.07	0.16	0.15
LF	0.19	0.27	0.32	0.20	0.26	0.35	0.05	0.29	0.25
SP	0.10	0.31	0.44	0.14	0.21	0.59	0.17	0.17	0.38
GB	0.10	0.27	0.33	0.18	0.47	0.41	0.05	0.24	0.33
GS	0.09	0.20	0.47	0.08	0.24	0.36	0.15	0.17	0.18
DB	0.08	0.10	0.20	0.06	0.26	0.60	0.03	0.15	0.19
Mean	0.10	0.21	0.32	0.14	0.26	0.40	0.11	0.22	0.27
S.E.	0.02	0.03	0.04	0.02	0.04	0.05	0.03	0.04	0.03

Acetyl-CoA

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	8.48	9.52	18.02	15.93	8.69	20.16	7.43	11.22	18.60
DK	23.88	21.22	43.52	38.04	31.51	41.03	19.20	14.53	17.18
MR	7.54	7.40	10.31	21.91	24.62	13.22	8.22	11.81	26.01
LF	4.18	10.84	20.83	28.26	14.56	21.25	10.02	12.65	17.12
SP	8.91	14.71	21.59	22.87	18.63	20.68	5.46	9.63	25.04
GB	6.51	11.86	18.78	16.23	15.68	35.20	11.05	16.70	15.77
GS	6.41	11.16	17.28	20.12	22.50	23.47	15.95	9.57	16.95
DB	5.21	11.37	16.33	19.62	23.71	24.30	6.42	10.23	17.86
Mean	8.89	12.26	20.83	22.87	19.99	24.91	10.47	12.04	19.32
S.E.	2.36	1.57	3.70	2.75	2.68	3.38	1.82	0.95	1.48

Acetylcarnitine

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	2.06	3.82	7.84	8.55	4.26	10.71	2.38	3.17	7.89
DK	11.88	11.92	12.33	17.79	16.92	16.38	9.22	6.32	7.01
MR	1.70	1.48	3.15	10.11	11.02	6.04	1.83	3.34	8.59
LF	2.43	3.11	8.09	11.35	7.70	10.29	6.23	6.89	8.87
SP	3.45	3.64	9.18	11.03	8.56	9.11	0.82	2.97	11.97
GB	3.01	3.45	9.95	8.09	7.85	17.60	3.24	5.22	8.36
GS	1.53	3.45	7.32	11.98	10.52	10.66	7.24	6.45	6.97
DB	1.54	2.59	6.86	9.31	7.99	10.72	0.98	2.26	7.25
Mean	3.45	4.18	8.09	11.03	9.35	11.44	3.99	4.58	8.37
S.E.	1.31	1.21	1.00	1.15	1.39	1.43	1.19	0.70	0.61

Glucose

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	2.55	2.47	4.19	2.16	5.66	10.23	2.19	2.28	6.60
DK	2.70	7.18	7.90	3.78	6.04	4.54	3.99	3.10	3.16
MR	2.41	3.64	9.33	2.17	5.71	2.82	1.30	6.40	6.22
LF	1.67	9.88	5.20	9.82	3.89	3.53	1.42	11.72	6.06
SP	2.21	4.75	4.65	3.40	5.20	7.82	1.42	1.52	4.22
GB	3.69	6.47	3.78	3.28	4.23	6.84	6.92	1.91	6.90
GS	0.79	3.19	2.42	1.55	2.88	2.00	2.30	10.31	4.54
DB	1.64	1.560	4.15	1.02	12.07	10.16	1.83	2.27	3.85
Mean	2.21	4.90	5.20	3.40	5.71	5.99	2.67	4.94	5.19
S.E.	0.33	1.05	0.86	1.04	1.05	1.22	0.73	1.53	0.53

G-6-P

Subject	CON			ACE			HYP		
	0	30	120	0	30	120	0	30	120
PW	0	0.55	1.37	0.72	0.17	0.63	0.13	1.50	1.32
DK	0.14	0.68	1.13	0.18	0.58	2.14	0.08	0.45	1.14
MR	0.21	0.48	0	0.23	1.06	0.73	0.12	1.42	1.00
LF	0.19	0.44	1.73	0.51	1.18	3.22	0.35	1.32	0.95
SP	0.19	3.68	2.36	0.69	0.65	0.87	0.15	0.57	2.16
GB	0.13	1.31	1.51	0.87	2.04	2.43	0	1.65	1.16
GS	0.31	2.93	4.13	0.81	5.65	3.38	1.33	1.56	2.97
DB	0.32	1.02	1.58	0.61	1.49	2.90	0.67	1.66	1.22
Mean	0.19	1.38	1.73	0.58	1.60	2.04	0.35	1.27	1.49
S.E.	0.04	0.47	0.44	0.09	0.66	0.43	0.17	0.18	0.27

F-6-P

Subject	CON			ACE			HYP		
	0	30	120	0	30	120	0	30	120
PW	0	0.08	0.20	0	0.05	0.13	0	0.24	0.20
DK	0.01	0	0.14	0	0.16	0.29	0.13	0.19	0.19
MR	0	0.08	0.10	0.04	0.15	0.14	0.09	0	0.14
LF	0	0.08	0.27	0.24	0.28	0.53	0.17	0.88	0.22
SP	0.03	0.59	0.43	0.23	0.33	0.36	0.06	0.03	0.40
GB	0	0.09	0.13	0.03	0.20	0.20	0.02	0.13	0.12
GS	0.14	0.43	0.63	0.29	0.71	0.51	0.32	0.22	0.41
DB	0.08	0.12	0.24	0.76	0.68	0	0	0.10	0.23
Mean	0.03	0.19	0.27	0.20	0.32	0.27	0.10	0.22	0.24
S.E.	0.02	0.08	0.07	0.10	0.09	0.07	0.04	0.10	0.04

G-3-P

Subject	CON			ACE			HYP		
	0	30	120	0	30	120	0	30	120
PW	2.31	3.36	3.55	1.47	4.87	3.39	0.95	4.74	3.54
DK	0.75	1.97	3.65	0.75	2.65	1.93	0	1.32	1.69
MR	1.58	3.57	3.82	1.65	0.19	0.86	0.15	1.69	2.54
LF	3.58	3.28	3.32	0	0.95	2.11	0	2.82	2.36
SP	1.19	6.24	9.47	1.16	0.30	7.27	0.91	2.97	1.23
GB	0	2.13	0	4.83	6.13	7.94	1.14	2.92	0.91
GS	0	2.10	0	0	3.23	1.95	0.52	5.87	1.64
DB	0	0.65	2.77	0	0.30	2.05	1.46	0.20	1.21
Mean	1.18	2.91	3.32	1.16	2.33	3.44	0.61	2.82	1.89
S.E.	0.49	0.62	1.11	0.63	0.86	1.01	0.22	0.69	0.33

G-1-P

Subject	CON 0	CON 30	CON 120	ACE 0	ACE 30	ACE 120	HYP 0	HYP 30	HYP 120
PW	0.03	0.10	0.11	0.19	0.04	0.13	0.06	0.13	0.16
DK	0.02	0.20	0.18	0	0.07	0.24	0.05	0.14	0.07
MR	0.06	0.06	0	0.03	0.01	0.07	0.07	0.20	0.03
LF	0.19	0.50	0.15	0.12	0.19	0.24	0.13	0.80	0.20
SP	0.06	0.30	0.31	0.19	0.28	0.18	0	0.12	0.14
GB	0.04	0.08	0.13	0.02	0.20	0.03	0	0.08	0.05
GS	0.05	0.05	0.16	0	0.27	0.34	0.04	0.02	0.27
DB	0.05	0.05	0.16	0	0.56	0	0.05	0.16	0.13
Mean	0.06	0.17	0.15	0.07	0.20	0.16	0.05	0.21	0.13
S.E.	0.02	0.06	0.03	0.03	0.07	0.04	0.02	0.09	0.03

Glycogen (rest)

Subject	CON	ACE	HYP
PW	336.7	340.6	411.9
DK	350.1	332.9	440.5
MR	410.5	448.8	433.7
LF	380.4	514.0	407.0
SP	489.9	727.6	436.2
GB	410.3	400.6	390.1
GS	486.9	500.4	405.7
DB	371.4		309.3
Mean	404.5	466.4	404.3
S.E.	21.8	55.3	16.0

Anaerobic ATP Yield

Subject	CON 0-30	CON 30-120	ACE 0-30	ACE 30-120	HYP 0-30	HYP 30-120
PW	42.1	50.2	26.7	97.7	53.6	34.4
DK	58.9	71.6	27.2	67.9	17.9	19.8
MR	13.1	9.2	10.9	1.6	25.9	7.6
LF	30.3	44.8	20.1	58.6	73.9	4.8
SP	68.5	5.1	24.5	45.3	48.9	53.4
GB	37.6	53.2	68.6	38.9	47.4	8.5
GS	59.7	10.7	64.9	7.9	79.3	23.7
DB	30.4	69.3	79.0	49.4	69.4	37.9
Mean	42.6	39.3	40.2	45.9	52.0	23.8
S.E.	7.0	10.3	9.9	11.8	8.3	6.5