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# EFFECTS OF NH4CI INGESTION ON PHOSPHOCREATINE METABOLISM DURING MODERATE- AND HEAVY-INTENSITY PLANTAR-FLEXION EXERCISE

Tyler A. Churchward-Venne
*Western University*

# EFFECTS OF $NH_4Cl$ INGESTION ON PHOSPHOCREATINE METABOLISM DURING MODERATE- AND HEAVY-INTENSITY PLANTAR-FLEXION EXERCISE

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Tyler A. Churchward – Venne

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## CERTIFICATE OF EXAMINATION

Supervisor

Dr. Greg D. Marsh

Supervisory Committee

Dr. John M. Kowalchuk

Dr. Greg D. Marsh

Examiners

Dr. John M. Kowalchuk

Dr. R. Terry Thompson

Dr. C.W. James Melling

The thesis by

**TYLER ASHLEY CHURCHWARD - VENNE**

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Date_____

Chair of the Thesis Examination Board

## ABSTRACT

Eight male subjects performed moderate- and heavy-intensity plantar-flexion exercise in both a control (CON) and $NH_4Cl$ ingestion (ACID) trial. Intracellular metabolism was examined using $^{31}P$-magnetic resonance spectroscopy. During the middle and late stages of heavy-intensity exercise, ACID resulted in a lower ($P<0.05$) intracellular pH (middle: ACID 6.63 vs. CON 6.70; late: ACID 6.64 vs. CON 6.70). Phosphocreatine [PCr] ($P<0.05$) was lower in ACID during the early [ACID 18.14 vs. CON 20.40 mmol/l] and middle [ACID 14.12 vs. CON 16.73 mmol/l] stages of heavy-intensity exercise. ACID did not affect the magnitude of the PCr slow component [ACID 2.7 vs. CON 4.0 mmol/l] ($P>0.05$). Fundamental phase PCr breakdown kinetics demonstrated greater amplitude ($P<0.05$) during heavy-intensity exercise in ACID [ACID: 14.54 vs. CON: 11.31 mmol/l] with no difference in the time constant. In summary, $NH_4Cl$ ingestion increased PCr breakdown during heavy-intensity exercise with no affect on the PCr slow component.



## KEYWORDS

$^{31}P$-magnetic resonance spectroscopy; skeletal muscle metabolism; phosphocreatine; acidosis; exercise; intracellular pH.

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"Traveller there is no path; paths are made by walking"
-Unknown

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| | |
|---|---|
| $^{1}H$ | $^{1}$Proton |
| $^{31}$P-MRS | $^{31}$Phosphorus Magnetic Resonance Spectroscopy |
| 3.0-T | Three Tesla |
| α-ATP | Alpha Adenosine Triphosphate |
| β-ATP | Beta Adenosine Triphosphate |
| γ-ATP | Gamma Adenosine Triphosphate |
| τ | Time Constant (tau) |
| Acetyl-CoA | Acetyl Coenzyme-A |
| ACID | $NH_4Cl$ Ingestion Condition |
| ADP | Adenosine Diphosphate |
| AMP | Adenosine Monophosphate |
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| ATP/ADP x Pi | Phosphorylation Potential |
| CK | Creatine Kinase |
| CON | Control Condition |
| Cr | Creatine |
| DCA | Dichloroacetate |
| e-CK | Extra-mitochondrial Isoform of Creatine Kinase |
| ETC | Electron Transport Chain |
| FID | Free Induction Decay |
| $\Delta G_{ATP}$ | Gibb's Free Energy of ATP Hydrolysis |

| | |
|---|---|
| $H^+$ | Hydrogen Ion |
| $H_2O$ | Water |
| $HCO^-_3$ | Bicarbonate |
| Hz | Hertz |
| IMRIS | Innovative Magnetic Resonance Imaging Systems |
| Kg | Kilogram |
| Km | Michaelis Constant |
| mi-CK | Mitochondrial Isoform of Creatine Kinase |
| Min | Minute |
| mmol/l | Millimoles per liter |
| MR | Magnetic Resonance |
| $mVO_2$ | Muscle Oxygen Uptake |
| $NAD^+$ | Nicotinamide Adenine Dinucleotide (oxidized) |
| NADH | Nicotinamide Adenine Dinucleotide (reduced) |
| $NADH/NAD^+$ | Redox Potential |
| $NaHCO^-_3$ | Sodium Bicarbonate |
| $NH_4Cl$ | Ammonium Chloride |
| $O_2$ | Oxygen |
| PCr | Phosphocreatine |
| PDE | Phosphodiesters |
| PDH | Pyruvate Dehydogenase |
| pHi | Intracellular pH |
| Pi | Inorganic Phosphate |

| | |
|---|---|
| $PIO_2$ | Intracellular Oxygen Pressure |
| PME | Phosphomonoesters |
| ROM | Range of Motion |
| SC | Slow Component |
| SD | Standard Deviation |
| SMIS | Surrey Medical Imaging Systems |
| T1 | Longitudinal Relaxation |
| T2 | Transverse Relaxation |
| TpHi | Intracellular pH Threshold |
| TCA | Tricarboxylic Acid Cycle |
| $VO_2$ | Oxygen Uptake |
| W | Watt |

# CHAPTER 1: INTRODUCTION

## 1.1 The Control of Muscle Oxygen Uptake

During a step increase in power output, such as the transition from rest to moderate-intensity constant load exercise, adenosine triphosphate (ATP) utilization increases immediately to provide the necessary energy for muscle contraction (Barstow et al., 1994a). However, muscle oxygen consumption ($mVO_2$) increases at a relatively slower rate, following an approximately mono-exponential pattern (Tschakovsky and Hughson, 1999). As a result, anaerobic mechanisms such as the depletion of high-energy phosphate stores from phosphocreatine (PCr) and anaerobic glycolysis (with lactate accumulation) provide supplementary energy for muscle contraction during this time (Rossiter et al., 2003). This anaerobic component of energy provision during the initial stages of exercise has been termed the oxygen ($O_2$) deficit (di Prampero and Margaria, 1968). In general, the study of "oxygen uptake kinetics" ($VO_2$) is aimed at understanding the mechanisms behind this delayed adjustment in oxidative phosphorylation to the immediate increase in ATP demand (Grassi, 2005).

In 1940, Hill reported that $O_2$ consumption in a frog sartorius muscle after tetanus demonstrated a simple time course (Hill, 1940). It was reported that the off-transient of muscle $O_2$ consumption was well fit by a single exponential whose time constant ($\tau$) was similar for all tetani of ~2-20 seconds duration (Mahler, 1985). However, since this time a number of metabolic control models have been proposed (e.g. Chance and Williams, 1956; Jeneson et al., 1995; Mahler, 1985; Meyer, 1988; and Wilson, 1994), and as such, the precise mechanism(s) involved in the control of oxidative phosphorylation are still debated. Adding further complexity to the issue, some authors have suggested that the

kinetics of skeletal muscle $O_2$ consumption during the on-transient to exercise may not be strictly exponential. For example, it has been suggested that more than a single factor may act independently as a limitation at different times during the course of activation of oxidative phosphorylation (Chung et al., 2005; Korzeniewski and Zoladz, 2006), or that single mechanisms (e.g. adenosine diphosphate (ADP)) control oxidative phosphorylation in a second (or higher) order manner (Jeneson et al., 1996).

The reaction that describes the process of oxidative phosphorylation is:

$$NADH + {}^{1}/_{2}O_2 + H^{+} + 3ADP + 3Pi \Rightarrow 3ATP + NAD^{+} + H_2O$$

Based on this reaction, any of the above substrates could act to control or limit the rate of oxidative phosphorylation during the non-steady exercise state. Indeed, the mechanisms that limit or regulate $VO_2$ kinetics during the adjustment to a higher workload have been debated for many years, mainly by those who favor the concept of a delayed metabolic activation or "metabolic inertia", and those who favor the idea of an $O_2$ delivery limitation (Grassi, 2005).

Respiratory control in skeletal muscle has traditionally been explained by a kinetic limitation of cytoplasmic [ADP] (Chance and Williams, 1956). The classic kinetic control model proposed by Chance and Williams evolved out of investigations of isolated mitochondria. In these studies it was found that the rate of $O_2$ consumption was dependent on [ADP] in a Michaelis-Menton fashion, when $O_2$, substrate, and phosphate were still present in the medium (Chance and Williams, 1956). While this relationship may be the case in an in-vitro model involving isolated mitochondria, it is not clear if this scenario accurately describes processes occurring in the intact muscle (Meyer and Foley, 1996). As Meyer and Foley point out, the simple kinetic model of ADP control has

several limitations (Meyer and Foley, 1996). One problem with the simple ADP kinetic model is that it requires [ADP] in resting muscle to be less than 10% of its Michaelis constant ($K_m$), otherwise it cannot explain the ~tenfold or higher increase in the steady state respiration rate that has been observed in active muscle (Hochachka and Matheson, 1992). On average, resting [ADP] in muscle is approximately 5-25 μM, and this is more than 25% of the apparent $K_m$ (3-30 μM) measured in isolated mitochondria (Meyer and Foley, 1996). Further bringing into question the simple kinetic model are the observations of Balaban and colleagues (Balaban et al., 1986). Using nuclear magnetic resonance spectroscopy to study the response of dog cardiac muscle in vivo, they reported increases in the rate of oxidative phosphorylation with no changes in free [ADP] (Balaban et al., 1986). Lastly, similar rates of oxidative flux have been observed despite significant differences in free [ADP] concentration. For example, during sodium bicarbonate ($NaHCO^-_3$) induced alkalosis, free [ADP] concentration was reduced compared to control conditions during heavy-intensity isotonic forearm exercise with no difference between conditions in the rate of oxidative flux (Forbes et al., 2005).

The creatine-shuttle hypothesis of respiratory control (Bessman and Geiger, 1981) is a variation of the simple kinetic model mentioned above. In this model, any [ATP] changes in the cytosol are prevented by the extra-mitochondrial isoform of creatine kinase (e-CK). Creatine (Cr) enters the mitochondrion through the outer mitochondrial membrane, and the resulting increase in [Cr] leads to ADP production through the mitochondrial isoform of CK (mi-CK). mi-CK is functionally coupled to oxidative phosphorylation through its close proximity to an ADP/ATP translocator located at the inner mitochondrial membrane. ADP enters the mitochondrial matrix and is reconverted

to ATP via oxidative phosphorylation. This ATP is then transported to the inner mitochondrial membrane by the translocase and interacts with Cr to resynthesize PCr. PCr can then leave the mitochondrion and enter the cytoplasm (Grassi, 2005). Thus, the basic feature of this model is that mi-CK is intimately connected with the nucleotide translocase in the inner mitochondrial membrane, such that diffusion of cytosolic Cr is the kinetically limiting acceptor that controls respiration. ADP is thought to be the principle driving force of respiration in a Michaelis-Menten fashion, however ADP supply is limited by Cr availability at the mitochondria (Grassi, 2005).

Other models (e.g. Jeneson et al., 1995; Meyer, 1988; and Wilson et al., 1994) proposed to explain the control of oxidative phosphorylation in skeletal muscle are based on thermodynamic control. In the near-equilibrium hypothesis of thermodynamic control, the mitochondrial redox potential ($[NAD^+]/[NADH]$), reflecting the degree of cytochrome c reduction, and the phosphorylation potential ($[ATP]/[ADP]\bullet[Pi]$) determine the rate of oxidative phosphorylation (Tschakovsky and Hughson, 1999). However, it is not the absolute concentration of substrates, but instead the ratio of substrate to product that is the important determining factor (Tschakovsky and Hughson, 1999). It is important to note that of the models mentioned thus far, this is the first having a role for the intramitochondrial reducing power ($[NAD^+/NADH]$ ratio) and inorganic phosphate (Pi) (Wilson, 1994). In Meyer's model of non-equilibrium thermodynamic control, oxidative phosphorylation is driven by the difference between the cytoplasmic energy of ATP hydrolysis ($\Delta G_{ATP}$) and the intramitochondrial energy potential (Meyer, 1988). If the mitochondrial potential remains relatively constant and $O_2$ availability is not a limiting factor, then the $\Delta G_{ATP}$ will be related to oxidative phosphorylation in a linear

fashion, with the oxidative flux for a given $\Delta G_{ATP}$ being dependent on the total Cr content and on the number and properties of mitochondria (Meyer, 1988). Indeed, in a recent in-vitro study by Glancy and colleagues, isolated skeletal muscle mitochondria were energetically linked to varying levels of total Cr by the addition of the CK enzyme and subjected to increases in ATP demand (ATP splitting system of glucose and hexokinase) (Glancy et al., 2008). The $\tau VO_2$ in response to the increase in ATP demand varied linearly with the total Cr present in the incubation (Glancy et al., 2008). Further, the $\tau VO_2$ was also linearly and inversely related to the mass of the mitochondrial protein added to the incubation (Glancy et al., 2008).

It has been suggested that substrate delivery to the electron transport chain (ETC) in the form of nicotinamide adenine dinucleotide (NADH) may exert feedforward control on the rate of oxidative phosphorylation (Timmons et al., 1996). The enzyme pyruvate dehydrogenase (PDH) determines the availability of substrate for the tricarboxylic acid cycle (TCA) and subsequently, the ETC. The enzyme PDH catalyses the irreversible reaction by which pyruvate is decarboxylated to acetyl coenzyme A (acetyl-CoA) (involving $NAD^+$ and Co-A) (Greenhaff et al., 2002). A greater PDH activity may be expected to result in an increase in acetyl-CoA, potentially leading to a faster delivery of NADH to the ETC. Timmons and colleagues have suggested that PDH may act as a 'stenosis' at the level of pyruvate processing (Timmons et al., 1996). This suggestion was based on observations of increased (~4 and ~10 fold) PDH activity and acetylcarnitine availability in canine skeletal muscle at rest following pharmacological activation of PDH with dichloroacetate (DCA). Activation of PDH with DCA resulted in reduced PCr breakdown and reduced lactate accumulation during subsequent intense contraction

(Timmons et al., 1996). Further, in the xenopus single muscle fibre preparation, PDH activation with DCA resulted in a significant decrease in the $\tau$ of intracellular $O_2$ pressure ($PIO_2$), indicating an acceleration of the rate of $O_2$ consumption in the muscle fibre (Howlett and Hogan, 2003). Other studies, using human in-vivo experimental models (Jones et al., 2004; Koppo et al., 2004; and Rossiter et al., 2003) have not found an increased rate of muscle $O_2$ consumption during the non-steady state exercise transition following pharmacological activation of PDH with DCA. However, a recent investigation by Gurd and coworkers examined the adaptation of pulmonary $VO_2$, activation of mitochondrial PDH, muscle metabolite concentration, and muscle de-oxygenation status in both young and old adults during the transition to moderate-intensity exercise (Gurd et al., 2008). It was found that the slowed pulmonary $VO_2$ kinetics observed in the old compared to the young at the onset of moderate exercise were accompanied by a slower activation of PDH during the first 30 seconds of exercise and by a greater change in deoxyhemoglobin for a given $VO_2$ (Gurd et al., 2008). These results suggest a slower activation of the metabolic machinery (in the form of PDH) and a slower adaptation of bulk $O_2$ delivery in older adults (Gurd et al., 2008).

Aerobic metabolism by definition is dependent on the presence of $O_2$, as it is the final hydrogen acceptor at the end of the ETC. Whether $O_2$ transport to the active muscle is a limitation to $VO_2$ at the onset of exercise has been a topic of controversy for some time, however it is generally agreed that at some point altering $O_2$ delivery will have an impact on $VO_2$ kinetics (Poole et al., 2008). An $O_2$ transport limitation implies that there is a rate-limiting step in $O_2$ transport somewhere between the lungs and the mitochondria (Poole et al., 2008). Investigations employing diverse experimental models have found

support for the general thesis that a reduced $O_2$ delivery causes a slowing of the kinetics of $VO_2$. In humans for example, it was reported that the phase II $\tau$ of the pulmonary $VO_2$ response was slower when heavy-intensity arm cranking exercise was performed above the level of the heart, where the local arterial pressure is lower (Koppo and Bouckaert, 2005). In another investigation looking at the effect of hypoxia during heavy-intensity cycling exercise, there was a reported increase in the $\tau$ of phase II $VO_2$ (Engelen et al., 1996). Lastly, beta-adrenergic receptor blockade has been shown to slow $VO_2$ kinetics at the onset of pedaling exercise (Hughson, 1984). Other studies however have found that $O_2$ does not appear to be a limiting factor at the onset of exercise. Grassi and colleagues (Grassi et al., 1998a; Grassi et al., 1998b), using an in-situ preparation involving isolated canine gastrocnemius muscle, demonstrated that both a faster adjustment of $O_2$ delivery to the working muscle (Grassi et al., 1998a), and an enhancement of peripheral $O_2$ diffusion (Grassi et al., 1998b) did not affect $mVO_2$ on-kinetics during electrically-stimulated contractions at intensities of ~60-70% of peak $VO_2$. In the same model however, it was reported that a faster $O_2$ delivery resulted in speeding of $VO_2$ on-kinetics during electrically stimulated isometric contractions corresponding to the muscles peak $VO_2$ (Grassi et al., 2000). Human studies in which $O_2$ delivery has been increased by breathing a hyperoxic gas mixture have not found a significant effect on the phase II $VO_2$ response (Hughson and Kowalchuk, 1995; Wilkerson et al., 2006). Thus, many factors seem to be important in determining the role of $O_2$ availability in the activation of oxidative phosphorylation. For example, exercise intensity (Grassi et al., 1998ab; Grassi et al., 2000), upright vs. supine posture (Jones et al., 2006), upper vs. lower body exercise (Koppo et al., 2002), and age of the population studied (Gurd et al., 2008; Scheuermann

et al., 2002) all appear to be important factors in determining the role of $O_2$ availability on the activation of oxidative phosphorylation. Overall, a slower rate of adjustment of $VO_2$ following a change in work rate will necessitate a greater reliance on substrate level phosphorylation to meet the energetic demands.

The creatine kinase (CK) reaction is directly linked to an increase in [ADP] (and a decrease in the phosphorylation potential and $\Delta G_{ATP}$) such that the enzyme facilitates the breakdown of PCr following a rapid increase in ATP demand.

$$PCr + ADP + H^{+} \Leftarrow CK \Rightarrow ATP + Cr$$

Mitochondrial respiration is intimately tied to the rate of ATP hydrolysis in skeletal muscle, and one or more of the reactants of this process (eg. [ADP], [Pi], phosphorylation potential, [PCr], [Cr]) have been proposed to be involved in the activation of oxidative phosphorylation through feedback control mechanisms (e.g. Chance et al., 1985; Mahler, 1985; Meyer, 1988; and Poole et al., 2008). Support for this proposal comes largely from investigations that have provided evidence of a close dynamic correlation between PCr breakdown and $mVO_2$ (e.g. Mahler, 1985), or its proxy pulmonary $VO_2$ (e.g. Barstow et al., 1994ab; McCreary et al., 1996; and Rossiter et al., 1999). Specifically, several researchers have provided evidence that phase II of pulmonary $VO_2$ reflects that of intramuscular [PCr] breakdown (and by implication that of $mVO_2$) during the transition from rest to both moderate- (Barstow et al., 1994ab; McCreary et al., 1996; and Rossiter et al., 1999) and heavy-intensity exercise (Rossiter et al., 2002ab).

Many of the biochemical metabolites thought to be important in the control of $mVO_2$ during exercise can be studied using 31-phosphorus magnetic resonance

spectroscopy ($^{31}$P-MRS). Work by Barstow and colleagues (Barstow et al., 1994a) showed that the τ describing the fundamental fall in [PCr] measured during plantar-flexion exercise and the increase in pulmonary $VO_2$ measured during cycle ergometry were similar during non-steady state exercise in humans (Barstow et al., 1994ab). However, a limitation in the study by Barstow and coworkers was the use of different muscle groups (gastrocnemius vs. quadriceps) and different modes of exercise (plantar-flexion vs. (knee/hip extension). The use of different forms of exercise involving very different muscle groups (leading to different metabolic rates), make inferences with regards to metabolic control mechanisms very difficult. However, a similar relationship between the τ for [PCr] breakdown (τPCr) and the τ for pulmonary $VO_2$ ($\tau VO_2$) was demonstrated using the same mode of exercise (plantar-flexion) involving the same muscle group (gastrocnemius) (McCreary et al., 1996). While this was a step forward for inferences with regards to control mechanisms, this study was limited due to the small increment in pulmonary $VO_2$ (~100 ml/min $^{-1}$) seen during plantar-flexion exercise (McCreary et al., 1996). Such a small increment in pulmonary $VO_2$ can make subsequent differences in $\tau VO_2$ values difficult to distinguish with confidence. Thus, using $^{31}$P-MRS, Rossiter and coworkers demonstrated a tight coupling (within 10%) between the fundamental phase of both τPCr breakdown and phase II pulmonary $\tau VO_2$ when measured simultaneously during both moderate- (Rossiter et al., 1999) (below lactate threshold) and heavy- (above lactate threshold) intensity quadriceps exercise (Rossiter et al., 2002ab). Interestingly, the work by Rossiter and coworkers also showed a strong dynamic relationship between the $VO_2$ and PCr slow component, such that their onset was shown to match temporally to within ~30 seconds (Rossiter et al., 2002). Further, the

magnitude of the PCr slow component was shown to be ~91% of that of the $VO_2$ slow component when normalized to the amplitude of the fundamental phase (Rossiter et al., 2002).

Further support for the notion that factors relating to the CK reaction are involved in controlling the rate of oxidative phosphorylation come from studies that have examined the effects of acute CK inhibition in single isolated myocytes (Kindig et al., 2005) and studies involving CK knockout mice (Roman et al., 2002). Kindig and colleagues reported that inhibition of the CK enzyme in xenopus laevis single muscle cells (using the drug iodoacetamide) resulted in a significantly faster fall $PIO_2$, indicative of accelerated $VO_2$ onset kinetics (Kindig et al., 2005). These data suggest that the CK catalyzed breakdown of PCr at the onset of isometric tetanic contractions moderates the rise in [ADP] in such a way that it acts as a temporal buffer for the immediate increase in ATP demand. Thus, the CK reaction may act to slow the rate of oxidative phosphorylation by delaying important energetic control signal(s) (e.g. ADP) between the sites of ATP breakdown and the mitochondria (Walsh et al., 2005). Inhibition of CK with iodoacetamide has resulted in similar findings in isolated rabbit hearts, reducing the on-transient $\tau$ of $mVO_2$ by 40-50% (Harrison et al., 1999; Harrison et al., 2003). Lastly, in a kinetic modeling study involving CK knockout mice, the initial rate of PCr depletion was dramatically reduced in the CK knockout vs. wild-type mice. The results were taken to suggest that cytoplasmic [ADP] increased more rapidly, and as a result, oxidative phosphorylation was activated faster at the onset of contractions in the knockout compared to the wild-type mice (Roman et al., 2002).

Overall, there is considerable evidence that products of the CK reaction related to PCr hydrolysis (e.g. [ADP], phosphorylation potential, or $\Delta G_{ATP}$) are involved in the control of oxidative phosphorylation through feedback control mechanisms. This is supported by the observed close kinetic coupling between pulmonary $VO_2$ and PCr breakdown (Barstow et al., 1994ab; McCreary et al., 1996; Rossiter et al., 1999; and Rossiter et al., 2002) and by consistent reports that a faster rate of increase in [ADP] results in a faster rate of $O_2$ consumption in the muscle (Harrison et al., 1999; Harrison et al., 2003; Kindig et al., 2005; and Roman et al., 2002).

### 1.2 Acid-Base Status and Oxidative Phosphorylation

Investigations into the effects of acid-base status on the control of oxidative phosphorylation have been carried out with in-vitro studies involving the examination of isolated mitochondria, and in-vivo studies, with examinations of intact human subjects. This section will focus on acid-base status and how perturbations to pH homeostasis influence the rate of oxidative phosphorylation.

One important factor that may act to limit sustained $O_2$ consumption is the metabolic acidosis that results from the accumulation of hydrogen ions ($H^+$) during heavy-intensity exercise above the lactate threshold (Jubrias et al., 2003). Proposed mechanisms by which [$H^+$] could reduce ATP supply include an indirect effect via reductions in the signals proposed to drive oxidative phosphorylation (e.g. [ADP] or phosphorylation potential), or a direct effect on mitochondrial function itself (Jubrias et al., 2003). Several in-vitro studies of isolated mitochondria have reported that acidosis impairs the maximal rate of oxidative phosphorylation (e.g. Fry et al., 1980; and Hillered

et al., 1984), while other examinations of isolated mitochondria have not found an effect (e.g. Suleymanlar et al., 1992). However, the physiological conditions under which isolated mitochondria are studied may be of importance as acidosis has been reported to have different effects when induced on non-phosphorylating vs. phosphorylating mitochondria (Tonkonogi and Sahlin, 1999). For example, when lactic acid was added to non-phosphorylating mitochondria, the maximal rate of $O_2$ consumption was reduced (Tonkonogi and Sahlin, 1999). However, lactic acidosis did not show the same effect on mitochondria during maximal ADP stimulated respiration. When mitochondria were actively phosphorylating, the maximal respiration rate was maintained at the same level as control conditions (no acidosis) (Tonkonogi and Sahlin, 1999). It has also been suggested that the type of mitochondria studied (i.e. skeletal muscle vs. cardiac muscle) may be of importance as acidosis is a more frequent phenomenon in skeletal muscle than in cardiac muscle, thus skeletal muscle mitochondria may be better protected from the effects of acidosis than cardiac mitochondria (Walsh et al., 2002).

Studies involving skinned muscle fibres have also reported reductions in the rate of oxidative phosphorylation under low pH conditions. For example, in skinned rat soleus muscle, a physiologically relevant acidosis (pH 6.6; similar to that seen during heavy-intensity exercise) was found to reduce the rate of oxidative phosphorylation when submaximal levels of ADP were present (Walsh et al., 2002). However, it was found that this reduced rate of oxidative phosphorylation could be compensated for when ADP levels were increased. In the presence of acidosis, oxidative capacity was restored by increasing [ADP] (Walsh et al., 2002). In the study by Walsh and coworkers it was suggested that a greater ADP stimulus was required during acidosis (pH 6.6) in order to

attain the same rate of oxidative phosphorylation as seen in the absence of acidosis (pH 7.0) (Walsh et al., 2002). Further, it was suggested that the mechanism(s) by which mitochondrial respiration was reduced at a low pH were the result of a dissociation of CK from the mitochondrial membrane, resulting in a reduced [ADP] due to deterioration of the Cr shuttle (Walsh et al., 2002).

In corroboration with the suggestions of Walsh (Walsh et al., 2002), Forbes and colleagues found that $NaHCO^{-}_{3}$ induced alkalosis reduced intracellular acidosis and this was associated with a reduction in [ADP] during heavy-intensity forearm exercise (Forbes et al., 2005). These findings support the notion that the inhibiting effects of acidosis on oxidative phosphorylation can be overcome by a greater quantity of the signal, as evidenced by the finding of a greater [ADP] in the lower pH condition (control condition) despite similar levels of oxidative flux (Forbes et al., 2005). Interestingly, other studies that have examined the effect of acid-base status on skeletal muscle metabolism have reported $NH_4Cl$ induced acidosis being associated with a reduced [ADP] accumulation during cycling exercise performed at 75% of $VO_2$ max, woth no effect on absolute $VO_2$ levels (Hollidge-Horvat et al., 1999).

Support in humans for the notion that a reduced intracellular pH limits oxidative ATP supply comes from Jubrias and colleagues (Jubrias et al., 2003). Using an in-vivo exercise model, it was reported that the highest oxidative flux during sustained exercise occurred in the absence of acidosis and that exercise that resulted in a drop in pH (< 6.88) prevented increases in oxidative flux (Jubrais et al., 2003). Their results further demonstrated that in the absence of acidosis, an increase in [ADP] resulted in the exercising muscles approaching their estimated oxidative capacities (Jubrais et al., 2003).

However, their results are not in agreement with the notion that acidosis indirectly inhibits the rise in oxidative flux by limiting the rise in [ADP] via a shift in CK equilibrium. Instead their findings support a direct effect of pH on mitochondrial function, indicated by the inability of oxidative flux to increase in an exercise condition that resulted in an acidosis despite a significant increase in [ADP] (Jubrais et al., 2003). A direct effect of acidosis has also been reported in the observed lower $O_2$ consumption at a given [ADP] in isolated perfused cat soleus muscle (Harkema and Meyer, 1997a).

Several studies examining the effect of acid-base balance have used $NaHCO^-_3$ to induce a metabolic alkalosis during exercise (e.g. Kolkhorst et al., 2004; Zoladz et al., 2005). These studies commonly measure the kinetics of $VO_2$ at the mouth, as it is a proxy for $mVO_2$ and as such, the results of these studies can provide valuable insight into the control of oxidative phosphorylation. For example, Zoladz reported a speeding of the phase II pulmonary $VO_2$ response (reflecting a faster rate of $mVO_2$) during the transition to heavy-intensity cycling exercise following $NaHCO^-_3$ ingestion (Zoladz et al., 2005). This speeding of $VO_2$ kinetics was attributed to an increased [ADP], due to either a shift in CK equilibrium or to a slowed rate of glycolysis (Zoladz et al., 2005). However, Kolkhorst and coworkers found the opposite effect on $VO_2$ kinetics following $NaHCO^-_3$ ingestion and attributed the increased phase II $\tau VO_2$ to a left-shift in the oxygen-hemoglobin dissociation curve, resulting in reduced $O_2$ delivery to the muscle (Kolkhorst et al., 2004).

An attenuated acidosis has been associated with greater fatigue resistance during exercise. For example Raymer and colleagues showed that $NaHCO^-_3$ ingestion reduced acidosis during forearm exercise above the intracellular pH threshold and this was

associated with a greater time to fatigue and greater peak power output (Raymer et al., 2004). Similarly, $NH_4Cl$ induced acidosis has been associated with a reduced peak power output during a progressive incremental exercise test (Kowalchuk et al., 1984), and a reduced endurance time when cycling at 95% of peak power output (Jones et al., 1977) compared to control and alkalosis conditions. However, no differences in absolute $VO_2$ were evident between the conditions in these studies (Kowalchuk et al., 1984; Jones et al., 1977).

The 'slow component' of pulmonary $VO_2$ seen during heavy-intensity exercise is associated with metabolic acidosis, in that both are a common feature of exercise performed above the lactate threshold (Jones et al., 2007). The transition to heavy-intensity exercise results in the dynamics of $VO_2$ departing from the simple monoexponential response seen during moderate-intensity exercise. Superimposed on the simple fundamental component is a slow to develop supplementary component of $VO_2$ with a delayed onset (Whipp et al., 2005). This dynamic response has been termed the $VO_2$ slow component and represents excess $VO_2$ (Whipp et al., 2005). As mentioned earlier, the [PCr] response to heavy-intensity exercise also displays a slow component. When measured simultaneously, [PCr] has been shown to continuously decline with a similar time course and magnitude to that of the $VO_2$ slow component (Rossiter et al., 2002ab). This tight coupling between both the primary and slow components of pulmonary $VO_2$ and PCr breakdown support the notion of feedback control in oxidative phosphorylation being linked to PCr hydrolysis via the CK reaction (Rossiter et al., 2002ab).

The slow component of $VO_2$ is an interesting characteristic of heavy-intensity exercise that has become vital to understanding the control of skeletal muscle $VO_2$ (Rossiter et al., 2005). While the exact mechanisms responsible for the $VO_2$ slow component are unknown, acidosis is a characteristic of heavy-intensity exercise, and has been shown to slow the rate of oxidative phosphorylation (Jubrias et al., 2003). A slowed rate of oxidative phosphorylation may be expected to result in PCr hydrolysis in order to maintain ATP supply, thus giving rise to the PCr slow component (Forbes et al., 2005). In agreement with this notion, $NaHCO^-_3$ induced alkalosis has been shown to reduce the magnitude of the PCr slow component during heavy-intensity forearm exercise, and this was proposed to be due to a reduced ATP demand (from substrate level phosphorylation) as a consequence of a reduced intracellular acidosis (Forbes et al., 2005). However, it has been reported that acidosis has no effect on the ATP cost of contraction, as measured in cat fast- and slow-twitch skeletal muscle (Harkema et al., 1997b). Other investigations into the effects of $NaHCO^-_3$ induced alkalosis on the slow component of pulmonary $VO_2$ have reported a delayed onset (Berger et al., 2006), and no effect (Santalla et al., 2003; Zoladz et al., 1997). However, $NH_4Cl$ induced acidosis has been reported to increase the magnitude of the $VO_2$ slow component during cycling exercise in humans (Zoladz et al., 1998).

If feedback control via products of the CK reaction are involved in the control of oxidative phosphorylation, as supported by the observed tight coupling between the kinetics of pulmonary $VO_2$ uptake and those of [PCr] breakdown (e.g. Rossiter et al., 1999; Rossiter et al., 2002a,b), then the observed increase in the magnitude of the $VO_2$ slow component following $NH_4Cl$ induced acidosis (Zoladz et al., 1998) might be

expected to be associated with greater PCr breakdown and a concomitant increase in the magnitude of the PCr slow component. Further, a falling intramuscular pH, as seen during exercise performed above the lactate threshold would be expected to drive the CK reaction in the direction of PCr breakdown, thereby generating a PCr slow component and maintaining ADP provision to the mitochondrion (Rossiter et al., 2005). However, investigations examining the effect(s) of acid-base status in which [PCr] has been measured have reported acidosis being associated with no changes (e.g. Hollidge-Horvat et al., 1999; Hultman et al., 1985) a lower (e.g. Sahlin et al., 1983) or a greater [PCr] (e.g. Meyer et al., 1991).

Overall, the effects of acid-base status on the products of PCr hydrolysis via the CK reaction and on the control of oxidative phosphorylation are not fully understood and warrant further study. However, it appears that acidosis acts to slow the rate of oxidative phosphorylation in human skeletal muscle during exercise (Jubrais et al., 2003). Only one other study (Hood et al., 1988) has examined effects of $NH_4Cl$ ingestion on skeletal muscle metabolism during exercise using $^{31}$P-MRS. However, in this experiment exercise involved the study participants squeezing a ball, making differences between conditions difficult to discern due to the inability to accurately quantify work. Further, no study has examined the effect(s) of $NH_4Cl$ ingestion on the PCr kinetic response to exercise.

### 1.3 Phosphorus Magnetic Resonance Spectroscopy ($^{31}$P-MRS)

$^{31}$Phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) allows for the continuous, non-invasive, in-vivo measurement of phosphorus containing metabolites and intracellular acid-base status. Specifically, phosphate compounds (with quantities greater

than ~ 0.1 mmol/kg wet weight) such as PCr, Pi, ATP, phosphomonoesters (PME), and phosphodiesters (PDE) are present in the spectrum and can be measured with a relatively high temporal resolution using $^{31}$P-MRS (Heerschap et al., 1999). Further, certain metabolite concentrations with known stoichiometry can be calculated such as [ADP] and adenosine monophosphate (AMP]. Intracellular pH (pHi) changes can be estimated based on the chemical shift (in parts per million) of Pi relative to PCr (Taylor et al., 1983), and $[H^+]$ then calculated from pHi.

MRS works by manipulating and measuring the gyromagnetic properties of nuclei. The nuclear precession frequency and the net nuclear magnetic moment are particularly important properties of nuclei. The movement of nuclei in a magnetic field can be divided into two parts: spin and precession. Spin refers to the movement of a nucleus around its own axis. However, the axis of the nucleus will also tip at an angle less than perpendicular to, for example, a flat surface. Thus, the axis of the nucleus will rotate at that angle in a motion referred to as precession. The effect of a magnetic field on the precession of a nucleus is of primary interest in MRS. The rate that nuclei precess will primarily be dependent upon the isotope examined and the strength of the magnetic field. The purpose of the magnet is to cause all the nuclei to precess and to orient all of the nuclear magnetic moments in the direction of the magnetic field. A very short pulse of radiofrequency (~ 3 milliseconds) similar to an FM radio wave is then transmitted perpendicular to the magnetic field generated by the magnet. This causes the net magnetic moment of the nuclei precessing at the same frequency as the radiofrequency to be deflected from the direction oriented by the magnet. After cessation of the radiofrequency pulse, the nuclei begin to emit FM radio waves in the direction of their

new orientation in the process of relaxing and returning to their original orientation. A radiofrequency receiver measures the FM radio waves emitted from the nuclei that were deflected. The signal sent to the receiver during this time period is referred to as a free induction decay (FID). The free induction decay quantitatively reflects the number of a certain type of nuclei in the sample (e.g. phosphorus), thus it can provide important information about the concentration and kinetics of high-energy phosphate compounds during rest, exercise, and recovery. $^{31}$P-MRS can be a valuable investigative tool to study the bioenergetics of skeletal muscle, as it allows for the measurement of important metabolites involved in the energy producing pathways of the human body.

### 1.4 Ammonium Chloride ($NH_4Cl$)

Several human studies (e.g. Hollidge-Horvat et al., 1999; Hood et al., 1988; Hultman et al., 1985; and Jones et al., 1977) investigating the effect of acid-base status on skeletal muscle metabolism during exercise have used $NH_4Cl$ to induce a metabolic acidosis. $NH_4Cl$ is a white, odorless, crystalline powder when consumed orally. Once ingested it gets absorbed in the gastrointestinal tract. The ammonium ion is converted into urea in the liver; the anion when liberated into the bloodstream and extracellular fluid causes a metabolic acidosis (Martindale: The complete drug reference, 1999).

### 1.5 Purpose

The purpose of this thesis was to examine the effects of $NH_4Cl$ ingestion on PCr metabolism during moderate- and heavy-intensity plantar-flexion exercise using $^{31}$P-MRS. As many of the variables proposed as potential controllers of oxidative

phosphorylation can be discerned in the $^{31}$P-MRS spectra during both rest and exercise, a further aim of this study was to provide greater insight into the effect of acid-base status on the control of oxidative metabolism during different intensities of exercise.

### 1.6 Hypotheses

Four hypotheses were tested in this study:

1. During resting conditions prior to exercise, $NH_4Cl$ ingestion would have no effect on pHi, consistent with previous reports that $NH_4Cl$ ingestion does not affect pHi at rest relative to control conditions (Hultman et al., 1985; Hollidge-Horvat et al., 1999).
2. During moderate-intensity exercise performed below the intracellular pH threshold, pHi would remain close to resting levels. In the absence of intracellular acidosis there would be no difference between conditions in pHi or [PCr].
3. During heavy-intensity exercise above the intracellular pH threshold, pHi would be lowered in both conditions but to a greater magnitude following $NH_4Cl$ ingestion.
4. The lower pHi seen during heavy-intensity exercise following $NH_4Cl$ ingestion would be associated with greater PCr breakdown relative to the control trial. This increase in PCr breakdown would be associated with an increase in the magnitude of the PCr slow component.

# CHAPTER 2: METHODS

## 2.1 Subjects

Eight (N=8) adult male subjects volunteered to participate in this study (age = 25.5 (SD 3.01) yr; body mass = 76.5 (SD 11.1) kg). All of the subjects were healthy and moderately active but none were highly trained. Prior to starting the study, the experimental procedures and any potential risks were explained (Appendix C) to each subject. An informed written consent document was signed by each participant prior to the study (Appendix D). The study was approved by The University of Western Ontario Ethics Review Board for Health Sciences Research Involving Human Subjects (Appendix A) and by The Lawson Health Research Institute Clinical Research Impact Committee (Appendix B).

## 2.2 Experimental Protocol and Procedures

Each subject was studied on three different occasions. During the first visit, subjects performed an incremental ramp plantar-flexion exercise protocol to volitional fatigue (Marsh et al., 1991). This protocol was used to determine the work rates that corresponded to each subjects moderate- and heavy-intensity exercise domain. Each subject was then studied during a constant-load isotonic plantar-flexion exercise protocol in both a control (CON) and $NH_4Cl$ ingestion condition (ACID).

The plantar-flexion exercise was performed with the subject lying supine on a table inside the bore of a 3.0-T superconducting magnet (Figure 1). The participants legs were fully extended and positioned inside a custom-built magnetic resonance compatible ankle exercise ergometer (Figure 2), the details of which have previously been reported

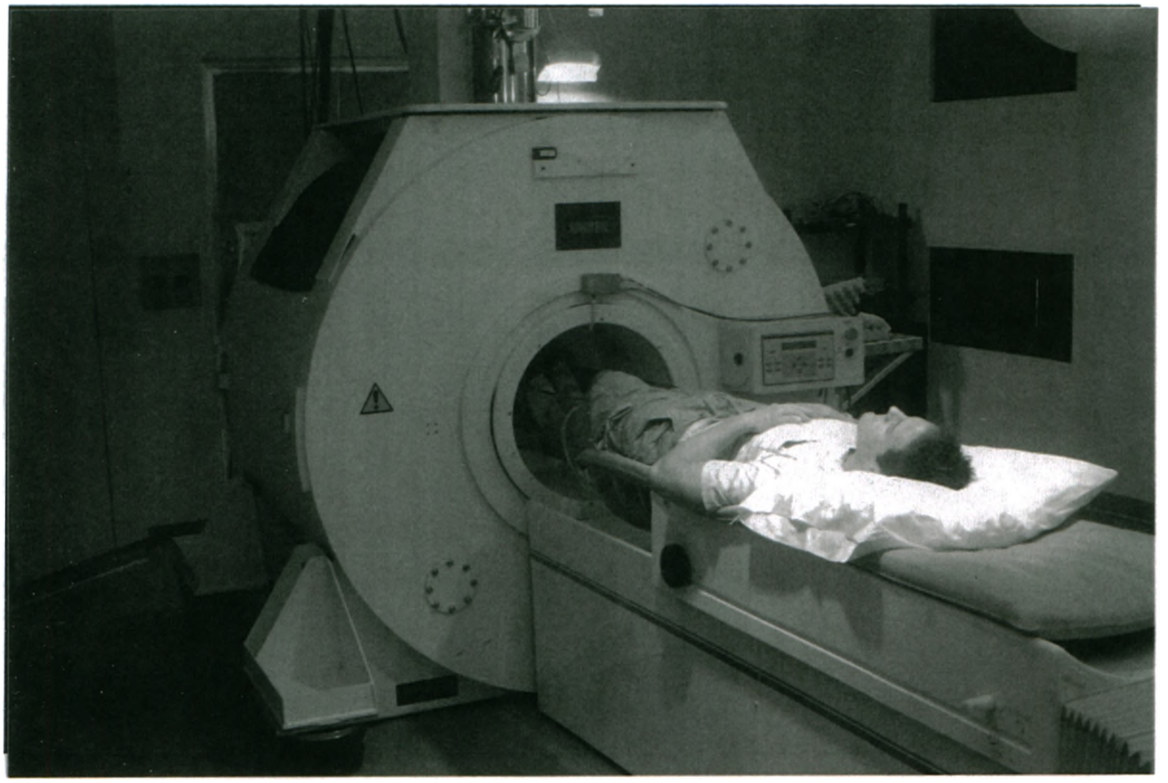

**Figure 1.** Positioning of the subject inside the bore of the 3.0-T MR scanner during the experimental protocol.

in detail (Raymer et al., 2006). The ankle ergometer used a cable and pulley system that raised and lowered a suspended reservoir of water. The right foot of each subject was securely fastened to the footplate of the apparatus prior to the start of exercise. Depression (plantar-flexion) of the footplate resulted in raising a water reservoir, the resistance of which could be changed during the constant-load exercise tests by adding known quantities of water. For the initial ramp test, water was added continuously at a constant rate using a roller pump (Cole-Parmer Instruments, Chicago, IL).

The plantar-flexion exercise involved the participant repeatedly depressing the footplate of the exercise ergometer through ~35° range of motion (ROM). To ensure appropriate exercise cadence during plantar-flexion exercise, a metronome was set

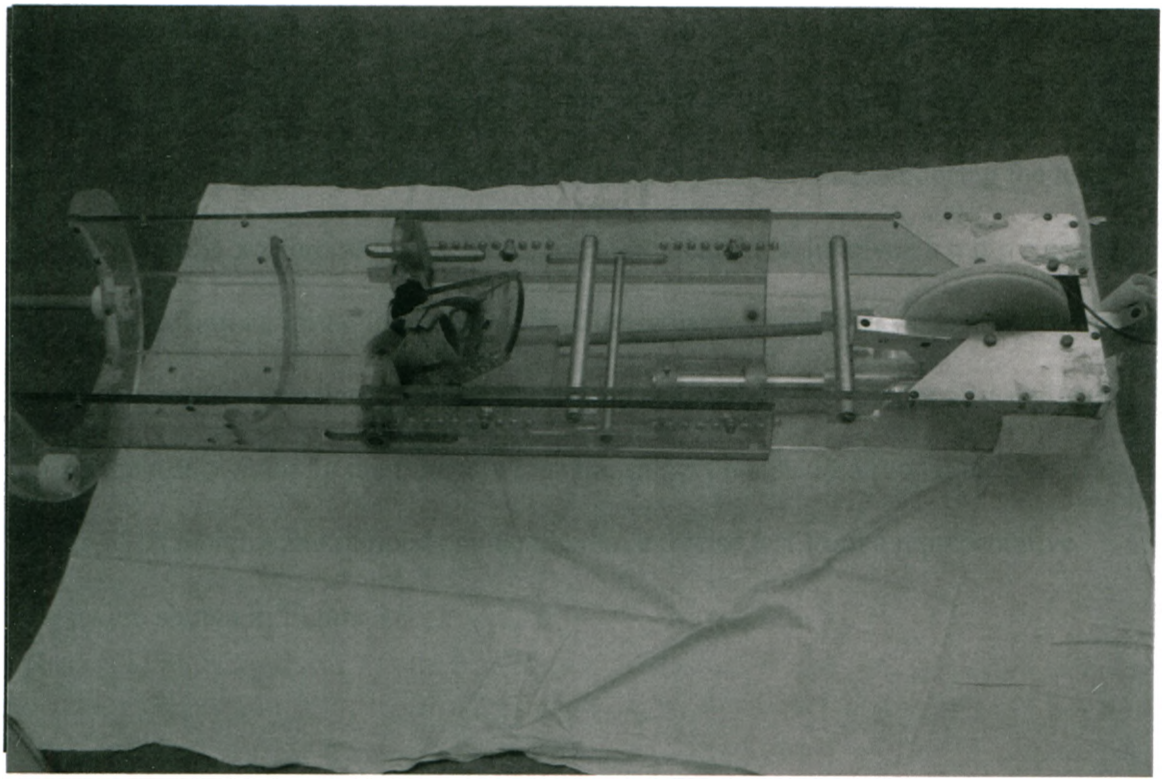

**Figure 2.** Custom-built MR-compatible ankle exercise ergometer used during the experimental protocol.

at 0.50 Hz (1 second contraction – 1 second relaxation) to provide an auditory cue for the subject. To ensure that subjects maintained a consistent ROM with each muscular contraction, the ergometer was connected to a computer, allowing a light emitting diode to signal the start (0°) and end (35°) of plantar-flexion ROM to the subject. Contraction frequency and ROM data were displayed on a computer screen visible to the investigator and acquired using a commercial computer software program (Spike2 software, version 4.13, Cambridge Electronic Design, Cambridge, UK).

During the initial incremental ramp test, subjects began by resting on the table inside the bore of the 3.0-T magnet while resting measurements were recorded. After this period of resting data collection, subjects began plantar-flexion exercise. At the same time that exercise commenced, water began to flow continuously into a reservoir at a rate

of ~1.4 kg/min. The calculation of power output was performed using the known repetition rate (0.5 Hz) during exercise, displacement of the water reservoir per contraction (0.08 m), and the weight of the reservoir plus water added during the test [1.7 kg + (1.4 kg/min x exercise time)]. Thus, the incremental test involved a ramp slope of ~0.6 W/min from an initial load of ~0.7 W. The actual rate at which water was pumped into the reservoir was calculated after each ramp test as the total volume of water added during the test divided by the time at which the subject reached volitional fatigue. The results of an incremental ramped plantar-flexion exercise test from a representative subject are shown in Figure 3.

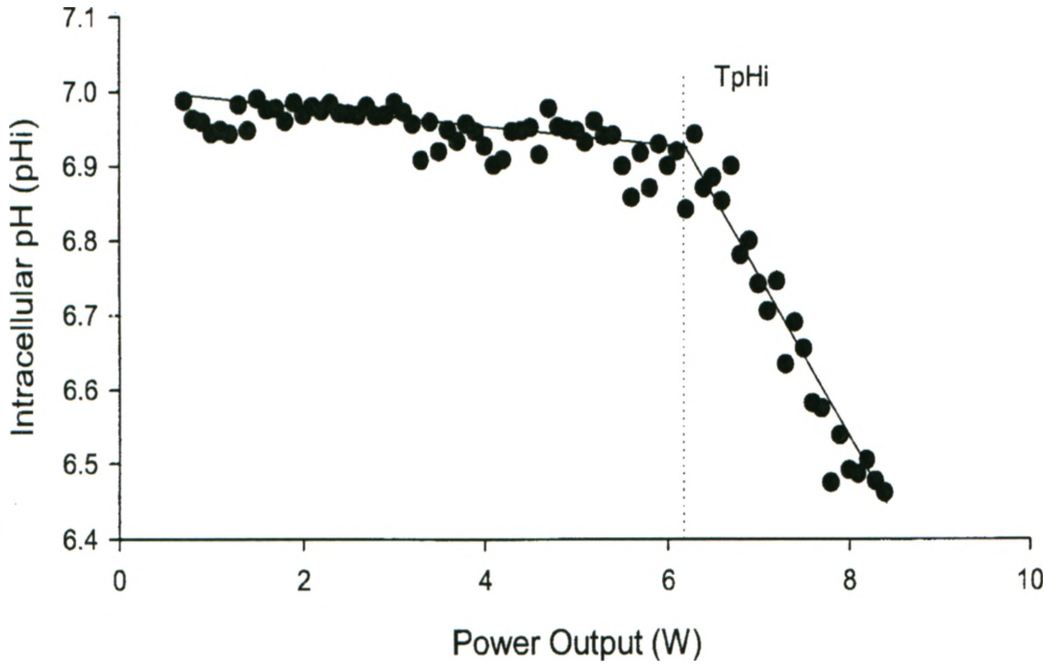


**Figure 3.** Intracellular pH (pHi) plotted over power output in a representative subject during an incremental ramp plantar-flexion exercise test. Note the intracellular pH threshold (TpHi) or onset of acidosis ~6.2 watts.

The workloads corresponding to the moderate- and heavy-intensity exercise domain were calculated from the results of each subject's incremental ramp exercise test. For each subject, pHi was plotted over power output and a piecewise linear regression algorithm was applied to determine the intracellular pH threshold (TpHi) or onset of intracellular acidosis. From the estimation of TpHi, 75% of the power output corresponding to TpHi was used during moderate-intensity exercise and 125% of the power output corresponding to TpHi was chosen as the heavy-intensity workload (Table 1).

| | Power Output at TpHi, W | Moderate Workload, W | Heavy Workload, W | Peak Power Output, W |
|---|---|---|---|---|
| Mean (SD) | 4.66 (2.17) | 3.50 (1.63) | 5.83 (2.71) | 9.07 (1.83) |

TpHi, Intracellular pH threshold.

**Table 1.** Power output at intracellular pH threshold (TpHi) during a ramped plantar-flexion exercise test, and mean moderate- and heavy-intensity workloads used during the experimental trials.

Prior to both the CON and ACID experimental exercise trials, subjects reported to the laboratory having abstained from caffeine, alcohol, and lower body exercise for at least 24 hours. For the ACID trial, subjects consumed gel caps containing ammonium chloride powder ($NH_4Cl$) (oral dose of 0.3 g/kg body mass) in equally divided doses over a three-hour period prior to the exercise test. No placebo was given before the CON trial as the side effects of $NH_4Cl$ can include nausea, gastric distress, and bloating and would

be recognizable to the subjects. The experimental trials were separated by at least one week.

In both the CON and ACID exercise trials, three minutes of resting data were collected prior to the initiation of warm-up exercise. Warm-up exercise involved the participant repeatedly depressing the footplate to elevate the empty water reservoir (mass = 1.7 kg). Following three minutes of warm-up exercise, subjects began nine minutes of moderate-intensity exercise. At the nine-minute point of moderate-intensity exercise, subjects immediately transitioned into nine minutes of heavy-intensity exercise, followed by a fifteen minute recovery period. The step increase in power output from warm-up to moderate, and from moderate- to heavy-intensity exercise was achieved by the investigator adding known quantities of water to the cable-pulley system of the exercise ergometer.

### 2.3 $^{31}$P- Magnetic Resonance Spectroscopy

Phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) was used to study intracellular muscle metabolism both at rest, and during exercise conditions. Data were collected using a 64-cm-bore, 3.0-Tesla superconducting magnet interfaced with a SMIS/IMRIS console (Surrey Medical Imaging Systems, Guilford, UK; Innovative Magnetic Resonance Imaging Systems, Winnipeg, Canada). A 4-cm square $^{31}$P surface coil was positioned on the exercising leg (right leg) over the lateral belly of the gastrocnemius muscle. The $^{1}$H signal was used for shimming to adjust the magnet homogeneity and improve the spectral resolution. Shimming continued until the proton

signal from water resulted in a peak with a full width at one half maximum of ~20-25 Hz and displayed a Lorentzian shape.

All $^{31}$P-MRS spectra collected during the experimental trials were collected continuously with a 10 second repetition delay. Spectra were collected with a 3-millesecond, 90°– adiabatic radio–frequency pulse, a 3.33 kHz receiver bandwidth, and 4,096 complex data points. An example of $^{31}$P-MRS spectra and changes in PCr and Pi during exercise are shown in Figure 4.

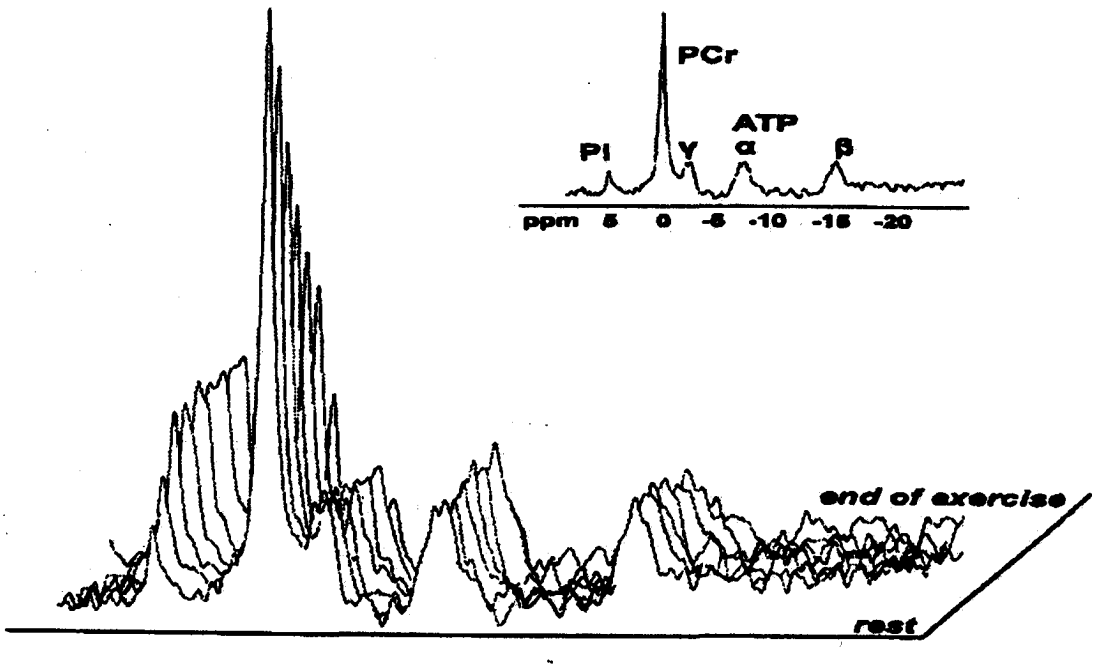


**Figure 4.** Example of sequential $^{31}$P-MRS spectra during exercise.
Note the decrease in PCr and increase in Pi from rest through exercise.

### 2.4 Data Analysis

Quantification of the $^{31}$P-MRS skeletal muscle metabolite data was performed in the time domain by fitting each raw $^{31}$P free induction decay (FID) to a sum of damped sinusoids which could be varied in terms of amplitude, phase, delay time, damping

constant, and frequency. This method used a priori knowledge and a nonlinear least squares algorithm (Marquardt, 1963) to iteratively reduce the difference between the data and the experimental model (Bartha et al., 1999). The concentrations of the phosphate resonances (Pi, PCr, and α-, β-, γ-ATP) were determined from the amplitudes of the exponential model function at time zero (0). Phosphate peaks were corrected for partial saturation (Taylor et al., 1983).

Prior to beginning the experimental protocol, baseline spectra were collected that consisted of an average of four acquisitions having a repetition time of 30 seconds each. Calculation of the longitudinal relaxation (T1) correction factors was carried out using the difference in amplitude of the $^{31}$P metabolites in the baseline spectrum (no T1 effects present) and the resulting spectra collected during the resting period of the experimental protocol (18 spectra acquired with a 10 second repetition time (T1 saturated)). Absolute PCr concentration values were calculated by using the PCr/ATP ratio, and by assuming a resting ATP concentration of 8.2 mM (Harris et al., 1974). The pHi was determined from the chemical shift (in parts per million) of Pi relative to PCr (Taylor et al., 1983). ADP concentration ([ADP]) was calculated using Equation 1 by assuming a total creatine concentration of 42 mmol/l (Harris et al., 1974) and that the CK reaction was at equilibrium.

**Equation 1.**

$$[ADP] = [ATP]\,[Cr] / [PCr]\,[H^+]\,Keq$$

where, Keq is the equilibrium constant for the CK reaction.

## 2.5 PCr Kinetic Analysis

[PCr] kinetic responses were fit with a mono-exponential model using nonlinear least squares regression techniques (SigmaPlot 2000). The formula is described in Equation 2. Responses for each work transition (i.e. transition to moderate- and transition to heavy-intensity exercise) were analyzed separately.

**Equation 2.**

$$\Delta [PCr]_{(t)} = [PCr]_0 - \Delta[PCr]_{ss} (1 - e^{-t/\tau})$$

In the above formula, $[PCr]_0$ is the steady state [PCr] before the step increase in work-rate (rest to moderate- or moderate- to heavy-intensity exercise), $\Delta[PCr]_{ss}$ is the amplitude change from baseline ([PCr]) to the [PCr] plateau (steady state) in which the simple exponential phase projects, and $\tau$ is the time constant of the response (i.e. time taken to reach 63% of the final amplitude in an exponential function). During the transition to moderate-intensity exercise, the data were fit starting at the time corresponding to the transition in work rate and continued until the end of exercise (9 minutes). During the transition to heavy-intensity exercise, the data were again fit from the time corresponding to the transition in work rate but continued only to the estimated onset of the PCr slow component (~1.5 - 3.5 min depending on the subject).

For analysis of the PCr kinetic response during heavy-intensity exercise, the methods used were similar to those reported by Forbes and colleagues (Forbes et al., 2005). The entire PCr response (9 min) was not included in the data field for the fit, as was the case for moderate exercise. Instead, the PCr data were fit beginning at the time corresponding to the start of heavy exercise and continued to initially, 60 seconds. The

data were then fit with the inclusion of each subsequent data point until the exponential model fit showed a discernable deviation from the measured response. Once the exponential model fit began to diverge (discernable drop in R-squared value) from the measured PCr data, the fitting field was moved back in time by one data point to ascertain the fundamental component of the response and the best fit mono-exponential. The goodness of fit was determined by 1) maintenance of a flat residual profile, and 2) the highest correlation (R value) obtained between the measured data and the simple exponential model fit. Therefore, the fit that resulted in a discernable drop in the R-squared value was used to establish the onset of the PCr slow component. The magnitude of the PCr slow component was then estimated from the difference between the projected steady state [PCr] from the simple exponential model fit and the actual measured [PCr] at end (9 min) of heavy-intensity exercise.

**2.6 Statistical Analysis**

Statistical analyses were performed using SigmaStat version 3.1. statistical software (Systat). Before analyses, the intracellular metabolic data (PCr, pHi, and free ADP) were each binned and averaged over 3 minute periods: rest, warm-up, early, middle, and late stages of moderate- and heavy-intensity exercise. Intracellular measurements were analyzed for condition, time, and interaction effects by two-way repeated measures analysis of variance (ANOVA). Further, PCr kinetic parameter estimates were analyzed for condition, intensity, and interaction effects. Differences in the magnitude of the PCr slow component between conditions were tested using a 1-way ANOVA with repeated measures. When a significant F-ratio was found, a post hoc

analysis was performed using a Student-Newman-Keuls post hoc test at specific time points. Significance was set at the $P < 0.05$ level for all comparisons. Data presented are reported as means (SD).

# CHAPTER 3: RESULTS

## 3.1 Intracellular pH (pHi)

During pre-exercise resting conditions (post $NH_4Cl$ ingestion in ACID), pHi was not different between ACID (pHi 6.96 (SD 0.02)) and CON (pHi 6.95 (SD 0.01)). No differences in pHi were found between conditions during warm-up exercise (ACID pHi 6.97 (SD 0.02); CON pHi 6.96 (SD 0.02)) or during moderate-intensity exercise (ACID pHi 6.93 (SD 0.01); CON pHi 6.93 (SD 0.02)). During heavy-intensity exercise, no differences ($P > 0.05$) between conditions were found in pHi during the Early (0-3 min) stages (ACID pHi 6.78 (SD 0.05); CON pHi 6.81 (SD 0.06)), however during the Middle (3-6 min) and Late (6-9 min) stages of exercise, pHi was lower ($P < 0.05$) in ACID (pHi 6.63 (SD 0.04) (Middle stages), (pHi 6.64 (SD 0.06) (Late stages)) than CON (pHi 6.70 (SD 0.08) (Middle stages), (pHi 6.70 (SD 0.09) (Late stages)) (Figure 5).

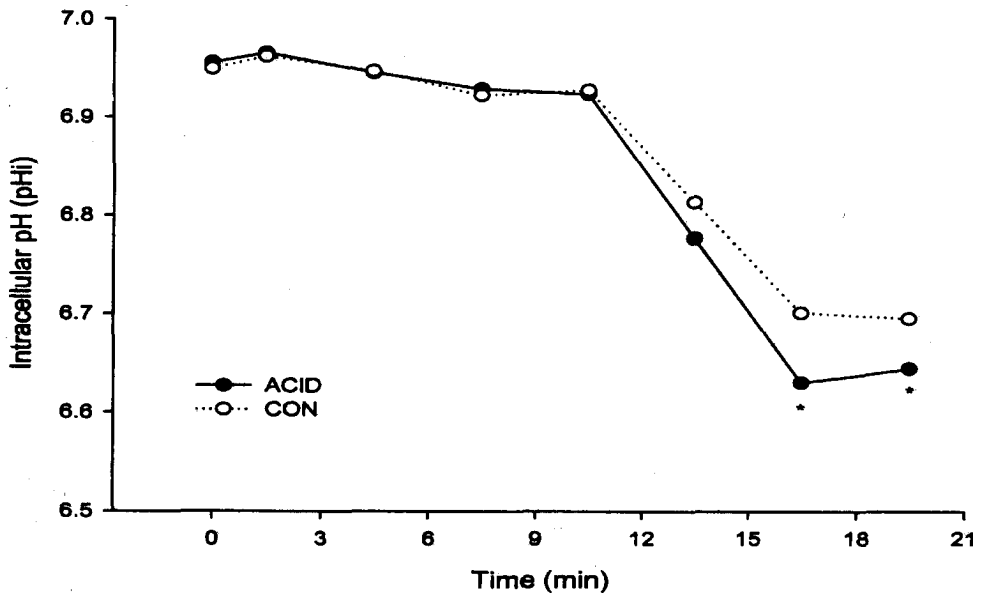


**Figure 5.** Intracellular pH (pHi) during ACID and CON conditions at rest, warm-up, moderate-, and heavy-intensity exercise. Values are means. * Difference between conditions ($P < 0.05$).

### 3.2 Phosphocreatine (PCr)

[PCr] changes in a representative subject (control condition) during rest, warm-up, moderate-, heavy-intensity exercise, and recovery can be seen in Figure 6. [PCr] was not different ($P > 0.05$) between conditions during rest (ACID [PCr] 37.5 mmol/l (SD 1.95), CON [PCr] 38.0 mmol/l (SD 2.35)), warm-up (ACID [PCr] 34.46 mmol/l (SD 2.10), CON [PCr] 35.16 mmol/l (SD 2.93)) or moderate-intensity exercise (ACID [PCr] 28.61 mmol/l (SD 2.95), CON [PCr] 29.72 mmol/l (SD 3.34)). During the Early (0-3 min) and Middle (3-6 min) stages of heavy-intensity exercise [PCr] was lower ($P < 0.05$) in ACID ([PCr] 18.14 mmol/l (SD 5.14) (Early stages), [PCr] 14.12 mmol/l (SD 5.41) (Middle stages)) than CON ([PCr 20.42 mmol/l (SD 5.43) (Early stages), [PCr 16.73 mmol/l (SD 6.03) (Middle stages)) (Figure 7). No differences ($P > 0.05$) in [PCr] between conditions were evident during the Late stages of heavy-intensity exercise (ACID [PCr] 13.50 mmol/l (SD 6.46), CON [PCr] 14.90 mmol/l (SD 6.26)).

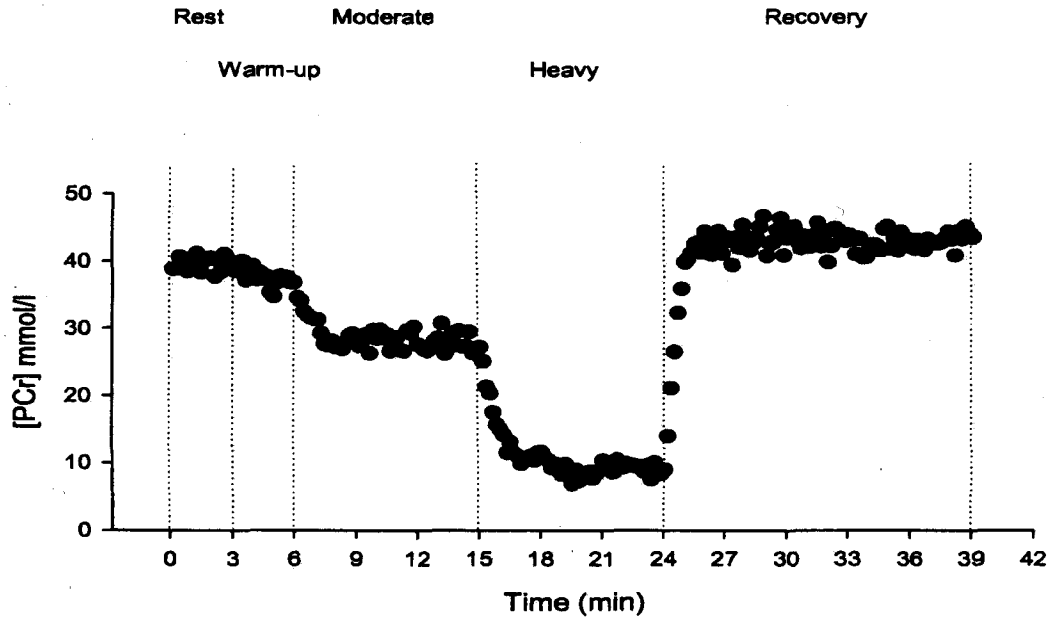


**Figure 6.** Phosphocreatine [PCr] response of a representative subject (control condition) during rest, warm-up, moderate-, heavy-intensity exercise, and recovery.

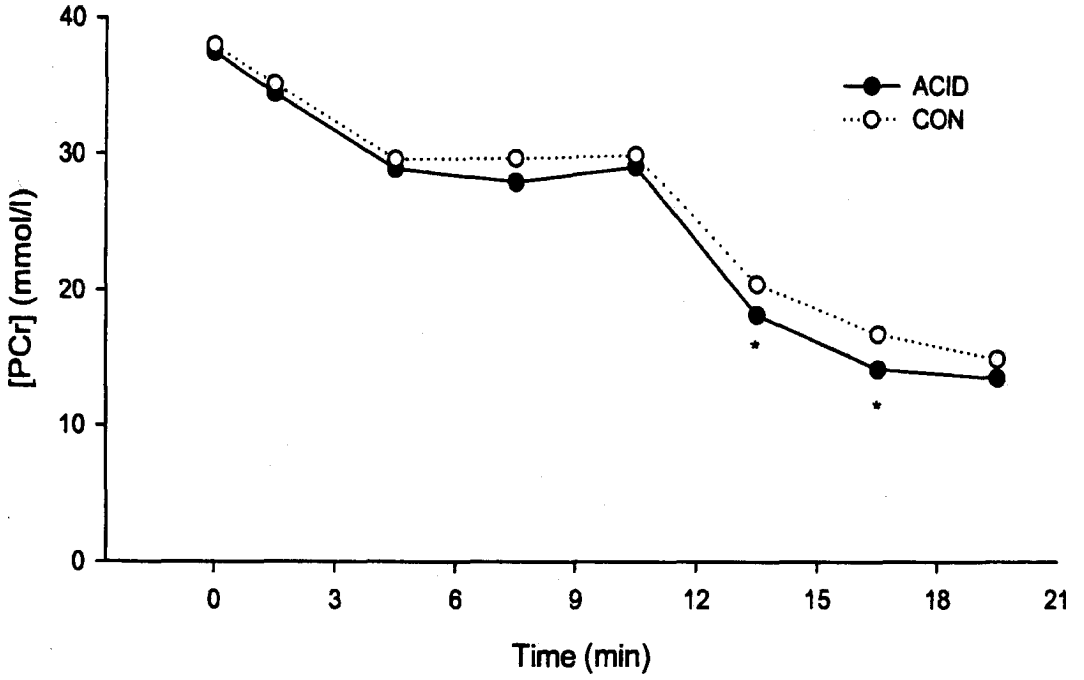


**Figure 7.** Phosphocreatine concentration [PCr] during ACID and CON conditions at rest, warm-up, moderate-, and heavy-intensity exercise. Values are means.
* Difference between conditions ($P < 0.05$).

### 3.3 Phosphocreatine Kinetic Response

To gain further insight into the PCr response during heavy-intensity exercise, the time course of adaptation of [PCr] was analyzed during both the transition to moderate-intensity exercise, and during the transition from moderate- to heavy-intensity exercise. There was no difference in the $\tau$ of PCr breakdown between conditions during the transition to moderate- or heavy intensity-exercise ($P > 0.05$) (Table 2). However, there was a significant ($P < 0.05$) difference in the $\tau$PCr between the transition to moderate- and to heavy-intensity exercise for both conditions (Table 2). There was no difference between ACID and CON in the amplitude of the primary component of PCr breakdown during the transition to moderate-intensity exercise ($P > 0.05$) (Table 2). However, the amplitude of the primary component of PCr breakdown during the transition from

moderate- to heavy-intensity exercise was significantly greater ($P < 0.05$) in ACID than CON (Table 2). There was no difference in the time of onset, or in the magnitude of the PCr slow component between ACID and CON ($P > 0.05$) (Table 2).

| | | Control | Acidosis |
|---|---|---|---|
| Transition 1 | Primary component τ, sec | 29.68 (15.29) | 27.26 (14.26) |
| | Primary amplitude, mmol/l | 8.63 (2.66) | 9.22 (3.57) |
| Transition 2 | Primary component τ, sec | 50.79 (19.98)† | 58.16 (22.16)† |
| | Primary amplitude, mmol/l | 11.31 (4.79) | 14.54 (5.79)* |
| | SC amplitude, mmol/l | 4.01 (2.59) | 2.66 (1.22) |
| | SC delay time, sec | 225.0 (72.0) | 217.5 (92.0) |

**Table 2.** Phosphocreatine (PCr) kinetic parameters during the transition to moderate-intensity exercise (transition1) and during the transition from moderate- to heavy-intensity exercise (transition 2).

Values are means (SD). Phosphocreatine (PCr) kinetics are expressed as time constant (τ) and amplitude change during the primary and slow component (SC) phase.
* Significantly different ($P < 0.05$) from control.
† Significantly different ($P < 0.05$) from transition 1.

### 3.4 Free Adenosine Diphosphate (ADP)

Calculated free cytosolic [ADP] was not different between ACID and CON during pre-exercise resting conditions (ACID [ADP] 0.02 mmol/l (SD 0.006), CON [ADP] 0.02 mmol/l (SD 0.008)), warm-up (ACID [ADP] 0.03 mmol/l (SD 0.01), CON [ADP] 0.03 mmol/l (SD 0.01)), or moderate-intensity exercise (ACID [ADP] 0.06 mmol/l (SD 0.01), CON [ADP] 0.06 mmol/l (SD 0.02)). During heavy-intensity exercise, free [ADP] increased in both ACID and CON, however, during the Late stages of heavy-intensity exercise [ADP] was greater ($P < 0.05$) in ACID ([ADP] 0.31 mmol/l (SD 0.12) than CON ([ADP] 0.25 mmol/l (SD 0.12)) (Figure 8).

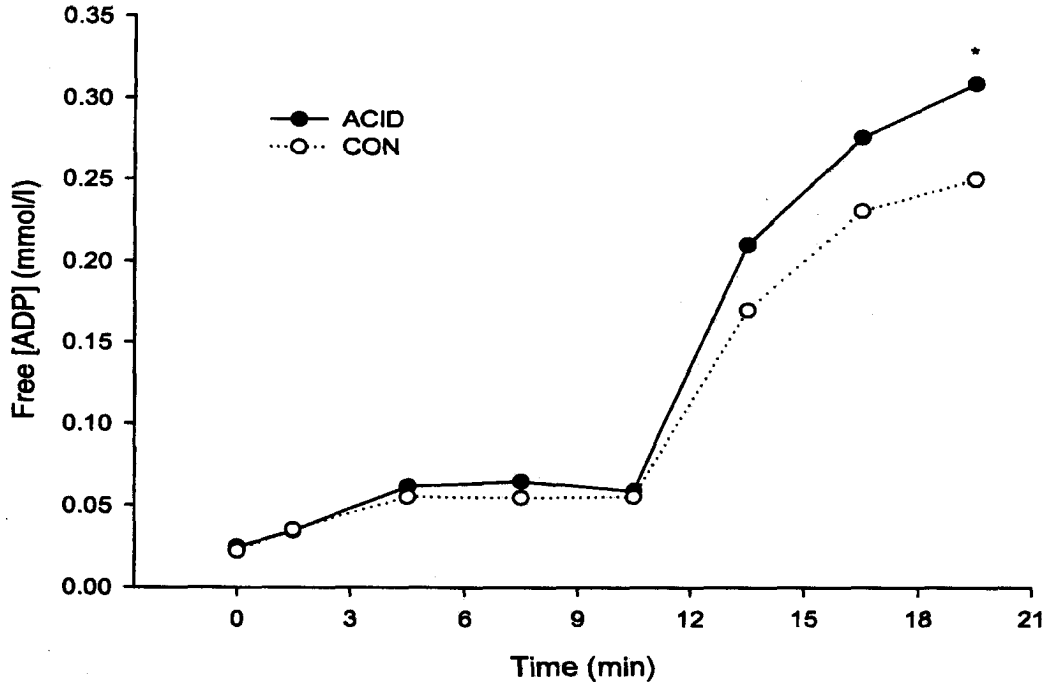


**Figure 8.** Calculated free cytosolic adenosine diphosphate concentration [ADP] during ACID and CON conditions at rest, warm-up, moderate-, and heavy-intensity exercise. Values are means. * Difference between conditions ($P < 0.05$).

## CHAPTER 4: DISCUSSION

The present study examined the metabolic response(s) to both moderate- and heavy-intensity constant load plantar-flexion exercise following acute $NH_4Cl$ ingestion. $^{31}$P-MRS was used to non-invasively monitor changes in skeletal muscle pHi, [PCr], and calculated free [ADP] at rest, during the exercise protocol, and during recovery from exercise. Consistent with the findings of Hollidge-Horvat and colleagues who reported lower pHi following $NH_4Cl$ induced acidosis during cycling exercise at 75% (ACID: 6.62 ± 0.04 vs. CON: 6.82 ± 0.04), but not at 30% or 60% $VO_2$ max (Hollidge-Horvat et al., 1999), $NH_4Cl$ ingestion in the present study was associated with a lower pHi during heavy-intensity exercise conditions (Middle and Late stages) compared to CON. There were no differences between ACID and CON in pHi at rest (post $NH_4Cl$ ingestion) or during moderate-intensity exercise. Consistent with the hypothesis that [PCr] would be reduced following $NH_4Cl$ ingestion during heavy exercise performed above TpHi, the lower pHi (acidosis) observed during the Middle (3-6 min) stages of heavy-intensity exercise was associated with greater PCr hydrolysis. [PCr] was reduced in ACID relative to CON during the Early (0-3 min) and Middle (3-6 min) stages of heavy-intensity exercise. Further, $NH_4Cl$ ingestion resulted in a greater amplitude of the fundamental component of PCr breakdown during the transition from moderate- to heavy-intensity exercise. Taken together, these findings are in agreement with the hypothesis that a greater intracellular acidosis during heavy-intensity exercise following $NH_4Cl$ ingestion would be associated with greater PCr breakdown relative to CON during exercise above the estimated TpHi. However, in contrast to this hypothesis, $NH_4Cl$ ingestion was not associated with an increase in the magnitude of the PCr slow component during heavy-

intensity exercise. Lastly, calculated free [ADP] was increased during the Late (6-9 min) stages of heavy-intensity exercise in ACID compared to CON. This may be explained by a falling pHi at exercise intensities above TpHi in combination with $NH_4Cl$ ingestion driving the CK reaction in the direction of PCr breakdown, thus allowing for a constant rate of ADP provision to the mitochondrion to drive oxidative phosphorylation (Conley et al., 2001).

**4.1 Pre-exercise Resting Conditions**

The finding of no significant effect on measured pHi following $NH_4Cl$ ingestion during resting conditions is in agreement with other investigations that have used the same dose of 0.3g/kg body mass over a three hour period prior to exercise (e.g. Hollidge-Horvat et al., 1999; Hultman et al., 1985). The lack of any significant effect on pHi following $NH_4Cl$ ingestion during resting conditions has been taken to suggest that initially, intracellular buffers can sustain the amount of added protons (Hultman et al., 1985). During resting conditions prior to exercise, measured blood pH and bicarbonate ($HCO^-_3$) values have been reported to be consistently lower following $NH_4Cl$ ingestion (e.g. Hood et al., 1988; Hollidge-Horvat et al., 1999; and Hultman et al., 1985). Indeed, an acknowledged limitation of the present investigation is that no blood samples were collected from the study participants to confirm that $NH_4Cl$ ingestion did in fact result in an acidosis of the blood. However, the present investigation employed an identical dosage protocol that has been used in previous investigations (0.3g/kg body mass of $NH_4Cl$ over a three hour period prior to exercise) and has been reported to result in a significant metabolic acidosis, with venous blood pH values falling anywhere between 0.08 - 0.16 pH units (Hollidge-Horvat et al., 1999; Hultman et al., 1985; Jones et al.,

1977; and Kowalchuk et al., 1984). For example, Hollidge-Horvat reported that following $NH_4Cl$ ingestion (0.3 g/kg bodyweight), venous blood pH was reduced from 7.37 ± 0.01 – 7.21 ± 0.01 mmol/l (Hollidge-Horvat et al., 1999). In the present study, the measured intramuscular metabolites did not appear to be affected by $NH_4Cl$ ingestion during resting conditions, as there was no difference in [PCr] or calculated free [ADP] during this time period. This finding is consistent with those of Hollidge-Horvat and coworkers who also reported no difference in PCr following $NH_4Cl$ ingestion (PCr ACID: 83.7 ± 5.2 mmol/kg vs. PCr CON: 87.4 ± 4.5 mmol/kg) (Hollidge-Horvat et al., 1999). In the present investigation it is worth noting that the estimated absolute [PCr] in mmol/l (ACID: 37.5 (1.95) mmol/l vs. CON: 38.0 (2.35) mmol/l) is consistent with previous reports of estimated [PCr] in the human calf muscle measured using $^{31}$P-MRS (for review see: Kemp et al., 2007). Correction factors for saturation effects in the present study were as follows: PCr 1.31-1.57; γ-ATP 1.10-1.31; α-ATP 1.18-1.21; β-ATP 1.02-1.15.

**4.2 Moderate-intensity Exercise**

During the transition to moderate-intensity exercise performed below TpHi, pHi values remained stable in both the CON and ACID trials. This finding is similar to that reported by Hollidge-Horvat (Hollidge-Horvat et al., 1999) who reported no change in pHi following $NH_4Cl$ ingestion during cycling at 30% and 60% of subjects $VO_2$ max. The TpHi (also referred to as the 'lactate threshold') typically occurs during exercise at 45%-60% $VO_2$ max and can be as high as 80% in trained athletes (Jones and Poole, 2005). Moderate-intensity exercise encompasses all work-rates below the lactate threshold (Jones and Poole, 2005), thus exercise corresponding to 30% $VO_2$ max, and possibly exercise at 60% $VO_2$ max (as used by Hollidge-Horvat et al., 1999) would fall

into the moderate-intensity exercise domain. The finding of stable pHi during moderate-intensity indicates that the energy equivalent to the $O_2$ deficit incurred prior to reaching steady state $VO_2$ levels was met primarily via PCr hydrolysis. As mentioned previously, the fundamental component of the PCr kinetic response has been shown to provide a reasonable estimate of the adaptation of muscle $O_2$ utilization during the on-transient to moderate-intensity exercise (McCreary et al., 1996; Rossiter et al., 1999). Given that there were no differences between ACID and CON for both the $\tau$ and amplitude of the fundamental component of PCr breakdown during the transition to moderate-intensity exercise (see Table 2), the results suggest that the rate of increase in oxidative phosphorylation and its contribution to energy production were not appreciably different between the two trials. The present investigation is the first study to examine the effect(s) of $NH_4Cl$ ingestion on the kinetic response of PCr during exercise. Another investigation (Zoladz et al., 1998) examining pulmonary $VO_2$ during cycling exercise below the lactate threshold (40% of $VO_2$ max) following $NH_4Cl$ ingestion found no difference in absolute pulmonary $VO_2$, however the kinetic response was not measured in that study (Zoladz et al., 1998). Similarly, calculated free [ADP] was not different between ACID and CON at any time point during moderate-intensity exercise (see Figure 8), suggesting a similar quantity of the signal thought to play a key role in the control of oxidative phosphorylation. This finding is consistent with those of Hollidge-Horvat who reported no difference in free [ADP] concentration during cycling exercise at 30% and 60% of subjects $VO_2$ max following $NH_4Cl$ ingestion (Hollidge-Horvat et al., 1999).

**4.3 Heavy-intensity Exercise**

As can be seen from Figure 5, pHi was lower in ACID compared to CON and reached statistical significance during the Middle and Late stages of heavy-intensity exercise. Similarly, Hollidge-Horvat and coworkers reported a lower intramuscular pH following $NH_4Cl$ induced acidosis during cycling exercise at an intensity corresponding to 75% of each subjects $VO_2$ max (Hollidge-Horvat et al., 1999). Venous plasma pH values in this study were reduced following $NH_4Cl$ ingestion, and were significantly lower during exercise intensities corresponding to 30%, 60%, and 75% $VO_2$ max (Hollidge-Horvat et al., 1999). Hultman and colleagues also reported that intramuscular pH was reduced during electrical muscle stimulation following $NH_4Cl$ ingestion relative to control conditions (Hultman et al., 1985). Other studies however, have found no difference in end-exercise pHi values following $NH_4Cl$ ingestion compared to control conditions (Hood et al., 1988). However, in the study by Hood and coworkers, exercise involved the study participants squeezing a rubber ball until exhaustion, thus the quantity of work performed in each condition was not measured and cannot be compared with accuracy (Hood et al., 1988). The ingestion of $NH_4Cl$ appears to affect primarily the extracellular space, with secondary effects on the intracellular space becoming evident at higher intensities of exercise. $NH_4Cl$ ingestion has been reported to be associated with reductions in lactate during exercise, and this appears to due to reduced lactic acid generation (Hood et al., 1988) and reduced lactate accumulation and efflux resulting from a reduced lactate gradient (Hollidge-Horvat et al., 1999). No lactate measurements were made in the present study, however the reduced pHi seen following $NH_4Cl$ ingestion has been suggested to be due to an acidotic inhibition of the lactate transporter due to an

altered $H^+$ gradient or to reduced extracellular [$HCO^-_3$] (Hollidge-Horvat et al., 1999). Lactate transport across the muscle membrane is enabled by a monocarboxylate lactate-proton cotransport protein, and therefore limits the rate of lactate efflux (Hollidge-Horvat et al., 1999). Diffusion of lactic acid (undissociated form of lactate) is thought to account for only 20% of lactate efflux, whereas the monocarboxylate protein carrier is thought to account for 70-90% of lactate transport across the physiological range of lactate concentration (Hollidge-Horvat et al., 1999). Elevated [$H^+$] within the muscle (lower pHi) is thought to increase the proportion of lactate in the associated form and thus reduce the availability of lactate to the transporter (Hollidge-Horvat et al., 1999). Several investigations have also found that $NH_4Cl$ ingestion reduces extracellular [$HCO^-_3$] (e.g. Hollidge-Horvat et al., 1999; Hood et al., 1988; Hultman et al., 1985; and Kowalchuk et al., 1984). There is evidence that under low external [$HCO^-_3$] conditions in isolated muscle preparations, there is a reduced lactate efflux (Hirche et al., 1975). While lactate and [$HCO^-_3$] were not directly measured in the present study, the aforementioned investigations suggest a possible mechanism for the lower pHi seen during the Middle and Late stages of heavy-intensity exercise following $NH_4Cl$ ingestion.

Reduced [PCr] was observed during the Early and Middle stages of heavy-intensity exercise following $NH_4Cl$ ingestion (Figure 7) compared to CON. An investigation by Sahlin and coworkers examining the effect of acidosis on isolated rat skeletal muscle, reported a decrease in [PCr] to 44% of the control value following electrically stimulated tetanic contractions (Sahlin et al., 1983). Alternatively, other studies (Hollidge-Horvat et al., 1999; Hood et al., 1988) have reported no difference in [PCr] following $NH_4Cl$ ingestion during cycling exercise performed at different

intensities relative to calculated $VO_2$ max (Hollidge-Horvat et al., 1999) or during forearm exercise that involved study participants squeezing a rubber ball (Hood et al., 1988). However, in the study by Hood and coworkers there was also no difference in end exercise pHi relative to control conditions following $NH_4Cl$ ingestion (Hood et al., 1988). Another study by Forbes and coworkers reported that $NaHCO^-_3$ ingestion and the resulting alkalosis was associated with a reduction in PCr breakdown during heavy-intensity forearm exercise (Forbes et al., 2005). The reduced PCr breakdown following induced alkalosis, was suggested to be due to a reduced ATP demand from substrate level phosphorylation in the absence of acidosis (Forbes et al., 2005). The attenuated acidosis following $NaHCO^-_3$ ingestion in the study by Forbes and coworkers was also associated with a reduced [ADP] accumulation during the final six minutes of heavy-intensity exercise, however there were no differences in the estimated level of oxidative flux (Forbes et al., 2005). Similarly, in the present study, acidosis was associated with a greater [ADP] during the Late stages of heavy-intensity exercise, consistent with the notion that at submaximal exercise intensities, the inhibitory effect of acidosis on the rate of oxidative phosphorylation may be overcome by a greater quantity of the signal [ADP] (Walsh et al., 2002; Forbes et al., 2005). Sahlin and coworkers reported similar findings, as acidosis was associated with an increase in [ADP] in isolated rat skeletal muscle (Sahlin et al., 1983). The increase in [ADP] reported by Sahlin and coworkers was observed when lactate concentration was similar to control muscles and very low in both groups (Sahlin et al., 1983). Thus, the increased [ADP] was suggested to be due to the effects of increased $[H^+]$ on phosphofructokinase (PFK) activity. Sahlin and coworkers suggested that the inhibitory effects of $[H^+]$ on PFK resulted in decreased rates of

pyruvate formation and thus a decrease in substrate supply to the tricarboxylic acid cycle from carbohydrates. Inadequate supply of substrate to the tricarboxylic acid cycle would be expected to decrease the rate of oxidative phosphorylation and cause a decrease in ATP and an increase in [ADP] (Sahlin et al., 1983). However, not all studies have reported higher [ADP] during acidotic conditions. Hollidge-Horvat and coworkers reported that free [ADP] was reduced relative to control conditions during cycling exercise at 75% of $VO_2$ max following $NH_4Cl$ induced acidosis (Hollidge-Horvat et al., 1999). These differences are difficult to reconcile as Hollidge-Horvat and coworkers also reported that $NH_4Cl$ ingestion reduced PFK activity and resulted in significantly reduced pyruvate production during exercise at 30%, 60%, and 75% $VO_2$ max (Hollidge-Horvat et al., 1999).

In the present study there was no difference between ACID and CON conditions in the $\tau$ of the fundamental component of PCr breakdown during heavy-intensity exercise, suggesting a similar response of muscle $O_2$ consumption between the two trials. However, $NH_4Cl$ ingestion was associated with a greater amplitude of the fundamental component of PCr breakdown during the transition from moderate- to heavy-intensity exercise. This is the first study to show that $NH_4Cl$ ingestion is associated with a greater amplitude of the fundamental phase of the PCr kinetic response during heavy-intensity exercise performed above the TpHi. The greater amplitude of the on-transient fundamental PCr response suggests that a greater ATP supply from substrate level phosphorylation occurred during the transition to projected steady state (see Methods) heavy-intensity exercise. Taken together, these data are in agreement with the findings of Jubrias and colleagues that acidosis causes a slowing of the rate of oxidative

phosphorylation (Jubrias et al., 2003). Further, the data are generally consistent with the notions of Conley and colleagues, that according to the CK reaction, under low pH conditions a greater PCr breakdown is necessary to provide a given [ADP] stimulus to maintain the required rate of oxidative phosphorylation (Conley et al., 2001). Thus in the present study, the greater PCr breakdown following $NH_4Cl$ ingestion suggests that there was an enhanced PCr related stimulus to maintain the same rate of oxidative phosphorylation.

Despite no significant difference between the ACID and CON trials in the $\tau$ of the fundamental component of PCr breakdown during the on-transient to either moderate- or heavy-intensity exercise, the $\tau$ was greater during the transition to heavy-intensity exercise (ACID: 58.16 (22.16) sec; CON: 50.79 (19.98) sec), than to moderate-intensity exercise (ACID: 29.68 (15.29) sec; CON 27.26 (14.26) sec) for both conditions. This was an unexpected finding, however it is in agreement with the findings of Jones and colleagues (Jones et al., 2008). In their study it was reported that the $\tau$ describing the kinetics of the initial exponential-like fall in [PCr] was significantly different between rest to moderate-, rest to heavy-, and moderate- to heavy-intensity exercise, such that heavy exercise initiated from a baseline of moderate-intensity exercise was associated with the largest $\tau$ value (Jones et al., 2008). In the present study, heavy-intensity plantar-flexion exercise was also initiated from a baseline of moderate-intensity exercise, and this resulted in the $\tau$ being significantly larger than during the transition to moderate-intensity exercise. As noted by Jones and colleagues (Jones et al., 2008), the finding of a significantly larger $\tau$ when heavy-intensity exercise is initiated from a baseline of moderate-intensity exercise suggests that the muscle metabolic responses related to

oxidative phosphorylation do not consistently show linear-first order behaviour (Jones et al., 2008). The exact mechanism(s) behind the longer τ describing the on-transient to heavy-intensity exercise from a baseline of moderate exercise are unknown, however the observed slower kinetics may be related to the metabolic properties of type II muscle fibres (Jones et al., 2008). There is evidence that type II fibres posses slower $VO_2$ kinetics compared to type I fibers (Crow and Kushmerick, 1982), have a greater ATP cost of force production (Crow and Kushmerick, 1982), have lower mitochondrial density and lower oxidative enzyme activity than type I fibres (possibly resulting in slower PCr kinetics (Meyer, 1988)), and finally, would predominantly be recruited during the transition from moderate- to heavy-intensity exercise (Jones et al., 2008). On the other hand, during moderate-intensity exercise, it might be expected that the large majority of the muscle power generated would be from the low-threshold, fatigue resistant type I muscle fibres (Jones et al., 2008). Interestingly, the PCr response during heavy-intensity exercise initiated from a baseline of moderate exercise has been associated with a reduction in the magnitude of the PCr slow component, such that the PCr response begins to return towards being strictly exponential in nature (Jones et al., 2008). In that study Jones and colleagues suggested that the transition to heavy exercise from a baseline of moderate-intensity exercise might involve the recruitment of muscle fibres with more homogeneous characteristics (type II fibres) than is the case during the transition from rest to heavy exercise (in which both type I and type II fibres might be recruited) (Jones et al., 2008). The present study also involved a transition to heavy exercise from a baseline of moderate exercise, however a clear slow component was evident in both CON and ACID conditions. Although not measured in the present study, the magnitude of the

PCr slow component may have been greater had heavy-intensity exercise been initiated from rest rather than moderate-intensity exercise. Forbes and colleagues also reported a clear PCr slow component in their study despite the transition to heavy exercise occurring from an elevated baseline (Forbes et al., 2005). Differences between the findings of the present study and those of Jones and coworkers may relate to the muscle group involved (e.g. gastrocnemius vs. quadriceps) or to the workload employed (125% of TpHi vs. 80% of peak work rate).

In contrast to one of the original hypotheses of this study, $NH_4Cl$ ingestion was not associated with an increase in the magnitude of the PCr slow component during heavy-intensity exercise. The close kinetic coupling between the [PCr] and pulmonary $VO_2$ slow component as measured during constant-load knee-extension exercise, has been taken to suggest that the majority of the $VO_2$ slow component is likely to originate within the exercising muscle (Rossiter et al., 2005). The continued fall in [PCr] during exercise performed above the lactate threshold for the same work rate is thought to be indicative of a progressive loss of muscle efficiency as essentially, there is a greater phosphate cost for the same level of muscle force production (Jones et al., 2008). This finding has been taken to be indicative that the slow component may be due to a higher ATP cost of force production. As pointed out by Rossiter and coworkers, an increased ATP requirement would likely lead to an increased requirement for oxidative ATP production, in turn giving rise to a demand for increased energy buffering by PCr, causing [PCr] to decrease (Rossiter et al., 2005). However, the exact mechanisms responsible for the [PCr] and $VO_2$ slow component are not known. The slow component has been suggested to be associated with the metabolic acidosis and to the recruitment of type II muscle fibres that

accompany exercise performed above the lactate threshold (Jones et al., 2008). Previous work has shown that an attenuated acidosis following $NaHCO^-_3$ ingestion was associated with a reduction in the magnitude of the PCr slow component during heavy-intensity forearm exercise (Forbes et al., 2005). Similarly, $NH_4Cl$ induced acidosis has been shown to increase the magnitude of the $VO_2$ slow component during cycling exercise at 75% $VO_2$ max (Zoladz et al., 1998). However, the slow component in the study by Zoladz and coworkers was simply calculated as the difference in pulmonary $VO_2$ at three minutes and six minutes of exercise (Zoladz et al., 1998). This method of measurement is inaccurate as it may overestimate the magnitude of the slow component if steady state is not reached by the three-minute mark. Ingestion of $NH_4Cl$ in the present study was associated with a greater amplitude of the fundamental PCr response during the transition to estimated steady state heavy-intensity exercise. PCr breakdown has been suggested to indicate an imbalance between ATP supply from oxidative phosphorylation and ATP demand (Conley et al., 2001). The greater breakdown of PCr in ACID indicates that ATP supply from oxidative phosphorylation was unable to match ATP demands, and this necessitated a greater ATP supply from substrate level phosphorylation than seen in CON. Interestingly, this did not result in a greater magnitude of the PCr slow component. This may be because [PCr] was significantly lower only during the Early and Middle stages of heavy-intensity exercise, but not during the Late stages (6-9 min) of exercise when the magnitude of the slow component is estimated (see Methods). This finding is in contrast to the findings of Forbes and colleagues who, examining the effects of $NaHCO^-_3$ induced alkalosis, reported greater PCr breakdown and an increased magnitude of the PCr slow component in the control condition during the Late stages (6-9 min) of heavy-

intensity forearm exercise when [$H^+$] was greater (Forbes et al., 2005). However, other studies have also reported exercise-induced acidosis having no significant affect on the magnitude of the PCr slow component. Forbes and colleagues recently showed that intracellular hydrogen ion concentration was not associated with the amplitude of the PCr slow component (Forbes et al., 2008). They examined the effect of different recovery times on PCr kinetics during repeated bouts of heavy-intensity plantar-flexion exercise. The level of intracellular acidosis at the onset of subsequent exercise was increased with shorter recovery times, but this did not effect the magnitude of the PCr slow component as it averaged ~2% change in [PCr] regardless of the initial level of acidosis prior to the exercise bout (Forbes et al., 2008). Similarly, prior heavy-intensity cycling exercise has been shown to eliminate the $VO_2$ slow component during a subsequent bout of exercise despite significantly elevated muscle and blood lactate concentrations resulting from the prior heavy exercise (Sahlin et al., 2005). Further, the control trial, during which muscle lactate and pH both remained stable, demonstrated a considerable $VO_2$ slow component (Sahlin et al., 2005). The findings of Sahlin and colleagues are consistent with the findings of the present study in that the incurred acidosis (from prior heavy-intensity exercise) resulted in increased PCr breakdown, a higher asymptote of phase II $VO_2$, and no difference in the $\tau VO_2$ during subsequent exercise (Sahlin et al., 2005). Lastly, as the asymptote of the phase II $VO_2$ response was higher following the bout of prior heavy-intensity exercise, it was suggested that the $VO_2$ slow component might have merged into a higher $VO_2$ asymptote and that the two phenomena were inter-related (Sahlin et al., 2005).

Overall, while it appears that acid-base balance does affect PCr breakdown, acidosis does not appear to be the sole cause of the PCr slow component. The $VO_2$ and PCr slow components appear to manifest only at work intensities performed above the lactate threshold, however the link between the $VO_2$ slow component and lactic acid appears to be more of a coincidence than a cause and effect relationship (Sahlin et al., 2005). Other factors such as the progressive recruitment of less efficient type II muscle fibres may play an important role in the development of the slow component, as they have been shown (in mice) to have a greater ATP cost of force production than muscles composed of primarily type I fibres (Crow and Kushmerick, 1982). Studies examining the relationship between $VO_2$ kinetics and the pattern of muscle recruitment, evaluated by transverse relaxation time (T2) magnetic resonance imaging (MRI), have reported a significant positive correlation between the $VO_2$ slow component and the variance of thigh muscle T2 values, supporting the progressive recruitment of muscles within the muscles engaged in external work as a major determinant of the magnitude of the $VO_2$ slow component (Endo et al., 2007). However, Scheuermann and coworkers reported that the slow component of $VO_2$ was not accompanied by changes in the muscle electromyography signal (EMG) during repeated bouts of heavy-intensity cycling exercise, indicative of a lack of additional motor unit recruitment during this time period (Scheuermann et al, 2001). Thus, the exact cause of the slow component of $O_2$ uptake remains to be fully elucidated.

### 4.4 Conclusions and Future Directions

The present study investigated the effect of $NH_4Cl$ ingestion on PCr metabolism during moderate- and heavy-intensity plantar-flexion exercise in healthy young human

males. $^{31}$P-MRS was used to non-invasively, and with relatively high temporal resolution, monitor changes in skeletal muscle [PCr], pHi, [ATP], and calculated free [ADP] during rest, exercise, and recovery. The effects of $NH_4Cl$ ingestion became apparent only during heavy-intensity exercise performed at ~125% of TpHi as no differences between ACID and CON trials were apparent at rest or during moderate-intensity exercise. During the Early and Middle stages of heavy-intensity exercise, there was a lower [PCr] in ACID vs. CON and this was associated with a greater amplitude of the [PCr] primary component during the transition from moderate- to heavy-intensity exercise. During the Middle and Late stages of heavy exercise, $NH_4Cl$ ingestion was associated with a lower pHi (more acidic). Further, calculated free [ADP] was greater following $NH_4Cl$ ingestion during the Late stages of heavy-intensity plantar-flexion exercise. An unexpected finding of this study was that heavy exercise initiated from a baseline of moderate-intensity exercise was associated with a larger $\tau$ describing the fundamental decline in [PCr] compared to the transition to moderate-intensity exercise. This finding suggests a slower rate of the adaptation of muscle $O_2$ consumption during heavy-intensity exercise. The lack of dynamic linearity in the [PCr] response suggests greater complexity in the control of oxidative phosphorylation during heavy-intensity exercise (Jones et al., 2008).

One limitation of the current study was that the kinetic analysis of PCr breakdown for each exercise intensity (Moderate vs. Heavy) and for each condition (ACID vs. CON) involved the study participants undergoing only one transition. The $\tau$PCr values and the amplitude of the fundamental component of PCr breakdown could potentially have been more accurate and been reported with greater confidence had a greater number of exercise transitions been undertaken and measured. However, the $\tau$PCr values describing

the transition to heavy-intensity plantar-flexion exercise are consistent with the values reported by Forbes and coworkers, who reported τPCr values in the range of 54-62 seconds following three transitions per exercise condition (Forbes et al., 2008). Further, all subjects in the present study reported some feelings of nausea and gastric distress following $NH_4Cl$ ingestion, so it was unclear whether subjects would be able to physically tolerate three exercise trials involving $NH_4Cl$ ingestion (ACID condition). Another limitation of the present study was that no blood samples were taken from the study participants. Blood samples would have provided confirmation that the $NH_4Cl$ dosage, the same dosage reported in the literature, did in fact result in an acidosis of the blood. Blood samples would have provided direct evidence that $NH_4Cl$ ingestion resulted in an induced metabolic acidosis, and in conjunction with $^{31}$P-MRS data, may have provided more mechanistic insight with regards to how $NH_4Cl$ and altered acid-base status affect the control of oxidative phosphorylation in exercising human skeletal muscle. There is evidence that heavy-intensity exercise initiated from a baseline of moderate exercise returns to, or again manifests the characteristics of a first order system (mono-exponential), as opposed to heavy exercise initiated from rest, in which a clear slow component manifests (Jones et al., 2008). Thus, while the [PCr] slow component was evident in this study during heavy-intensity exercise, it is possible that its magnitude was significantly reduced by the exercise protocol that was used. The protocol employed in this study has been used successfully during previous investigations (e.g. Forbes et al., 2005) examining acid-base status and skeletal muscle metabolism. The exercise protocol was chosen as it permits examination of the response to both moderate- and heavy-intensity exercise during the same trial. However, it is possible that a larger slow

component may have been evident had heavy-intensity exercise been initiated from rest; the result being greater confidence of the estimation of this unique parameter of $VO_2$. Lastly, as articulated by Zoladz and coworkers, $NH_4Cl$ ingestion and the resulting acidosis do not reflect the true physiology of exercise-induced acidosis. Exercise-induced acidosis is accompanied by enhanced lactate in the blood while $NH_4Cl$ ingestion causes acidosis with decreased levels of lactate in the blood (Zoladz et al., 1998). Further, $NH_4Cl$ ingestion affects primarily the extracellular space with secondary effects on the intracellular space, whereas exercise-induced acidosis has an intracellular origin (Zoladz et al., 1998). Thus, whether the effects of pharmacologically induced acidosis are representative of the physiological effects of exercise induced acidosis is not clear.

Overall, acid-base status does appear to impact skeletal muscle metabolism, particularly during heavy-intensity exercise. Currently, more research has focused on the effects of induced alkalosis (usually through $NaHCO^-_3$ ingestion), possibly because it appears to be associated with improved performance. Only one study has examined the effect of $NH_4Cl$ induced acidosis on the $\tau VO_2$ and magnitude of the phase II $VO_2$ kinetic response (Oren et al., 1982). However, as PCr and pulmonary $VO_2$ are tightly coupled, the results of the present study suggest that $NH_4Cl$ ingestion may be associated with a greater amplitude of the pulmonary $VO_2$ phase II response with no change in the $\tau VO_2$ during heavy-intensity exercise. Further it would be interesting to examine the effects of different levels of acidosis on skeletal muscle metabolism. It may be that acidosis approaching the lower physiological range (i.e. pHi of 6.3) may exert an even more pronounced effect than pHi changes that still remain well within the physiological range

(i.e. pHi of 6.6). Clearly, more research remains to be undertaken in the area of acid-base status and its influence on the control of skeletal muscle bioenergetics.

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## Office of Research Ethics

The University of Western Ontario
*Room 00045 Dental Sciences Building, London, ON, Canada N6A 5C1*
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics


### Use of Human Subjects - Ethics Approval Notice

**Principal Investigator:** Dr. G.D. Marsh
**Review Number:** 13470 **Review Level:** Full Board
**Review Date:** July 24, 2007
**Protocol Title:** Effects of NH4Cl induced acidosis on phosphocreatine metabolism during heavy intensity plantar flexion exercise
**Department and Institution:** Kinesiology, University of Western Ontario
**Sponsor:**
**Ethics Approval Date:** October 19, 2007 **Expiry Date:** July 31, 2008
**Documents Reviewed and Approved:** UWO Protocol, Letter of Information and Consent, Advertisement.
**Documents Received for Information:**

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald

| Ethics Officer to Contact for Further Information | | | |
|---|---|---|---|
| ☐ Janice Sutherland (jsutherland@uwo.ca) | ☑ Jennifer McEwen (jmcewen4@uwo.ca) | ☐ Grace Kelly (grace.kelly@uwo.ca) | ☐ Denise Grafton (dgrafton@uwo.ca) |

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**LAWSON HEALTH RESEARCH INSTITUTE**

**CLINICAL RESEARCH IMPACT COMMITTEE**

RESEARCH OFFICE REVIEW NO.: R-07-416

PROJECT TITLE: Effects of NH4CI induced acidosis on phosphocreatine metabolism during heavy intensity plantar flexion exercise

PRINCIPAL INVESTIGATOR: Dr. GD Marsh

DATE OF REVIEW BY CRIC: October 24, 2007

Health Sciences REB#: 13470

Please be advised that the above project was reviewed by the Clinical Research Impact Committee and the project:

**Was Approved**

**PLEASE INFORM THE APPROPRIATE NURSING UNITS, LABORATORIES, ETC. BEFORE STARTING THIS PROTOCOL. THE RESEARCH OFFICE NUMBER MUST BE USED WHEN COMMUNICATING WITH THESE AREAS.**

Dr. David Hill
V.P. Research
Lawson Health Research Institute

*All future correspondence concerning this study should include the Research Office Review Number and should be directed to Sherry Paiva, Room C210, Nurses Residence, South Street Campus.*

cc: Administration

**Letter of Information**

Effects of $NH_4Cl$ induced acidosis on phosphocreatine metabolism during heavy-intensity plantar flexion exercise

Principal Investigator: GD Marsh, PhD.

You are being invited to participate in a study that examines muscle metabolism during moderate and heavy intensity exercise. In this study we will be examining the breakdown of phosphocreatine or PCr (a compound that supplies energy for muscle contraction) during a control trial and during an ammonium chloride ($NH_4Cl$) induced acidosis trial. Specifically, we will be examining the effect of ammonium chloride induced acidosis on the PCr metabolism during heavy intensity plantar flexion exercise. The amount of PCr used during exercise may be related to an intracellular acidosis, a characteristic of heavy intensity exercise. An acidosis has been shown to slow the rate of oxidative phosphorylation, thus more PCr may be metabolized to help meet the demand for energy when it is not fully being met through oxidative phosphorylation.

Participation in this study involves visits to the laboratory at the Lawson Health Research Institute on a maximum of three different occasions. The first two visits will take a maximum of two hours, the third visit will take a maximum of four hours. If you decide to take part in this study, you will be one of approximately 12 subjects.

Two techniques will be used in this study. In order to look at the changes in metabolism during exercise we will use a non-invasive technique called Magnetic Resonance Spectroscopy (MRS). MRS involves high magnetic fields (about 200 times that of a fridge magnet) and radio waves (like an FM radio). In order to look at changes in blood pH and lactate, a venous catheter will be placed into a vein on the back of the hand allowing for blood sampling.

You can participate in this study if you are a:

- Healthy male (20 – 35 years old)
- Non-smoker

You CANNOT participate in this study if you:

- Are diagnosed with respiratory, cardiovascular, kidney, or metabolic disease.
- Obese, non-ambulatory, or suffering from arthritis.
- Have cardiovascular or neuromuscular limitations to exercise during the initial screening.
- Use any medications with side effects of dizziness, ataxia, or slowed reaction time.
- Use heart rate or blood pressure medications.
- Have severe heart disease (including susceptibility to arrhythmias)

- Have any skin ulcers or other non-healed skin areas or infections on your foot or lower leg
- Have any surgically implanted metal objects (e.g. Screws, plates, cardiac pacemakers, aneurysm clips, etc.) or wear metal braces on your teeth.
- Have any reason to believe that you may have metal fragments in your body.

Description of Research

If you agree to participate, you will be required to come to the laboratory for approximately 1 to 2 hours for the first test session, 1 to 2 hours for the second test session, and then approximately 4 hours for the last testing session. These exercise sessions will be separated by a minimum of 24 hours. Tests will occur at the Lawson Health Research Institute on the fifth floor in the 3.0T magnetic resonance imaging lab.

On your first visit prior to entering the magnet you will be asked about your medical history to confirm that you have no metal and to safely store any metallic objects. Upon arriving in the MRI suite you will be familiarized with the MRI machine, venous sampling equipment, and the leg exercise apparatus.

During the testing portion of each visit, you will be asked to lie on your back with your lower legs secured within the bore of the magnet. We will be studying the muscles in your leg that are involved in plantar flexion exercise (i.e. primarily the gastrocnemius). The dominant foot will be secured to a platform that is connected to a bucket through a pulley that will measure the work done during the experiments. On your first visit you will perform a ramped exercise test. The test will begin with 3 minutes of resting data collection, followed by three minutes of warm-up where you will raise and lower an empty bucket. After the three minutes of warm-up the "ramped" testing will begin. This "ramped" testing consists of constant plantar flexion at a rate of 0.5 Hz while the bucket that you are lifting is filled at a constant rate with water (~1 litre/min). You will be asked to keep contracting until you can no longer lift the bucket through the entire range of motion. After this we will continue to monitor your recovery from this exercise for an additional 15 minutes. The ramped exercise test will allow the investigators to determine the appropriate intensity for the subsequent exercise bouts (i.e. bouts of control and induced acidosis constant-load moderate and heavy intensity exercise).

During the control trial, $^{31}P$ -MRS data will be collected during 3 minutes of rest, 3 minutes of warm-up, 9 minutes moderate intensity exercise, 9 minutes heavy intensity exercise, and 15 minutes of recovery. Venous blood samples will be taken at the beginning of the rest period, the end of the warm up, the end of moderate intensity exercise, and the end of heavy intensity exercise.

The induced acidosis trial will involve ingesting gel caps of ammonium chloride at a dose of 0.3 g/kg body mass in divided doses over 180 minutes prior to the start of the trial. $^{31P}$-MRS data will then be collected in the same manner as the control trial.. Again, during 3 minutes of rest, 3 minutes of warm-up, 9 minutes moderate intensity exercise, 9 minutes heavy intensity exercise, and 15 minutes of recovery. Venous blood samples will be

taken at the beginning of the 180 minute dose period prior to the ingestion of any gel caps, at the end of the 180 dose period after all gel caps have been ingested, at the end of warm-up, the end of moderate intensity exercise, and the end of heavy intensity exercise. The control and the induced acidosis trial will be separated by a minimum of 24 hours.

Risks and Discomforts

The 31P-MRS research procedures used in this study are non-invasive and when used properly should pose no threat to your health. The Food & Drug Administration (USA) has indicated that for clinical diagnosis an 'insignificant' risk is associated with human MRI exposure at the intensities used in this project. Current Canadian guidelines follow the USA guidelines. Although very rare, injury and deaths have occurred in MRI units from unsecured metal objects being drawn at high speeds into the magnet or from internal body metal fragments of which the subject was unaware or had not informed MRI staff. To minimize this latter possibility it is essential that you complete a screening questionnaire. Other remote but potential risks involve tissue burns and temporary hearing loss from the loud noise inside the magnet. The latter can be avoided with ear headphone protection that also allows continuous communication between the subject and staff during the study.

If you have any history of head or eye injury involving metal fragments, if you have ever worked in a metal shop or been a soldier, if you have some type of implanted electrical device (such as a cardiac pacemaker), if you have severe heart disease (including susceptibility to arrhythmias), if you are wearing metal braces on your teeth, or [for women] if you could be pregnant, or have an intrauterine device, you should not have an MRI scan

There may be some discomfort during the exercise testing, particularly in the ramped exercise protocol that goes to volitional fatigue. You may experience some local muscle pain from the exercise, although this should not last for any length of time.

For the venous blood sampling you will be required to have a catheter placed into a superficial vein on the back of your hand. There may be some pain experienced when the catheter is placed into the vein (but no more than when you get a needle in your arm) after which you should feel no pain or discomfort. Minor localized bruising sometimes occurs following venous catheterization, but it generally fades away after a few days.

Ammonium chloride has been given to healthy volunteers in various doses as part of their participation in other small research studies. The number of participants in these studies range from 5 to 8 participants. Based on reports from these studies, possible side effects associated with ammonium chloride administration are gastrointestinal upset, nausea and diarrhea. It is believed that the dose you will be receiving for this study is the highest possible dose that can be administered without causing the gastrointestinal upset, however this is not guaranteed. Because the previous studies were based on small numbers of participants, it is possible that other more serious side effects may be noted

when larger groups of people are given ammonium chloride. If you experience any side effects after being given ammonium chloride please contact the study doctor.

Voluntary Participation

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions, or withdraw from the study at any time with no effect on your future employment or academic status. The investigator has the right to withdraw you from the study at any time for reasons related to you (e.g., not following the study-related directions); or because the entire study has been stopped.

Confidentiality

If the results of the study are published, your name will not be used and no information that discloses your identity will be released or published. Individual results will be held in strict confidence and all data will be placed in a locked cabinet. Only the investigators will have access to your records. You are encouraged to ask questions regarding the purpose of the study and the outcome of your test.

Inquiries Concerning the Study

If you have any questions about this study or your care/treatment please contact:

Dr. Greg Marsh, PhD. _____
Tyler Churchward Venne, B.A. _____

If you have questions about the conduct of this study or your rights as a research subject you may contact Dr. J. Gilbert, VP Research and Development at London Health Sciences Centre at _____

Note: You will be given a copy of this letter of information and consent form once it has been signed. You do not waive any legal rights by signing the consent form.

Letter of Informed Consent

Effects of $NH_4Cl$ induced acidosis on phosphocreatine metabolism during heavy-intensity plantar flexion exercise

Principal Investigator: GD Marsh, PhD.

I have carefully read the "Letter of Information", have had the nature of the study explained to me and I agree to participate. All my questions have been answered to my satisfaction.

_____

Name (please print)

_____

Signature

_____

Date

_____

Name of Person Obtaining Consent

_____

Signature

_____

Date

# WANTED:
## SUBJECTS FOR EXERCISE PHYSIOLOGY

**Advertisement**

## Effects of $NH_4Cl$ induced acidosis on phosphocreatine metabolism during heavy-intensity plantar flexion exercise

Principal Investigator: GD Marsh, PhD

We are seeking volunteers for a study that examines muscle metabolism during exercise and recovery. In particular, we will determine how blood acidity affects the ability to perform work and how the levels of phosphate containing compounds in the muscle change.

The experiment will be conducted at the Lawson Health Research Institute using non-invasive magnetic resonance spectroscopy techniques. Subjects will be required to come into the laboratory on three different occasions:

Session 1: The first visit will be approximately 2 hrs long and will involve a questionnaire, mandatory health and safety screening, and an exercise test for familiarization purposes.

Sessions 2-3: The second visit will each be approximately 2-hours in length, the third approximately 4 hours in length. Both will involve the different exercise protocols that are to be studied.

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**Volunteers must be healthy, non-smoking, males between the ages of 20 and 30.**

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Interested individuals are asked to contact:

Dr. Greg Marsh _____
Tyler Churchward-Venne _____
E-Mail: _____