THE INFLUENCE OF EXTRACELLULAR pH ON HUMAN SKELETAL MUSCLE METABOLISM

by

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A Thesis

Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

McMaster University

THE INFLUENCE OF EXTRACELLULAR pH ON HUMAN SKELETAL MUSCLE METABOLISM

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ABSTRACT

Moderate to high intensity exercise generates lactate and hydrogen ions which accumulate in both blood and muscle and are thought to contribute to the development of fatigue. Alteration in the extracellular pH has been shown to influence the appearance of lactate in plasma, acidosis decreasing and alkalosis increasing blood lactate concentration. The present research was designed to explore the potential mechanisms by which acid-base alterations influence lactate production and the associated metabolic changes in exercise.

Normal subjects took part in two studies. Acidosis induced by ingestion of ammonium chloride and alkalosis via ingestion of sodium bicarbonate were compared to control during rest and exercise. Needle biopsies of the vastus lateralis muscle, blood flow and arterial and femoral venous blood samples were taken. Acidosis resulted in decreased lactate production and efflux, secondary to decreased glycogenolysis from reduced transformation of glycogen phosphorylase to its active form and the lack of accumulation of positive allosteric modulators. Lower glycogen utilization resulted and was associated with an increase in free fatty acid (FFA) utilization from intramuscular stores. Pyruvate dehydrogenase activity was lower. Alkalosis had opposite effects: greater lactate production, efflux, glycogenolysis, pyruvate dehydrogenase activity and glycogen utilization, with lower FFA utilization. However, the increased glycogenolysis resulted from allosteric

activation of glycogen phosphorylase through increases in the concentration of its positive modulators adenosine monophosphate and substrate free inorganic phosphate.

These studies conclude that alterations in lactate production result entirely from a mismatch in the catalytic rates of glycogen phosphorylase and pyruvate dehydrogenase. Additionally, these studies identified for the first time the mechanisms responsible for the complex interplay of regulatory enzyme activity, fuel utilization and the importance of acid-base homeostasis in its control.

PERSONAL ACKNOWLEDGEMENTS

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got in the way of doing what you already knew I was capable of. I respect and admire you for your commitment to research, to your students and to quality. I only hope this is the start of a long and prosperous future of collaboration.

To Dr. Jones, I wish to thank you for your time, patience and challenging conversations. I never came away from a meeting having not learnt something. I have a great deal of respect for you and aspire to one day have the ability to meld basic science research and clinical research as you do so well.

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You are the most exceptional group of individuals I have ever had the pleasure of working with and I consider it an honor to be able to call you my friends and colleagues.

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Ivan, what can I say? Thanks for putting up with me, acting interested (even when I know you didn't have a clue what I was talking about) and most of all giving me the security, confidence and support to follow my dreams.

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Hello,

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Putman, C.T., N.L. Jones, E. Hultman, M.G. Hollidge-Horvat, A. Bonen, D.R. McConachie, and G.J.F. Heigenhauser. Effects of short-term submaximal training in humans on muscle metabolism in exercise. Am. J. Physiol. 275 (Endocrinol. Metab. 28): E132-E139, 1998.

L.Maureen. Odland, George J.F. Heigenhauser, Denis Wong, Melanie G. Hollidge-Horvat, and Lawrence L. Spriet. Effects of increased fat availability on fat-carbohydrate interaction during prolonged exercise in men. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R894-R902, 1998.

Howlett, Richard A., George J. F. Heigenhauser, Eric Hultman, Melanie G. Hollidge-Horvat, and Lawrence L. Spriet. Effect of dichloroacetate infusion on human skeletal muscle metabolism at the onset of exercise. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E18-E25, 1999.

The manuscript that has been accepted for publication is entitled:

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My supervisor is Dr. G.J.F Heigenhauser

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Thank-you for your cooperation,
Sincerely,

Melanie G. Hollidge-Horvat,
Ph.D. Candidate, McMaster University, Faculty of Health Sciences

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LIST OF SYMBOLS AND ABBREVIATIONS

acetyl-TPP	acetyl-thiamine pyrophosphate
acetyl-CoA	acetyl-Coenzyme A
ACID	metabolic acidosis trial
ADP	adenosine diphosphate
ADP_f	free adenosine diphosphate
ALK	metabolic alkalosis trial
AMP	adenosine monophosphate
AMP_f	free adenosine monophosphate
ATP	adenosine triphosphate
°C	degrees Celcius
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium
CHO	carbohydrate
CO_2	carbon dioxide
CON	control condition
CoASH	coenzyme A
DTT	dithiothreitol
E1	pyruvate decarboxylase (E.C. 1.2.4.1)
E2	dihydrolipoamide acetyltransferase (E.C. 2.3.1.12)
E3	dihydrolipoamide dehydrogenase (E.C. 1.6.4.3)
EDTA	ethylene diamine tetraacetic acid
ETC	electron transport chain
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
F-1,6-P	fructose 1,6, bisphosphate
FFA	free fatty acid
F-6-P	fructose-6-phosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
H^+	hydrogen ion
$[H^+]$	hydrogen ion concentration
HCO_3^-	bicarbonate
HSL	hormone sensitive lipase
$KHCO_3$	potassium bicarbonate
l	liter
LDH	lactate dehydrogenase
Liquid N_2	liquid nitrogen
LPL	lipoprotein lipase

LIST OF SYMBOLS AND ABBREVIATIONS

Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
min	minutes
mM	millimoles per liter
mmol	millimoles
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaF	sodium fluoride
$NaHCO_3$	sodium bicarbonate
NH_4Cl	ammonium chloride
nmol	nanomoles
O_2	oxygen
PCA	perchloric acid
PCr	phosphocreatine
PDH_a	active form of the pyruvate dehydrogenase complex
PDH_b	inactive form of the pyruvate dehydrogenase complex
PDH_c	pyruvate dehydrogenase complex
Phos	glycogen phosphorylase
Phos *a*	glycogen phosphorylase in the active form
Phos b	glycogen phosphorylase in the less active form
PFK	phosphofructokinase
P_i	free inorganic phosphate
pH_e	extracellular pH
pH_i	intramuscular pH
P-NMR	phosphorous nuclear magnetic resonance imaging
SR	sarcoplasmic reticulum
TAG	triacylglycerol
TCA	tricarboxylic acid cycle
TPP	thiamine pyrophosphate
*u*l	microliter
*u*M	micomoles per liter
V_{max}	maximum enzyme activity
VCO_2	rate of carbon dioxide production
VO_2	rate of oxygen consumption
$VO_{2\,max}$	maximum oxygen uptakte

FORMAT AND ORGANIZATION OF THESIS

The present thesis was prepared in the "sandwich thesis" format as outlined in the School of Graduate Studies "Guidelines for the Preparation of Theses", Section 3, and the additional requirements set forth in the Medical Sciences Program "Guide to Graduate Studies", Section X, for the academic year 1998-1999. This thesis is comprised of two original research papers (Chapter 2,3) that have been submitted for publication to a peer reviewed journal.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Chapter 2

Publication

Hollidge-Horvat, M.G., Parolin, M.L., Wong. D., Jones, N.L. and G.J.F. Heigenhauser. The effect of induced metabolic acidosis on human skeletal muscle metabolism during exercise. *Accepted for Publication - American Journal of Physiology (Endocrinology and Metabolism) - October, 1999.*

Contributions

Experimental design was by Dr. G.J.F. Heigenhauser and M.G. Hollidge-Horvat. Organization and execution of the experiments was carried out by M.G. Hollidge-Horvat. Insertion of the radial arterial line and femoral venous thermodilution catheter was carried out by Dr. D. Wong. The maintenance of patency of those lines, sampling of blood and blood flow were a shared responsibility between M.G. Hollidge-Horvat and Dr. D. Wong. Dr. D. Wong and Dr. N.L. Jones also provided medical supervision during the induction of both metabolic acidosis/alkalosis. M.L. Parolin provided technical assistance in the collection, preparation and analysis of the blood samples. Dr. G. J. F. Heigenhauser was responsible for obtaining the muscle biopsies samples from the vastus lateralis from all subjects.

The primary supervisor for this study was Dr. G. J. F. Heigenhauser with the assistance of Dr. NL Jones. The data contained in this paper was the result of extensive biochemical analysis that was completed by M.G. Hollidge-Horvat in conjunction with the technicians from Dr. G. J. F. Heigenhauser's laboratory (T.M. Bragg and M. Ganaragah) and the summer research student (M.Matsos). The data summary, statistical analysis and writing of the manuscript were completed by M.G. Hollidge-Horvat.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Chapter 3

Publication

Hollidge-Horvat, M.G., Parolin, M.L., Wong. D., Jones, N.L. and G.J.F. Heigenhauser. The effect of induced metabolic alkalosis on human skeletal muscle metabolism during exercise. *Submitted for Publication - American Journal of Physiology (Endocrinology and Metabolism)*

Contributions

The experimental protocol was identical to the study presented in Chapter 2, and as such the design, organization, execution and sample collection were done by M.G. Hollidge-Horvat with the assistance of the co-authors. The primary supervisor for the study was Dr. G.J.F. Heigenhauser with medical supervision provided by Dr. D. Wong and Dr. N. L. Jones. The data contained in this paper was the result of extensive biochemical analysis that was completed by M.G. Hollidge-Horvat in conjunction with the technicians from Dr. G. J. F. Heigenhauser's laboratory (T.M. Bragg and M. Ganaragah) and the summer research student (M. Matsos). The statistical analysis into writing of the manuscript were completed by M.G. Hollidge-Horvat.

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Appendix A

Publication

Putman, C.T., Jones, N.L., Lands, L.C., Bragg, T.M., Hollidge-Horvat, M.G., and G.J.F. Heigenhauser. The Skeletal muscle pyruvate dehydrogenase activity during maximal exercise in humans. *Am. J.* Physiol. (Endocrinol. Metab.) 269: E458-E468, 1995.

Contributions

The experiments were designed by Dr. G.J.F. Heigenhauser and Dr. N.L. Jones. The experiments were executed and samples collected by C.T. Putman with the assistance of Dr. G.J.F. Heigenhauser who took the biopsies from each subject. The primary supervisor for this study was Dr. G.J.F. Heigenhauser. Medical supervision was provided by Dr. L.C. Lands and Dr. N.L. Jones. Biochemical analysis was completed entirely by C.T. Putman with the exception of PDH_a analysis in which M.G. Hollidge-Horvat assisted. Technical assistance was provided by T.M. Bragg and M.G. Hollidge-Horvat. The data were analyzed and the paper written by C.T. Putman.

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Appendix B

Publication

Putman, C.T., Jones, N.L., Hultman, E., Hollidge-Horvat, M.G., Bonen, A., McConachie, D.R. and G.J.F. Heigenhauser. The effects of short-term submaximal training in humans on muscle metabolism in exercise. *Am. J.* Physiol. (Endocrinol. Metab.) 275: E132-E139, 1998.

Contributions

Experiments were designed by Dr. E. Hultman and Dr. G.J.F. Heigenhauser. Organization and execution of the experiments was carried out by C.T. Putman. Insertion of the brachial arterial line and femoral venous thermodilution catheter was carried out by Dr. D.R. McConachie with the assistance of M.G. Hollidge-Horvat. The maintenance of patency of those lines, sampling of blood and blood flow were a shared responsibility between M.G. Hollidge-Horvat and Dr. D.R. McConachie. Dr. D.R. McConachie and Dr. N.L. Jones also provided medical supervision. Dr. E. Hultman and Dr. G.J.F. Heigenhauser shared the responsibility for obtaining muscle biopsy samples from the vastus lateralis of all subjects. Dr. A. Bonen provided the analysis of the glucose transporter. The primary supervisor for this study was Dr. G. J. F. Heigenhauser. The data were analyzed and the paper written by C.T. Putman.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Appendix C

Publication

Odland, L.M.,, Heigenhauser, G.J.F., Wong, D., Hollidge-Horvat, M.G., and L.L. Spriet. Effects of increased fat availability on fat-carbohydrate interaction during prolonged exercise in men. *Am. J. Physiol. (Regulatory Integrative Comp. Physiol.)* 275: R894-R902, 1998.

Contributions

The experiments were designed by Dr. L.L. Spriet and Dr. G.J.F. Heigenhauser. Organization and execution of the experiments was carried out by L.M. Odland. Insertion of the radial arterial and femoral venous lines were carried out by Dr. D. Wong with the assistance of M.G. Hollidge-Horvat. The maintenance of patency of those lines and sampling of blood were the responsibility of M.G. Hollidge-Horvat and L.L. Spriet. Dr. D. Wong also provided medical supervision. Dr. G.J.F. Heigenhauser was responsible for obtaining muscle biopsy samples from the vastus lateralis of all subjects. The primary supervisor for this study was Dr. L.L. Spriet. The data were analyzed and the paper written by L.M. Odland.

CONTRIBUTION TO MULTI-AUTHORED PAPERS

Appendix D

Publication

Howlett, Richard A., George J.F. Heigenhauser, Eric Hultman, Melanie G. Hollidge-Horvat, and L.L. Spriet. Effects of dichloroacetate infusion on human skeletal muscle metabolism at the onset of exercise. *Am. J. Physiol. 277 (Endocrinol. Metab. 40)*: E18-E25, 1999.

Contributions

The experiments were designed by Dr. L.L. Spriet, Dr. G.J.F. Heigenhauser and Dr. Eric Hultman. Organization and execution of the experiments was carried out by R.A. Howlett. Insertion of the intravenous catheter, administration of the dicholoacetate infusion and medical supervision during the experiments were carried out by M.G. Hollidge-Horvat. Dr. G.J.F. Heigenhauser was responsible for obtaining muscle biopsy samples from the vastus lateralis of all subjects. The primary supervisor for this study was Dr. L.L. Spriet. The data were analyzed and the paper written by R. A. Howlett.

CHAPTER 1

THE INFLUENCE OF pH ON MUSCLE METABOLISM

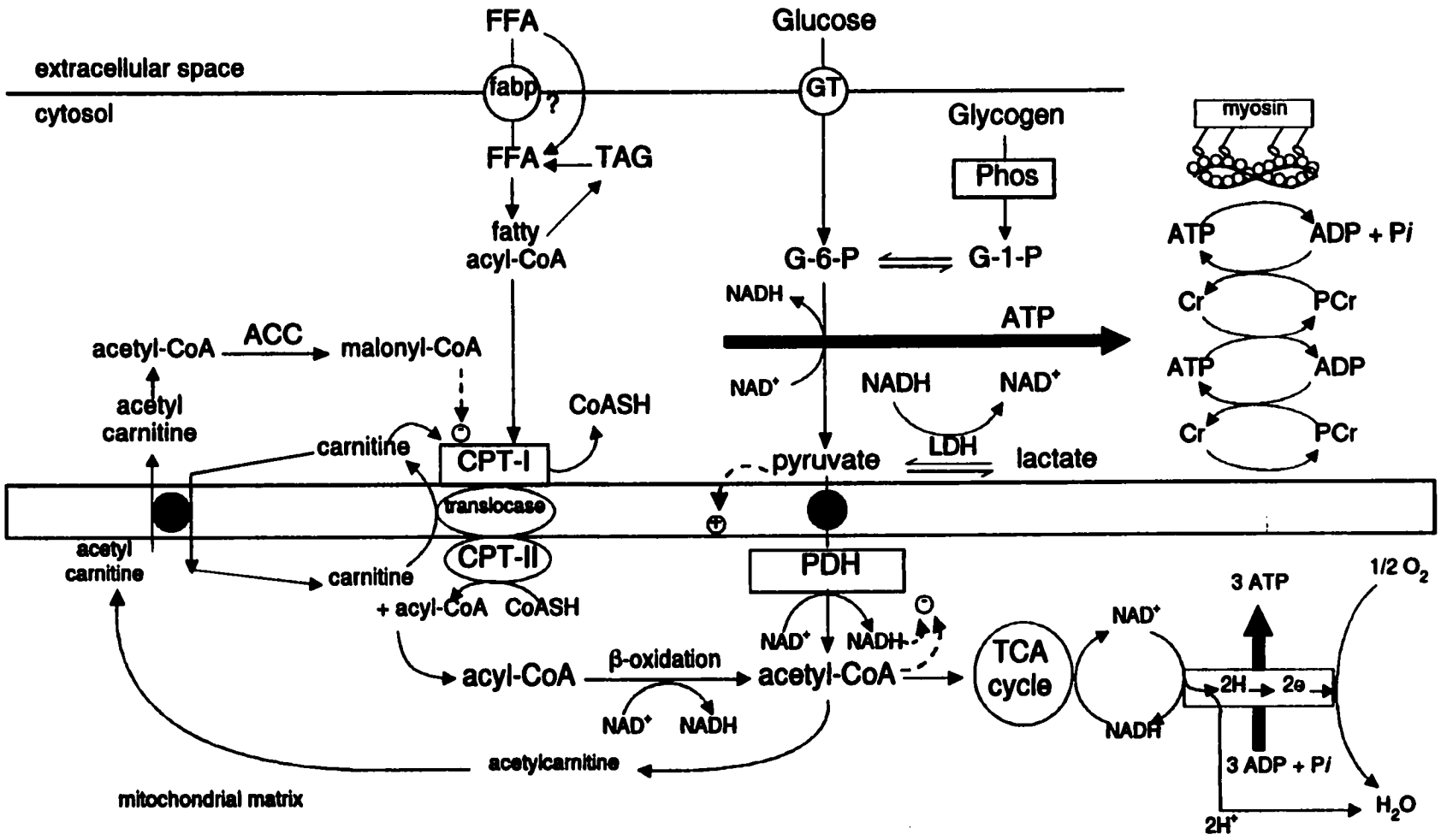
1.1 General Overview

Skeletal muscle is a highly adaptable tissue which comprises almost half of the total body mass in humans. The primary function of muscle is to generate movement to allow interaction with the environment. Exercise is one example of purposeful action that requires the coordination of a number of body and tissue specific systems. Contraction of skeletal muscle represents the mechanical process by which movement is achieved. This is intimately coupled to the chemical processes which provided energy in the form of ATP to the contractile apparatus of the myocyte. The provision of ATP is accomplished by the utilization of a variety of fuel sources that through various metabolic pathways oxidize a number of different substrates. The available pool of ATP within skeletal muscle is small and in order for contraction to continue, the demand for ATP must be matched by a comparable ATP production rate. The resynthesis of ATP can be accomplished by the pathways of both oxidative phosphorylation ((tricarboxylic acid cycle (TCA) and electron transport chain (ETC)) and the non-oxidative pathways of glycolysis and

phosphocreatine (PCr) degradation. The ability to dramatically alter ATP production rate to meet demand is accomplished by the combined ability of the metabolic pathways to operate in an integrated manner. Thus, mechanisms exist to control the rate of each metabolic pathway, ensuring an integrated and appropriate use of available fuels. The end result is the controlled release of chemical energy from carbohydrate (CHO), triacylglyceride (TAG) and other fuels as each is broken down by a series of linked reactions within the cytosol and mitochondria to produce ATP (Fig. 1). In skeletal muscle, enzyme regulation is the major means by which energy pathways are not only controlled to match ATP production to ATP demand but the means by which the selection of fuels are determined.

Exercise of moderate to high intensity produces lactate and hydrogen ion (H^+) which accumulates in both the muscle and blood as fuel utilization shifts from TAG to CHO. The intramuscular accumulation of H^+ has been implicated in the development of muscular fatigue by interfering with the contractile apparatus functioning and the activity of the key regulatory enzymes of both oxidative and non-oxidative metabolism. In a number of the metabolic pathways, H^+ plays an important role as both a substrate and product.

The appearance of lactate in the blood during exercise can be influenced by changing the extracellular pH such that an acidosis decreases and an alkalosis increases the blood lactate concentration. The mechanisms responsible for this alteration in lactate appearance have been suggested to be related to an alteration in the muscle lactate production rate secondary to the effect of a change in the


FFA
Glucose
extracellular space
cytosol
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Glycogen
Phos
myosin
FFA
TAG
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acetyl-CoA
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malonyl-CoA
acetyl
carnitine
carnitine
CPT-I
CoASH
translocase
CPT-II
PDH
acetyl
carnitine
carnitine
+ acyl-CoA
CoASH
NAD+
NADH
acyl-CoA
β-oxidation
acetyl-CoA
TCA
cycle
NAD+
NADH
3 ATP
1/2 O2
2H
2e
3 ADP + Pi
H2O
2H+
NAD+
NADH
acetylcarnitine
mitochondrial matrix

extracellular pH on the regulatory enzymes of metabolism and/or lactate transport. However, the majority have inferred this from blood measurements only, without any assessment of the intramuscular pH or metabolites during an extracelluar acidosis/alkalosis. Only a few studies have used more direct measurement techniques of the intramuscular compartment by either needle biopsy or phosphorous nuclear magnetic resonance imaging (P-NMR) to assess the intramuscular pH, lactate efflux and lactate production rate. The results of these studies have been both contradictory and inconclusive. The current research presented in chapters 2 and 3 was undertaken to investigate the mechanisms responsible for the differences in the appearance of lactate under conditions of an extracellular acidosis/alkalosis.

1.2 Introduction

The formation of lactic acid in exercising muscle was first noted in 1848 by Berzelius who observed that the accumulation of lactic acid was proportional to the extent to which the animal had previously been exercised (67). In the early part of the 1900's Parnas and Wagner and Meyerhof demonstrated that glycogen loss was proportional to lactic acid formation which led to the notion that glycogen was also the likely precursor of lactic acid (67). Hill and Lupton (71) then demonstrated that fatigue in isolated frog muscle was related to the accumulation of lactic acid within the muscle but that due to the lower pK_a of lactic acid it was found to exist primarily as H^+ and lactate ion. These researchers proposed that the formation of lactate resulted from a lack of oxygen. The formation of lactate was seen to represent an "oxygen

debt" to be paid following exercise. These authors were also the first to suggest that the buffering of H^+ within the exercising muscle could possibly counteract the fatigue associated with the lactic acid accumulation and therefore prolong exercise duration by increasing the "oxygen debt".

Around this time the alteration in the appearance of lactate in the blood with altitude and with acclimatization to altitude was observed. Acute hypoxia led to an increase in lactate in the blood but concomitant with ascent to altitude was an acute hyperventilation that led to a respiratory alkalosis. This combination of events led Edwards and Dill to question whether it was the lack of oxygen or the altered buffering capacity of the blood due to the respiratory alkalosis that was responsible for the increased blood lactate concentration at altitude. Edwards and Dill (49) conducted experiments under normoxic conditions with metabolic and respiratory alkalosis and observed similar increases in the blood lactate concentration. This led these authors to suggest that it was the enhanced buffering capacity of the blood and not the hypoxia with altitude that was responsible for the increased blood lactate concentration with ascent to altitude.

Following these initial studies researchers began to employ extracellular pH manipulation to examine the effects on exercise performance and the appearance of lactate in the blood. Denning et al (40) found that metabolic acidosis induced by ingestion of ammonium chloride (NH_4Cl) decreased the capacity to perform work and decreased the blood lactate concentration. Metabolic alkalosis induced by ingestion of sodium bicarbonate ($NaHCO_3$) had the opposite effects. However, the sample size

was exceedingly small (n=1). Other researchers repeated the Denning et al study but found conflicting results (3,88,114).

The subject remained virtually unexplored following these initial investigations until 1977 when Jones et al (95) confirmed the findings of Denning et al (ie decreased blood lactate concentration and exercise time with acidosis and the opposite with alkalosis). Renewed interest in the topic followed, but the majority of studies focused on the effects of an altered extracellular pH on exercise performance and the alteration in blood lactate concentration. The mechanisms responsible for these changes were thought to result from the effects of an altered extracellular pH on the intramuscular processes despite the lack of direct measurement of the constituents of this compartment. Many of the studies were conducted under conditions of long duration or high-intensity exercise resulting in situations of decreasing ATP turnover; making it difficult to attribute the altered exercise performance and blood lactate concentration to specific mechanism(s). The net result of almost one hundred years of study was still a less than clear picture of the mechanisms that contribute and/or are responsible for the universally observed alteration in blood lactate with extracellular acidosis/alkalosis.

The purpose of the present chapter is to review the literature and provide a theoretical framework for the design and interpretation of the original investigations presented in Chapters 2 and 3. The sheer number of metabolic systems involved and their integration precludes an exhaustive review of the literature in each area.

Therefore, in each section the information presented will be limited to the most pertinent research in the area, primarily focused on the research involving skeletal muscle, with special emphasis on studies utilizing human subjects. The present chapter reviews the pertinent literature regarding the known effects of pH on the metabolic energy producing systems within skeletal muscle and the theoretical concepts concerning lactate metabolism and transport. Finally, this chapter concludes with a description of the rationale for examining the effect of pH on human skeletal muscle metabolism.

1.3 Muscle pH and Exercise

As previously mentioned the majority of studies relating changes in the H^+ concentration ($[H^+]$) to metabolism and muscle fatigue have been based on measurements made in the plasma. Original intramuscular pH (pH_i) measurements on cat muscle homogenates demonstrated that muscle had a resting pH of ~7.0 and declined to ~6.3 at fatigue with electrical stimulation (125). However, human pH_i determination did not occur until the advent of the muscle needle biopsy technique. Analysis of muscle pH by a pH meter following homogenizing either the wet or dry biopsy sample has determined that the resting pH_i is also ~ 7.0. During heavy exercise (ie at intensities approaching $VO_{2\ max}$ or sprint exercise) when the resynthesis of ATP occurs primarily by glycolysis the pH_i of human muscle has been shown to decline to 6.3-6.5 at exhaustion (69,78,117,170). More recently measurement of pH_i has been derived non-invasively by P-NMR which examines the

change in the high energy phosphate ratios. Additionally, estimation of pH_i can be derived from the intramuscular lactate concentration and PCr degradation. Comparison of pH_i methodology has yielded similar values. All of the studies examining pH_i have been done to determine the effect of exercise of various durations/intensities on pH_i and have universally demonstrated that the pH_i declines during moderate to high intensity exercise. The source of the increased H^+ was traditionally thought to result from increases in the intramuscular lactate concentration and increases in the partial pressure of carbon dioxide (PCO_2) (78). However, more recently, it has been demonstrated that the change in $[H^+]$ is more complex. Increases in the intramuscular lactate concentration and PCO_2 are contributing factors but decreases in the intramuscular PCr and potassium concentrations also appear to play an important role (see (91) for review). It is this accumulation of H^+ that has been implicated as the primary factor in the fatigue process by a number of different mechanisms. These include: disruption of excitation-contraction coupling; alteration of the cross-bridge cycling process and inhibition of the rate limiting glycolytic enzymes (78,92).

Hydrogen ions may interfere with both the excitation-contraction and cross-bridge cycling processes by effecting loci that involve Ca^{2+} and inorganic phosphate (P_i) by either competing for the calcium (Ca^{2+}) binding site on troponin C (56) or by altering the conformation of the tropomyosin molecule thereby indirectly affecting Ca^{2+} binding (51). Alternatively H^+ may increase the Ca^{2+} binding to the sarcoplasmic reticulum (SR) and/or reduce the Ca^{2+} affinity of troponin (7,124). The

combined results of these studies suggest that impaired performance in the presence of an elevated [H^+] may result from an altered Ca^{2+} cascade during contraction which ultimately reduces the number of actin-myosin interactions.

Hydrogen ion has also been implicated in the fatigue process by interfering with cross bridge cycling by altering the conformation of the contractile proteins. This leads to a decrease in the myosin ATPase activity and therefore an increase in the number of cross-bridges in the weakly bound state (32,39). Increases in the intramuscular [H^+] is also associated with increases in the ratio of the diprotonated ($H_2PO_4^-$) to monoprotonated (HPO_4^{2-}) forms of inorganic phosphate (P_i) as defined by the reaction:

(1) $$HPO_4^{2-} + H^+ \rightleftharpoons H_2PO_4^-$$

The total amount of phosphate in the diprotonated form doubles as the pH_i decreases from 7.0 at rest to 6.4 at exhaustion due to the pK_a of the reaction (129). To determine if the effects of increases in H^+ and P_i concentrations were additive in the fatigue process Wilson and associates (193) conducted a study to examine the relationship between force production and changes in the free P_i and H^+ concentrations. Using P-NMR, they preceded a maximal exercise bout with 2 minutes of submaximal exercise, and compared it to a control trial consisting of 4 minutes of maximal exercise. The relationship between the $H_2PO_4^-$ concentration and the reduction in force output was similar between trials, despite a significantly higher [H^+] with the control trial. This suggests that the two metabolites exert their effects independently.

A change in [H^+] can affect enzyme activity in two ways. First, the enzyme may be directly affected by a change in the charge of the ionizable groups of the enzyme, particularly at the sites containing histidyl residues. An alteration at these sites may result in a change in the enzyme's affinity for its substrate, product or result in an alteration in the natural conformation of the enzyme and therefore its catalytic activity. Second, an alteration in the [H^+] can change the ionic state of the substrate, product or the inhibitors/activators of the enzyme which would indirectly affect the enzyme's catalytic activity (164). The effects of a change in enzyme function could potentially be widespread and thereby not only affect the proteins involved in excitation-contraction coupling and/or cross-bridge cycling but also those proteins that make up the regulatory enzymes responsible for energy metabolism such as glycogen phosphorylase (Phos), phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH_c). As will be reviewed in detail in the following sections, H^+ has been shown to influence the activity of these key enzymes and therefore has the potential to affect lactate production and ultimately the appearance of lactate in the plasma.

1.4 Exercise Intensity and Substrate Utilization

Since the classical studies of the 1920's and 1930's it has been recognized that a shift toward greater carbohydrate utilization and lactate production occurs as exercise intensity increases (110). The two main fuels available for the production of ATP are CHO and TAG. The selection of fuel has a direct impact on the ATP

resynthesis rate as CHO can replenish ATP approximately twice as fast as TAG under aerobic steady state conditions (38,96). However, CHO stores are relatively small when compared to the available TAG stores, as lipids are approximately 9 x more efficient as an energy store, with respect to weight (81). Another difference between the two fuels is the time required to reach maximal ATP production rate: ~3 minutes for CHO and ~30 minutes for TAG under aerobic conditions (81). The large time delay with TAG utilization results from fact that the TAG stores in muscle and adipose tissue must first be hydrolyzed into free fatty acids (FFA). TAG hydrolysis is controlled by the sympathetic nervous system. Epinephrine activates TAG hydrolysis, and epinephrine levels rise gradually during continuous exercise. At the same time plasma insulin levels decline, which removes the stimulus for fatty acid esterification and storage. Increases in epinephrine/insulin ratio promote FFA release from TAG stores leading to a progressive elevation in plasma FFA concentration ([FFA]) as exercise duration progresses (57,65). In resting skeletal muscle, FFA oxidation provides ~60% of fuel for the average person consuming a mixed diet (16,62). During exercise, equal amounts of CHO and TAG are utilized at intensities of 35 - 40% VO_{2max} (62,81). As exercise intensity increases, the utilization of both fuels increases but the peak value for TAG utilization occurs at ~50% VO_{2max}. At higher exercise intensities, the absolute and relative contribution of TAG declines (15,79). At intensities above 65-75% VO_{2max}, CHO becomes the preferred substrate as the plasma [FFA] markedly declines (93). The reason for the reduction in the plasma [FFA] is not clear but may result from the inhibition of FFA release from

adipose tissue by the increasing H^+ (55) or lactate (86) concentrations and/or by a shift in blood flow away from adipose tissue (54). The ATP demands at exercise intensities greater than 75% $VO_{2\ max}$ are met primarily by the oxidation of intramuscular glycogen stores (79). Therefore, it is clear that a direct relationship exists between fuel utilization and exercise intensity in skeletal muscle (61,79).

The preceding discussion made no distinction between the contribution of intramuscular vs extramuscular CHO and TAG stores. Romijin and associates recently (155) examined the relative contribution of endogenous and exogenous CHO and TAG stores during exercise in humans utilizing stable isotope and indirect calorimetry in subjects exercising at 25, 65 and 85% $VO_{2\ max}$. The results indicated that both glucose uptake and glycogen utilization increased with exercise intensity. Adipose tissue lipolysis was maximally stimulated at 25% VO_{2max}, while intramuscular TAG lipolysis was stimulated only during the two higher intensities. Additionally, the results indicated that the contribution of plasma glucose and plasma FFA to caloric expenditure did not change with exercise, rather it remained constant. The authors concluded that the regulation of CHO availability is directly related to exercise intensity while the regulation of lipid metabolism appears to be more complex.

The following sections will discuss the provision of substrate with respect to the regulation of the key enzymes involved in the mobilization and utilization of both CHO and TAG and the effects of H^+ on those processes. Prior to this an introduction to the theoretical concepts in metabolic regulation is warranted. Figure 1 displays

the integration of the metabolic pathways responsible for both CHO and FFA utilization.

1.5 Theoretical Concepts in Metabolic Regulation

The theoretical principles of metabolic pathways and the identification of the type and function of the enzymes within pathways has been reviewed in recent years (126,127,129). Within a metabolic pathway, three types of enzymes exist: flux-generating, pseudo-flux generating and near-equilibrium. Generally, a metabolic pathway is initiated by a flux-generating enzyme and ends with the formation of a product that is either used as a substrate for a subsequent pathway; released into the environment or is transferred to a physiological reservoir (127).

The flux-generating enzyme of a pathway is often a multi-enzyme complex, generally saturated with substrate and regulated by external factors other than the substrate concentration. The reaction catalyzed by this enzyme is irreversible, has a large drop in the standard free energy, and requires another separate enzyme or set of enzymes to accommodate the reverse reaction. Also, because the enzyme is saturated with substrate at all times alteration in the catalytic activity often result in reciprocal changes in the concentration of its substrate. In addition, to control the overall rate of the pathway, its activity must be the lowest in the series of linked reactions. Two examples of flux-generating enzymes are Phos and PDH_c. The flux-generating enzyme is typically followed by a number of enzymes that catalyze near-equilibrium reactions linked in series. Near-equilibrium reactions are governed by

changes in their respective substrate and product concentrations as both can modify the direction and magnitude of the flux through that enzyme. Near-equilibrium enzymes are also sensitive to changes in enzyme co-factors which are important for metabolic control. However, these enzymes are not sites of allosteric regulation as changes in the co-factors are opposed by changes in the production or substrate concentration. The forward and reverse reactions catalyzed by these enzymes proceed at rates much greater than the rate of the flux-generating reactions. However, due to the nature and dependance of these reactions on both substrate and product concentrations the overall enzyme activity is therefore essentially controlled by the up-stream activity of the flux-generating enzyme and the down-stream rate of removal of the product at the end terminus of the pathway. An example of a near-equilibrium enzyme is lactate dehydrogenase (LDH).

Pseudo-flux generating enzymes or regulatory enzymes, as they will be referred to in this chapter, are controlled by external allosteric modulation but the reactions catalyzed by these enzymes are reversible and not generally saturated with substrate (127). Consequently, the enzyme catalytic activity is controlled by changes in its substrate and product concentrations. This provides another level of complexity and sensitivity to control flux through the metabolic pathway. The nature of the allosteric regulation of this type of enzyme is similar to those that regulate flux-generating reactions of the pathway, but differ in the sensitivity to the signal and remain highly dependant on the substrate concentration to determine catalytic activity. It should be noted that the allosteric regulation acts in an additive manner

with the changes in the substrate and product concentrations. For example, if the substrate increases and/or product concentration decreases, flux through the enzyme will proportionally increase, as enzyme activity increases in accordance with Michaelis-Menton kinetics. However, the addition of a positive modulator will change the catalytic activity and therefore allow the same increase in enzyme flux to occur at a lower substrate and/or higher product concentration. The reverse is also true. When a negative modulator is present in sufficient quantities to effect a decrease in catalytic activity, the decrease in substrate or increase in product concentrations that alone would be required to reduce enzyme activity are also attenuated. The advantage is the avoidance of large variations in the concentration of pathway intermediates and overall improved responsiveness of the pathway to a change in demand. PFK is one example of a key regulatory enzyme.

The specific strategic organization of enzymes in a particular pathway (ie flux-generating → near-equilibrium → pseudo-flux generating) determines the ability of the pathway to respond to changes in demand/flux and is both tissue and cell specific (127). In human skeletal muscle, flux through the metabolic pathways of glycolysis and the TCA cycle increase dramatically during the rest-to-work transition. For example, maximal exercise requires the ATP production rate to increase by greater than 100-fold over rest (73,192). If these pathways were governed by near-equilibrium enzyme reactions alone, it would necessitate a 100-fold increase in the concentrations of the pathway intermediates to achieve the required flux to meet the

ATP demand. The resulting change in osmolarity would have catastrophic affects within the cell (126,127). The flux-generating enzymes Phos and PDH_c and the regulatory enzyme PFK occupy key points in the metabolic pathways of skeletal muscle that allows the oxidation of CHO to ATP.

1.5.1 Cellular Energetics

The energy currency for the provision of work in human skeletal muscle is ATP. At rest ATP demand is low, but during exercise the utilization and therefore demand for ATP increases greatly. The primary consumer of ATP during muscular contraction is the actomyosin ATPase which converts the chemical energy of the phosphate bond in the ATP molecule to the mechanical energy of sarcomere shortening. In order for contraction to continue this ATP demand must be met, which is accomplished through an increase in the activity of the ATP synthesis pathways of glycolysis, PCr degradation, and oxidative phosphorylation. These processes must be increased quickly since the ATP concentration in skeletal muscle is low and can be rapidly depleted within seconds if not replenished (128). The ATP is regenerated by the following equation

(2) $ATP \rightleftarrows ADP + P_i + H^+ + energy$

The cytosolic ATP concentration and its products ADP and P_i when expressed as the ratio $[ATP]/[ADP]x[P_i]$ represents the energy state of the cell or the phosphorylation potential. (It is important to note that it is the free portions of these metabolites that participate in this equation). The above ratio together with the

[NADH]/[NAD^+] and the availability of O_2 regulates the rate of oxidative phosphorylation and therefore mitochondrial ATP production as represented in the equation (192):

$$(3) \quad NADH_i + 2c^{3+} + 2\ ADP_e + 2\ P_{i\,e} \leftrightarrow NAD^+_i + 2c^{2+} + 2\ ATP_e + H^+$$

(where c^{3+} and c^{2+} are the oxidized and reduced forms of mitochondrial cytochrome c respectively and the subscripts i and e refer to the intramitochondrial and extramitochondrial pools of reactants respectively)

Equation 3 represents the reversible part of oxidative phosphorylation which is under physiological control as the metabolite concentrations between the cytosol and mitochondrial spaces are in equilibrium. The irreversible, flux-generating step in oxidative phosphorylation is represented by the following equation which is catalyzed by cytochrome c oxidase:

$$(4) \quad 2c^{2+} + \tfrac{1}{2}\ O_2 + ADP_e + P_{i\,e} + 2\ H^+ \Longrightarrow 2c^{3+} + H_2O + ATP_e$$

Equation 3 and 4 operate in sequence and illustrate how the above mentioned metabolite ratios regulate the rate of oxidative phosphorylation.

The PCr system plays an important role in the regulation of oxidative phosphorylation and other metabolic processes as it serves as both an "energy buffer" and an "energy transport" system between the sites of ATP utilization and production (187) as expressed in the equation:

$$(5) \quad PCr + H^+ + ADP \rightleftharpoons creatine + ATP$$

The PCr system is very sensitive to changes in the intracellular free ADP (ADP_f) concentration, and serves to keep this concentration low by re-synthesizing ATP from

ADP when the ADP_f concentration increases thereby preventing the inactivation of the cellular ATPases and the net loss of cellular adenine nucleotides (187).

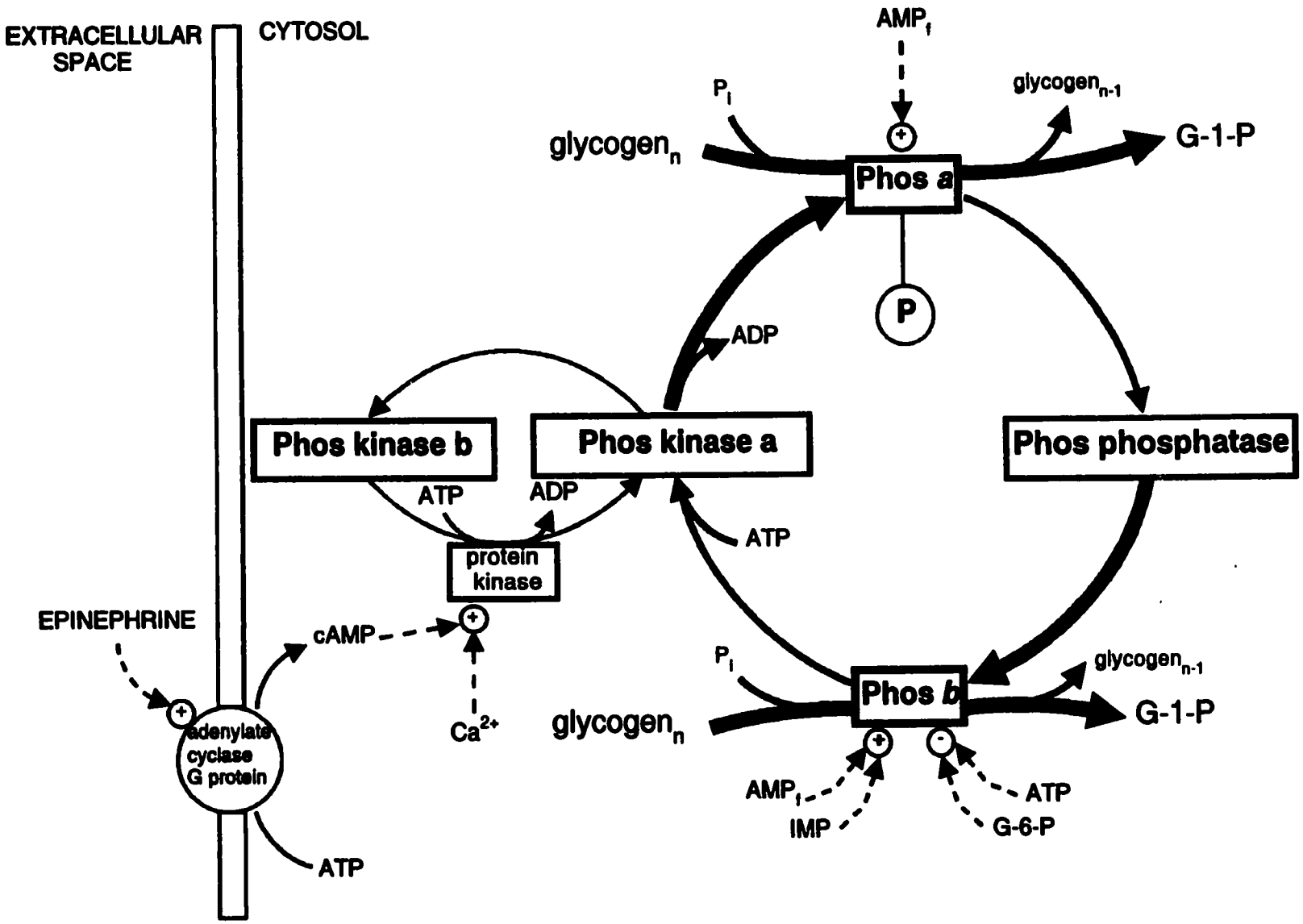
Simply increasing the rate of oxidative phosphorylation to match ATP demand is not enough, there must be an accompanying increase in the mobilization of substrate to the TCA cycle to provide reducing equivalents (NADH) for the ETC (as represented in equations 3 and 4). The provision of substrate supply is linked to the rate of oxidative phosphorylation by the metabolites ADP, AMP, P_i and NADH. Each of these has important effects on the catalytic activity of key flux-generating and regulatory enzymes in the pathways of CHO and TAG metabolism. For example, the phosphorylation state of the cell as reflected in the [ATP]/[ADP]x[P_i] ratio directly affects CHO and TAG utilization as increases in ADP, AMP and P_i concentrations have stimulatory effects on the catalytic activities of Phos, PFK and PDH_c (81,187,192). The net result is an increase in glycogenolysis, glycolysis and pyruvate entry into the TCA cycle and therefore mitochondrial synthesis of ATP from CHO. The integration of CHO and TAG utilization is mediated through changes in the activity of PDH_c which in turn is affected by the ATP/ADP and NADH/NAD^+ ratio; each of which is an important metabolite in oxidative phosphorylation as reflected in equations 3 and 4. Therefore, the phosphorylation state of the cell links fuel utilization and substrate supply by affecting the catalytic activity of rate-limiting enzymes. The integration of CHO and TAG metabolism and the control of the key flux-generating and regulatory enzymes is complex and warrants further discussion.

1.5.2 Glycogen Phosphorylase

Glycogen phosphorylase is the flux-generating enzyme responsible for glycogen degradation in skeletal muscle by catalyzing the sequential removal of glucosyl units from the terminal branches of the glycogen molecule through a phosphorylation reaction that produces glucose-1- phosphate (G-1-P) as described below:

$$(6) \quad (\text{Glycogen})_n + P_i \Longrightarrow (\text{Glycogen})_{n-1} + \text{G-1-P}$$

The near-equilibrium enzyme phosphoglucomutase then immediately converts the G-1-P produced into glucose-6-phosphate (G-6-P) (128). Phos is located in the myoplasm, is bound to glycogen, and is associated with an number of other proteins that are involved in glycogen metabolism including: debranching enzyme, glycogen synthase, phosphorylase kinase, several protein kinases and glycogen phosphatase (50,123). (Fig. 2) The complex has also been found to be closely associated with the SR and has been termed the "sarcoplasmic reticulum - glycogenolytic complex" (50). The close association of Phos with the SR provides two important functions: first, it coordinates contraction induced Ca^{2+} release from the SR with Phos transformation (discussed below) thus providing substrate for ATP synthesis by enhancing glycogenolysis; second, the resulting increase in ATP production is then also available to the SR ATPase to ensure adequate re-accumulation of Ca^{2+} stores into the SR for the maintenance of contractile activity (50,123).


EXTRACELLULAR SPACE
CYTOSOL
AMP$_f$
P$_i$
glycogen$_{n-1}$
glycogen$_n$
G-1-P
Phos a
P
ADP
Phos kinase b
Phos kinase a
Phos phosphatase
ATP
ADP
ATP
protein kinase
EPINEPHRINE
cAMP
Ca^{2+}
adenylate cyclase G protein
ATP
P$_i$
glycogen$_{n-1}$
Phos b
glycogen$_n$
G-1-P
AMP$_f$
IMP
ATP
G-6-P

1.5.2.1 Covalent Regulation of Glycogen Phosphorylase

As a flux-generating enzyme, Phos regulation is complex and involves control via substrate, product, allosteric and covalent mechanisms between two forms of the enzyme (Fig. 2). Phos exists in one of two interconvertible forms: Phos *a*, considered the active form, active in the absence of free AMP (AMP_f); and Phos *b*, considered the less active form, active only in the presence of AMP_f (22,53). Covalent modification is effected by transformation from the *b* to *a* forms which is catalyzed by phosphorylase kinase in an ATP dependent reaction. Phosphorylase kinase also exists in an inactive *b* form and an active, phosphorylated *a* form. The phosphorylation and thus activation of phosphorylase kinase *a* can be achieved by either an increase in epinephrine or cytosolic Ca^{2+} concentration via a cyclic AMP (cAMP) dependent and independent mechanism respectively (22,151). At rest it appears that the cAMP mediated Phos *a* transformation is more important, while the Ca^{2+} mediated transformation occurs during muscular contraction. However, it is important to note that during exercise when both epinephrine and Ca^{2+} concentrations increase within the myocyte, the effect on phosphorylase kinase activity and Phos *b* to *a* transformation is synergistic (53). Complete transformation of Phos into the *a* form can be observed when human or animal muscle is stimulated to contract either voluntarily or electrically (22,153). Previously it has been observed that soon after the initiation of exercise in isolated cells (25) and *in vivo* in humans (22) Phos *a* reverts back to the *b* form despite continued contractile activity. This phenomenon called into question the

previously accepted premise that the rate of glycogenolysis was determined only by the degree of Phos *a* transformation (53). Research investigating the rate of glycogenolysis and Phos *a* transformation has provided strong evidence that other factors are important in the control of glycogen degradation and glycogenolytic flux (19,90). Infusion of epinephrine in resting human subjects resulted in complete Phos *a* transformation but a low level of glycogenolysis, which led to the conclusion that the free P_i concentration was important in the regulation of glycogenolysis (21). Further investigation in humans during epinephrine infusion and electrical stimulation with and with out prior exercise to raise the free P_i concentration demonstrated that both the AMP_f and P_i concentrations were important in regulating Phos flux (18). It may seem paradoxical that Phos *a* transformation decreases while glycogenolysis is occurring but, it should be noted that Phos flux is always well in excess of Phos transformation (except during sprint exercise) as a result of the potent effects of post-transformation allosteric regulation by AMP_f and free P_i. In this way Phos flux and therefore glycogen utilization can be directly regulated to the demand for substrate by the energy status of the cell (76).

1.5.2.2 Substrate Regulation of Glycogen Phosphorylase

Glycogen phosphorylase catalytic activity is mediated by the availability of its substrates glycogen and P_i (53). Resting muscle glycogen concentration in humans ranges from 250 - 400 $mmol \cdot kg^{-1}$ dw in the sedentary subject to ~650 $mmol \cdot kg^{-1}$ dw in the athlete (36). Since the *in vitro* K_m of Phos for glycogen has been determined to be ~1 mM (128), the enzyme is rarely limited by the lack of

glycogen. However, controversy exists as to whether the pre-exercise glycogen concentration has any influence on the glycogenolytic rate. Muscle glycogen depletion prior to exercise is associated with fatigue and utilization is directly related to intensity (81). Helspel and Richter (70) examined the relationship between muscle glycogen content and glycogenolytic rate utilizing a diet manipulation with an isolated *in vitro* rat preparation and found a positive correlation between the pre-exercise glycogen concentration and glycogenolytic rate during 15 minutes of intermittent tetanic contraction. Additionally, in those animals with the highest pre-exercise glycogen concentration the Phos *a* transformation increased approximately two fold over that of the glycogen depleted muscle. Conversely, other researchers using diet manipulation with both *in vitro* (166) and *in vivo* (5,151) preparations have failed to find any correlation between force development, glycogenolytic rate and pre-exercise glycogen concentration. Difficulty exists in comparing these studies since each study used a different stimulation protocol and species differences are known to exist with respect to both fibre type and fuel utilization between rats and humans (79,81). Glycogen content may effect glycogenolytic rate by two mechanisms. First, glycogen concentration may affect glycogenolytic rate by altering the K_m of Phos *a* and *b* for free P_i and/or AMP_f as has been demonstrated *in vitro*. Using a rabbit muscle preparation it was observed that an increase in glycogen concentration lowered the K_m of Phos *b* for AMP_f while decreases in glycogen concentration had the opposite effect (68). Furthermore, there exists two forms of glycogen in skeletal muscle, termed pro glycogen and

macro glycogen (1) and it has been suggested that one of the two forms may have an effect on Phos flux.

The other substrate required by Phos is free P_i, which has a much more significant regulatory effect on glycogenolytic rate. The estimated *in vitro* K_m of Phos *a* for human muscle for P_i is 26.8 mM (22,151). This is well above the free P_i concentration found in resting skeletal muscle which is ~2 mM (19). During exercise the free P_i concentration increases as a function of power output as does the AMP_f concentration (2,151). *In vitro* studies have shown that physiological concentrations of AMP lower the K_m of Phos *a* for P_i substantially (151). In support of the importance of P_i in the regulation of muscle glycogenolysis is the observation that interventions which lower the intramuscular P_i accumulation such as endurance training (28), decrease the rate of glycogenolysis. However, the elevation of free P_i concentration alone is not sufficient to augment glycogenolysis, as Chasiotis and associates (18) observed in humans when they raised the free P_i concentration with blood flow occlusion prior to exercise. The presence of other positive allosteric modulators, particularly AMP_f is important.

1.5.2.3 Allosteric Regulation of Glycogen Phosphorylase

Post-transformational allosteric regulation of Phos *a* and Phos *b* by ATP, AMP_f, IMP, and G-6-P is an important mechanism by which the rate of glycogenolysis is matched to both the energy state of the cell and ATP demand. *In vitro* studies have demonstrated that Phos *b* is subject to greater allosteric control than Phos *a*. Phos *b* is activated by AMP_f and IMP and inhibited by ATP and G-6-P

(2,22,27,53,90). ATP and G-6-P have been shown *in vitro* to inhibit Phos *b* with K_i of ~2 mM and 0.3 mM respectively (53). Both Phos *a* and Phos *b* are activated by AMP_f. AMP_f activates Phos *a* by lowering the K_m for its substrate free P_i from 26.8 mM to 11.8 mM (2,151). AMP_f also activate Phos *b*, but with a higher K_m than Phos *a*. The differences in the K_m for AMP_f between the two forms of Phos accounts for the observation that only 10 *u*M of AMP_f was effective in increasing Phos *a* but not Phos *b* activity during tetanic stimulation (2). The action of AMP_f on Phos *a* and Phos *b* is important as it results in greater Phos flux at any given free P_i concentration in the presence of small amounts of AMP_f. In support of the close relationship between Phos flux and AMP_f concentration is the observation that glycogenolysis is blunted when the accumulations of AMP_f and free P_i are reduced as observed in studies utilizing endurance training (23), caffeine ingestion (24) and enhanced FFA availability (48,130).

IMP is another important activator of Phos *b* (2) with half maximal activation occurring at ~1.2 mM. IMP is produced from the deamination of AMP by AMP deaminase. AMP deaminase is activated by increases in AMP, ADP and H^+ concentrations (46,177). Increases in the IMP concentration in the range for activation of Phos *b* have been reported during both intense electrical stimulation and intense dynamic exercise, mediated primarily by activation of AMP deaminase as pH_i declines (2). However, Chasiotis et al (22) measured IMP accumulation during intense dynamic cycling and found that although the IMP concentration had increased, it was well below the K_m for Phos *b*. The contribution of IMP to Phos flux

in humans is not well quantified as very few studies have examined Phos flux, and IMP concentration simultaneously (2,53).

1.5.2.4 Effect of pH on Glycogen Phosphorylase

Decrease in pH_i have been shown to inhibit Phos *b* to *a* transformation. Chasiotis and colleagues (20), examined the effects of acidosis on Phos transformation in humans. Acidosis was induced by intense isometric contraction to failure that resulting in a pH_i of ~6.6. This exercise bout was followed by an epinephrine infusion which failed to significantly increase Phos *a*. Intramuscular cAMP levels were also measured and found to be decreased. The combined results demonstrate that decreases in pH_i inhibit Phos *a* transformation by decreasing both cAMP production and the conversion of phosphorylase kinase *b* (inactive) to phosphorylase kinase *a* (active). *In vitro* studies have also shown that phosphorylase kinase *a* activity is inhibited at low pH (109). The effect on Phos *a* may be amplified by the lack of available free P_i (as a substrate) as pH_i decreases. The active substrate for Phos *a* is HPO_4^{2-} (103) which accounts for ~61% of the total P_i at a pH of 7.0 but only ~ 33% at a pH of 6.5 as the $H_2PO_4^-$ form becomes more prevalent (18,22). Alterations in the availability of free P_i may therefore also play a role in the reduced Phos flux observed when pH_i declines.

1.5.3. Phosphofructokinase

Glycogen phosphorylase as the flux-generating enzyme determines the rate of glycogenolysis, while PFK determines the overall rate of glycolysis (81). PFK is classified as a regulatory enzyme as it is subject to substrate, end-product and

allosteric regulation. Following the conversion of Phos produced G-1-P to G-6-P by phosphoglucomutase, G-6-P is transformed to fructose-6-phosphate (F-6-P) by glucose isomerase, both via equilibrium reactions (128). PFK catalyzes the conversion of F-6-P to fructose-1,6,-bisphosphate (F-1,6,-P) by the following reaction:

$$(7) \qquad F\text{-}6\text{-}P^{2-} + ATP^{4-} \rightleftharpoons F\text{-}1,6,\text{-}P^{4-} + ADP^{3-} + H^{+}$$

In vitro it has been shown that the PFK complex is inhibited by ATP, citrate and H^+ and activated by ADP, AMP, F-6-P, glucose-1,6,-phosphate, F-1,6,-P, fructose-2,6,-phosphate and NH_4^+ (12,165,181-183,196). However, in contracting skeletal muscle the regulation of PFK activity is governed primarily by the F-6-P, ADP_f, AMP_f, free P_i and H^+ concentrations. Importantly, the ADP_f, AMP_f, free P_i and H^+ concentrations are also involved in the equations that reflect the energy status of the cell and oxidative phosphorylation (equations 2-5). This allows a link between PFK activity and substrate supply to the demand for ATP re-synthesis via oxidative phosphorylation (81,165,180,184).

The PFK complex is thought to exist in a tetramer that when unprotonated is active and subject to regulation by binding at high affinity activation sites for F-6-P, F-1,6-P, AMP_f, ADP_f and low affinity inhibitory sites for citrate and ATP. ATP is both substrate and inhibitor with a K_i of ~ 5 mM (182). Protonation of the tetramer increases the affinity of the ATP and citrate binding sites, reciprocally decreasing the affinity of the F-6-P and F-1,6-P binding sites (106). This

necessitates an increase in the concentration of these two substrates to offset the reduced affinity to provide continued enzyme activity (45,182). The initiation of contraction causes the removal of the ATP inhibition and an increase in the positive modulators ADP_f, AMP_f, free P_i, F-6-P and F-1,6,-P. It should be noted that the effects of each positive modulator is synergistic with the others. Increases in citrate concentration due to elevated FFA oxidation has previously been thought to be have a significant inhibitory influence on PFK. However, recent studies have found that citrate plays only a minor role. Rather the precise regulation of PFK and therefore glycolytic rate is more intimately tied to the energy state of the cell (48,130,137).

1.5.3.1 Effect of pH on Phosphofructokinase

Early *in vitro* studies on the effects of pH on PFK activity demonstrated that decreases in pH had a strong inhibitory effect with a total inhibition of activity observed at a pH of ~6.5 (45,181). Human *in vivo* studies utilizing exhaustive exercise protocols drew similar conclusions regarding reduced PFK activity with a decrease in pH_i (94,117). However, Spriet et al (172) utilized a repeated electrical stimulation protocol in men and found that glycolysis continued despite a reduction in pH_i to 6.3 - 6.4. This discrepancy can be explained on the basis that the PFK activity results from the combined effects of both inhibitory and stimulatory influences. In addition, the early *in vitro* studies did not simulate *in vivo* conditions with respect to: the physiological concentration of the metabolites seen with exercise; physiologically relevant pH and temperature; and physiological

concentration of the enzyme which is critically important as it has been shown that dilution of the enzyme results in dissociation of the enzyme into its inactive dimers (12,137,165). The mechanism responsible for the pH effect on PFK activity has been studied extensively. Increases in the H^+ concentration are thought to increase the affinity of the ATP binding site which reciprocally reduces the affinity of the enzyme for its substrate F-6-P (8,106,182,183). In addition, increases in the H^+ concentration are associated with protonation of the histidyl residues of the PFK tetramer which increases the tendency of the enzyme to dissociate into its inactive dimers (45,164). However, it has also been observed that the magnitude of the pH inhibition can be modulated by increases in the F-6-P, ADP_f, AMP_f and free P_i concentrations. Increases in the F-6-P concentration helps to overcome the reduced affinity of its binding site that occurs with an elevated H^+ concentration. Decreases in the ATP/ADP ratio due to increases in the ADP_f concentration with exercise, have been shown to substantially accelerate glycolysis *in vitro* due to the binding of ADP to a high affinity site (180,182,183). AMP also augments PFK activity by increasing the affinity of the enzyme for its substrate (12), while increases in free P_i of a little as 2 mM have been demonstrated to substantially increase PFK activity by enhancing the stimulatory effect of ADP (196).

In summary, a decrease in pH_i inhibits PFK activity at physiological pH, temperature and metabolite concentrations by affecting the affinity of the various binding sites. However, pH alone does not determine overall enzyme activity and the concentrations of the metabolites that reflect the energy status of the cell - ATP,

ADP_f, AMP_f and free P_i should be examined in conjunction with the availability of substrate to obtain an accurate reflection of enzyme activity and therefore glycolytic flux.

1.5.4 Pyruvate Dehydrogenase

The pyruvate dehydrogenase complex (PDH_c) is a flux generating multi-enzyme complex located on the inner mitochondrial membrane (matrix side). PDH_c catalyzes the decarboxylation of glycolytically derived pyruvate to acetyl-CoA and therefore reflects the rate of entry of CHO into the oxidative pathways of ATP production. The overall irreversible reaction is as follows (190):

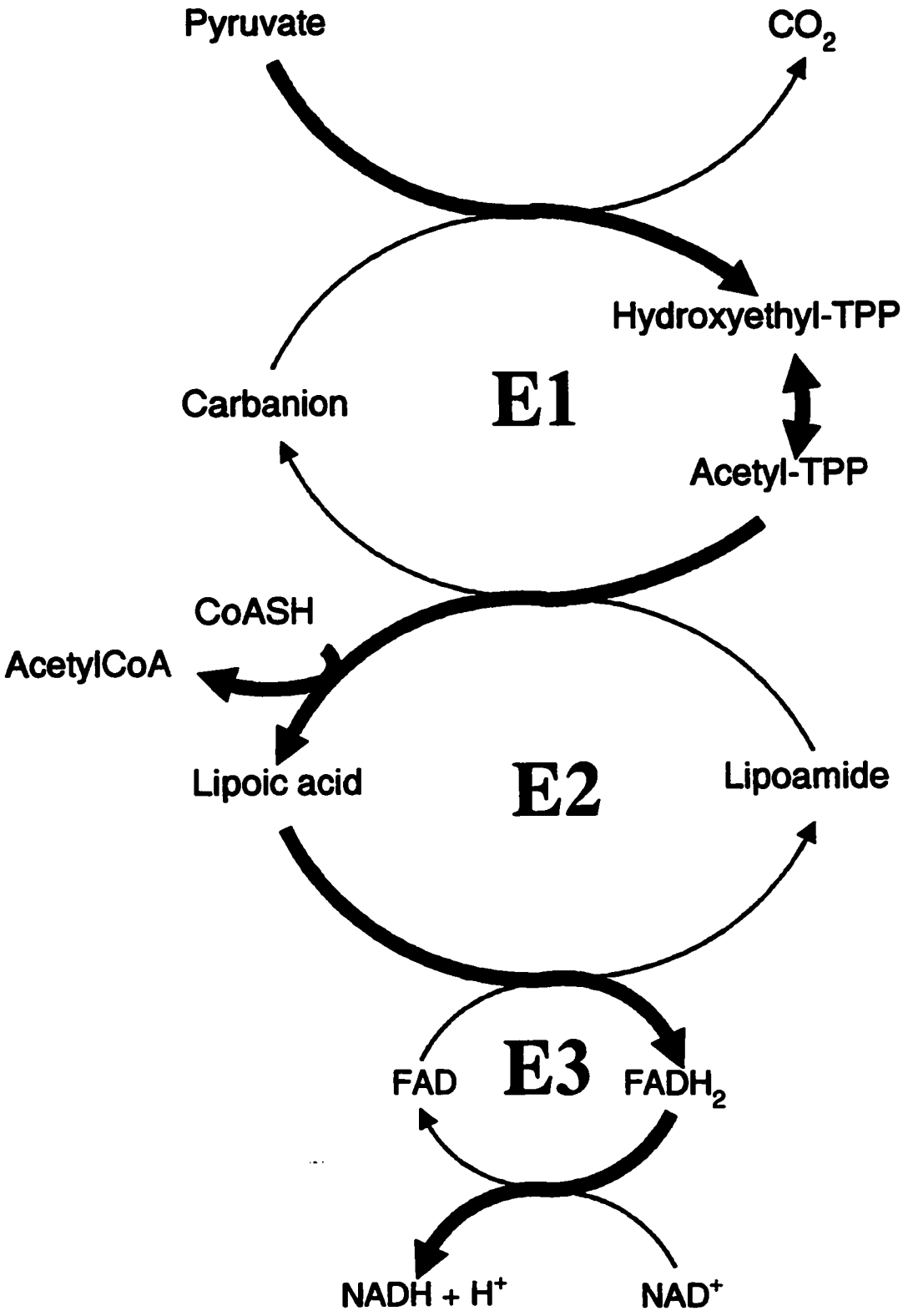
(8) $$\text{pyruvate} + NAD^+ + CoASH \Rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

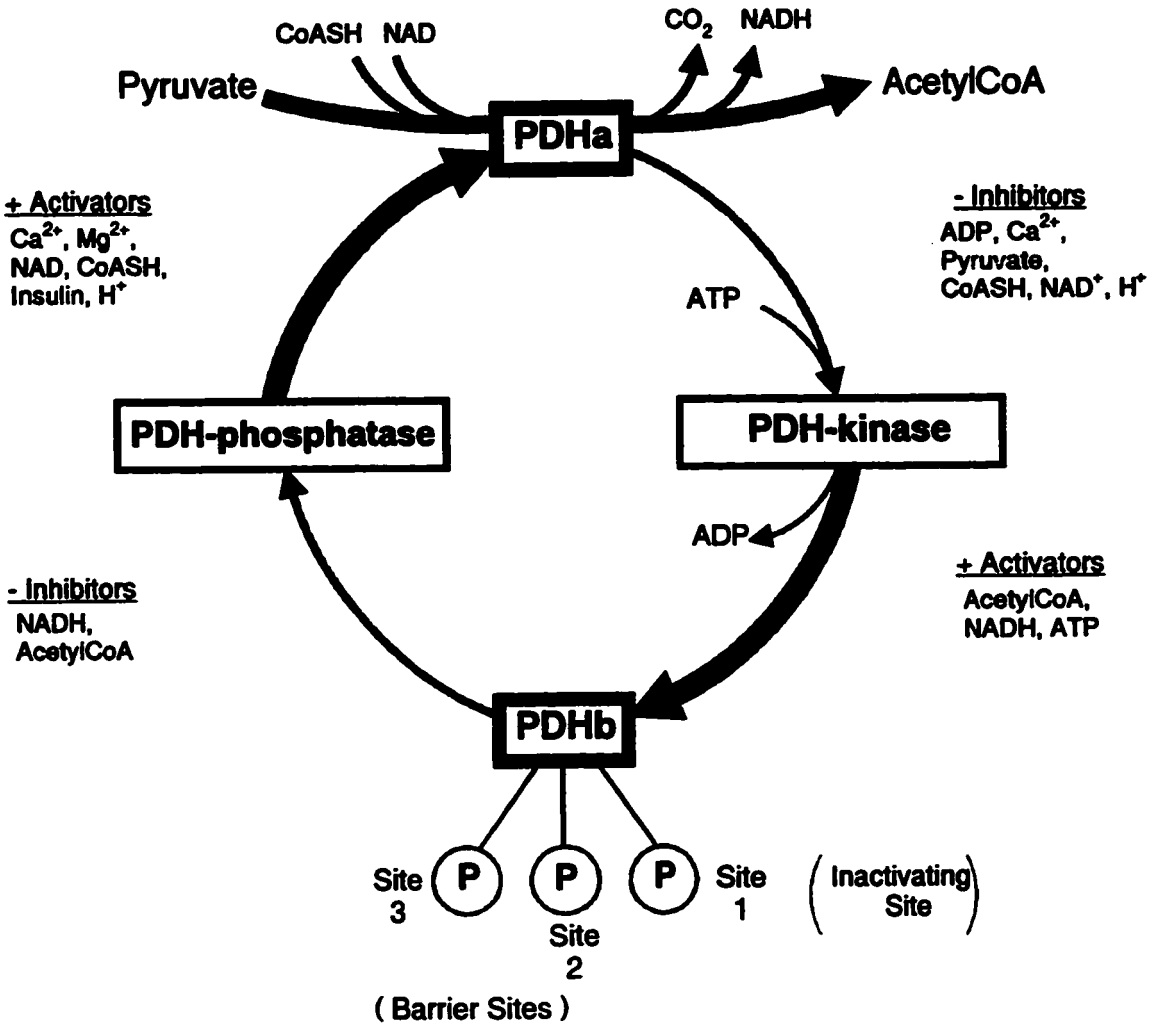
PDH_c is composed of three separate catalytic sub units: E1 (pyruvate decarboxylase), E2 (dihydrolipoamide acetyltransferase) and E3 (dihdydrolipoamide dehydrogenase). In addition, two regulatory proteins pyruvate dehydrogenase kinase (PDHK) and pyruvate dehydrogenase phosphatase (PDHP) are associated with the enzyme complex (190). The three catalytic sub units operate sequentially to produce acetyl-CoA from pyruvate. The E1 sub unit catalyzes the decarboxylation of pyruvate which is an irreversible reaction and therefore considered the rate limiting step for the enzyme complex (148). The E1 sub unit contains 3 phosphorylation sites and exists in two interconvertable forms; an unphosphorylated active form known as PDH_a and a phosphorylated inactive form known as PDH_b (138). The phosphorylation of only one site (site 1) confers

inactivation, while the other two phosphorylation sites serve as barriers to dephosphorylation or activation. It should also be noted that the phosphorylation of these inactivating sites is highly dependent on the cellular ATP concentration. The other two reactions catalyzed by the E2 and E3 sub units are reversible (146) (Fig. 3). E2 forms the core of PDH_c and catalyzes the addition of CoASH to the acetyl-thiamine pyrophosphate (TPP) (an essential enzyme cofactor) molecule produced from the E1 reaction, leading to the formation of acetyl-CoA. The E3 component then catalyzes an electron transfer from the reduced E2 component - lipoic acid, to FAD^+ producing $FADH_2$ and regenerating the disulfide form of the lipoyl group of E2. Transfer of the electrons to the reducing equivalent NAD^+ forms NADH + H^+ and regenerates FAD^+ (34) (Fig. 3).

As a flux-generating enzyme, PDH_c is subject to substrate, end-product and allosteric modulation which affects the phosphorylation of site 1 and therefore covalent transformation of the enzyme complex (Fig. 4). The overall catalytic activity of PDH_c is dependent upon the phosphorylation state of the complex which in turn is determined by the relative activities of PDHK and PDHP. The ratio of their activities determines the percentage of PDH in the active *a* form. In turn, the relative activities of PDHK and PDHP are mediated by the mitochondrial acetyl-CoA/CoASH, ATP/ADP, NADH/NAD^+ ratios, the availability of pyruvate and the concentrations of Ca^{2+} and H^+ (190).

Additionally, PDH_a can be regulated acutely or chronically. Acutely, the above mentioned modulators are important, however, chronic regulation is affected


Pyruvate
CO_2
Hydroxyethyl-TPP
Carbanion
E1
Acetyl-TPP
CoASH
AcetylCoA
Lipoic acid
E2
Lipoamide
FAD
E3
$FADH_2$
$NADH + H^+$
NAD^+


CoASH NAD
CO_2 NADH
Pyruvate
AcetylCoA
PDHa
+ Activators
Ca^{2+}, Mg^{2+},
NAD, CoASH,
Insulin, H^+
- Inhibitors
ADP, Ca^{2+},
Pyruvate,
CoASH, NAD^+, H^+
ATP
PDH-phosphatase
PDH-kinase
ADP
- Inhibitors
NADH,
AcetylCoA
+ Activators
AcetylCoA,
NADH, ATP
PDHb
Site 3
P
P
P
Site 1
Inactivating Site
Site 2
(Barrier Sites)

by phosphorylation of sites 2 and 3 which prevents activation of the enzyme. The chronic regulation of PDH_a is highly complex and is the beyond the scope of this thesis.

In order to determine the affects of exercise on PDH_c transformation numerous studies were undertaken by Constantin-Teodosiu utilizing isometric contraction, incremental exercise up to 65% VO_{2max} and prolonged exercise (29-31). These authors found that PDH_a was low at rest and gradually increased up to 65% VO_{2max}. Further investigation has demonstrated that increases in PDH_a occur with incremental exercise up to 75%-90% $VO_{2\ max}$ (76,140). Additionally, the initial activation rates reported by Constantin-Teodosiu are approximately an order of magnitude lower than those reported by Putman et al (140) and Howlett et al (76) and may be explained by a methodological problem since the analysis of PDH_a by Constantin-Teodosiu was carried out under conditions of substrate limitation.

1.5.4.1 Acetyl-CoASH/CoASH Concentration Ratio

The acetyl-CoA/CoASH ratio demonstrates regulatory aspects consistent with end-product inhibition and allosteric regulation of PDH_a by direct action of this ratio on PDHK and PDHP. Previous studies have shown that a high acetyl-CoA/CoASH ratio inhibits PDH_a by inhibiting the reversal of the CoASH transfer step at E2 by a high acetyl-CoA concentration (43,138). Allosteric regulation by a high acetyl-CoA concentration is mediated by a decrease in PDHP activity and an increase in PDHK activity, while a high CoASH concentration has the opposite effect (34,138). The net effect of an increase in this ratio is therefore

a lower PDH_a due to greater PDHK activity and a decrease in PDHP activity. A decrease in this ratio has the opposite effect and results in an increase in PDH_a. Recent studies have shown that the acetyl-CoA/CoASH ratio is important in regulating PDH_a at rest but may be less important during exercise. Research examining PDH_a during exercise and acetyl group accumulation has revealed that the acetyl-CoA/CoASH ratio is probably not inhibitory to PDH activation during exercise since the acetyl-CoA concentration markedly increases when PDH_a is highest (30,31). Further investigation into the importance of the acetyl-CoA/CoASH ratio on acute PDH_c activation by Putman et al (142) utilizing an acetate infusion revealed similar results. The acetate infusion was successful in raising the acetyl-CoA/CoASH ratio by increasing both the acetyl-CoA and acetylcarnitine concentrations at rest and during exercise and was considered to be responsible for the decreased PDH_a observed at rest. However, during exercise, despite continued elevation in the concentration of these metabolites, PDH_a increased to the same degree as control subjects. Other experiments using an intralipid infusion to artificially elevate the acetyl-CoA concentration and therefore the acetyl-CoA/CoASH ratio also had no effect on PDH_a during high intensity exercise (85% $VO_{2\ max}$) (48). Conversely, during low intensity exercise with increased FFA concentration, PDH_a was reduced despite the absence of any difference in the acetyl-CoA concentration during the exercise trial (130).

1.5.4.1 NADH/NAD⁺ Concentration Ratio

The regulatory action of the NADH/NAD⁺ ratio is two-fold, demonstrating characteristics of both end-product inhibition (154) and allosteric regulation (34,107,138). End-product inhibition by an elevated NADH concentration results from the displacement of NAD⁺ from its binding site on the E3 sub unit of PDH_c. Consequently, the electron transfer from $FADH_2$ does not occur nor does the re-generation of the lipoyl moiety of E2 by transfer of the reducing equivalents from lipoic acid to FAD⁺. The overall result is an inhibition of the PDH_c reaction. Allosteric regulation by the NADH/NAD⁺ ratio provides a more sophisticated level of control by affecting both PDHK and PDHP. NADH directly inhibits PDHP and activates PDHK, while NAD⁺ has the opposite effect (34,138,190). The net result of an increase in the ratio is a decrease in PDH_a while a decrease in the ratio results in an increase in PDH_a.

The NADH/NAD⁺ ratio is a potentially important regulator under conditions of increased FFA utilization such as low carbohydrate diets, intralipid infusion or fat feeding. The increased β-oxidation of FFA increases the NADH concentration and therefore can potentially effect PDH_c activation and the oxidative phosphorylation rate as NADH is a key metabolite in the two reactions that govern oxidative phosphorylation (Equations 3 and 5). Changes in the NADH concentration therefore play an integral role in the regulation of oxidative phosphorylation and the interplay of CHO and FFA utilization (4,144,145,192).

1.5.4.3 ATP/ADP Concentration Ratio

The ATP/ADP ratio unlike the previous two ratios effects PDHK only, via a competition between its substrates ATP and product ADP (33,107,112). Consequently an increase in the ratio results in increased PDHK activity which reduces PDH_a transformation while a decrease in the ratio has the opposite effect.

1.5.4.4 Regulation by Calcium

Regulation of PDH_a by the Ca^{2+} concentration is extremely important, particularly during exercise, as exercise results in Ca^{2+} release from the SR into the cytosol for the initiation of muscle contraction (118). Calcium effects PDH activity by simultaneously increasing the activity of PDHP and inhibiting PDHK by increasing and decreasing the affinity of each for its substrates (41,42,118). Estimates of the mitochondrial Ca^{2+} concentration within muscle have been shown it to be within the physiological range to cause half maximal activation (0.2 -0.3 *u*M) (41,42,44,118). Studies in humans using a variety of exercise conditions have demonstrated that PDH_a is low at rest and increases with exercise intensity up to ~75% $VO_{2\,max}$ where it appears to be maximally activated (29-31,76,140).

1.5.4.5 Regulation by Pyruvate

Pyruvate is the principal substrate for PDH_c and as such is an important determinant of transformation and flux. Pyruvate exerts its affect in two ways. First, through a direct affect of substrate binding to PDH_c, ensuring adequate availability of substrate and second, by allosteric regulation of PDHK. Pyruvate is inhibitory to PDHK with a K_i of 0.5 - 2.8 mM (33,77,112) which is within the physiological range

of the pyruvate concentration found at rest (143) and during exercise (140,143,170). As pyruvate concentration increases with muscular activity the inhibitory affect of pyruvate on PDHK also increases, resulting in PDH_a transformation to a higher level.

1.5.4.6 Effects of pH on PDH_a

Increases in the H^+ concentration have been shown to increase PDH_a and pyruvate oxidation in perfused rat hearts (136). A number of mechanisms have been proposed to explain this phenomenon. The change in pH may affect PDH_c function by altering its conformation, stability and/or the affinity for its regulatory metabolites (164). *In vitro* analysis of the pH optimum of PDHK and PDHP has found the kinase to have a pH optimum of 7.0 - 7.2, with increasing inhibition as pH declines, while the phosphatase has a pH optimum of 6.7 -7.1 (77). A low pH may also alter the distribution of pyruvate across the mitochondrial membrane such that the mitochondrial pyruvate concentration increases as cytosolic pH falls, thereby decreasing the activity of PDHK (6). The research to date suggests that increases in H^+ concentration, similar to those that accompany exercise have a potentially stimulatory effect on PDH activity. However, the *in vivo* effects in humans have not been studied.

1.6 Free Fatty Acid Metabolism During Exercise

As previously mentioned, glycogen is the preferred fuel source during high intensity exercise. However, FFA from both adipose tissue and intramuscular TAG pools are also important fuel sources and have a significant contribution during low, moderate and high intensity exercise and in situations when glucose availability is reduced (93,185). During high intensity exercise the intramuscular TAG stores are utilized rather than adipose tissue stores (to be discussed in the following sections). Intramuscular TAG content varies across species, fiber type, and is influenced by hormonal factors, diet and physical training (63,82). Exercise has been shown to increase both adipose tissue lipolysis and intramuscular TAG lipolysis (35,63).

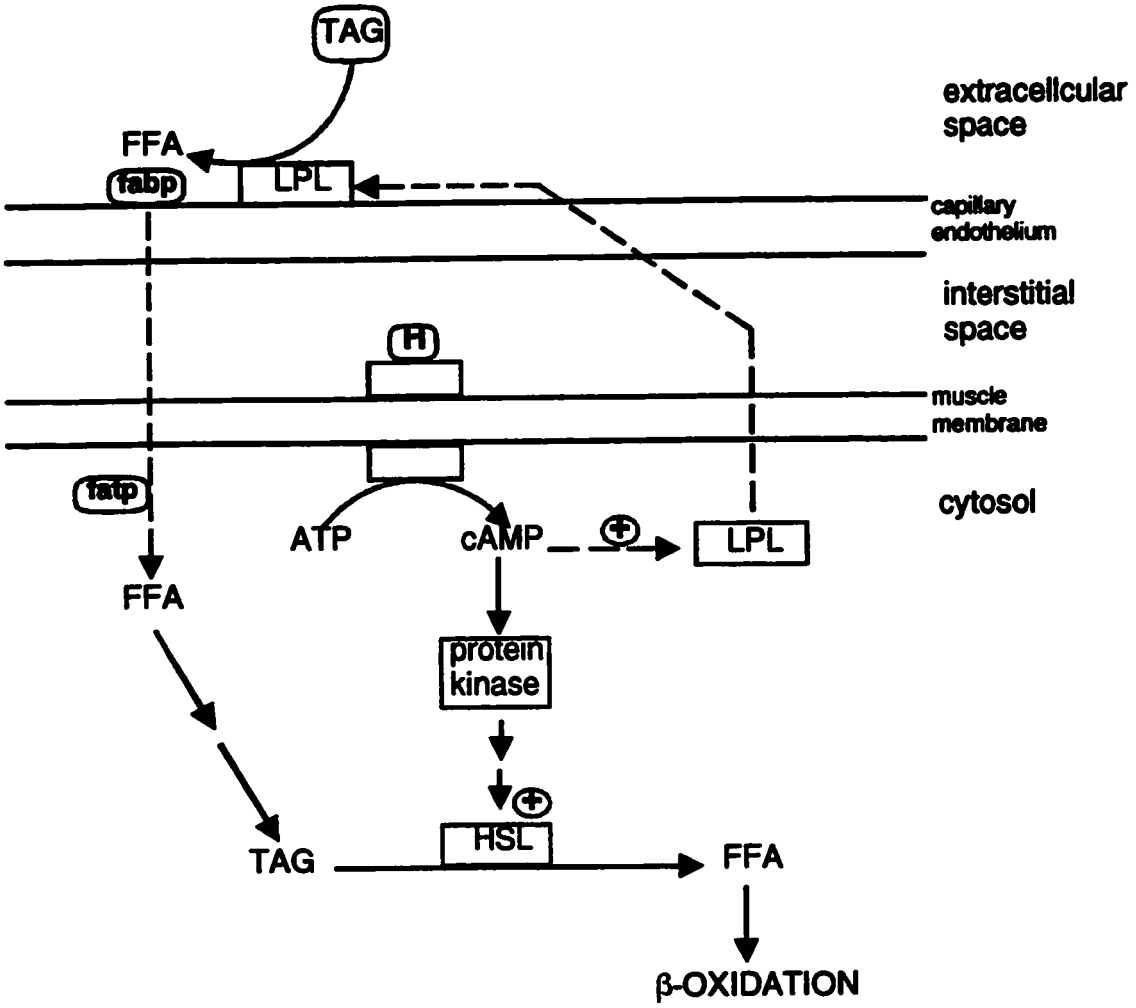
1.6.1 Adipose Tissue Lipolysis

Adipose tissue lipolysis occurs via a cAMP dependent activation of a hormone sensitive lipase (HSL) which is stimulated to breakdown TAG by catecholamines, and produce TAG for storage in response to insulin (35,63). Activation of the HSL is the rate-limiting, flux generating step in the mobilization of TAG from adipocytes. Hydrolysis of TAG releases both FFA and glycerol into the circulation. Another enzyme, lipoprotein lipase (LPL) is present on the lumenal side of capillary endothelium and functions to lyse the circulating TAG into FFA for tissue uptake. Once taken up by the tissue, the FFA can either be re-esterified into the TAG pool or oxidized (35,185). The presence of the LPL on the capillary membrane makes the estimation of adipose tissue lipolysis by the measurement of the plasma FFA concentration alone inaccurate (35). However, the plasma

glycerol concentration does provide a good estimate of adipose tissue lipolysis as the adipocytes lack glycerol kinase (35,185).

1.6.2 Intramuscular Lipolysis

The precise regulation of intramuscular lipolysis has not been fully elucidated. Three distinct LPL's have been discovered, each with a distinct maximal activation at a different pH: acid LPL at pH 5.0, neutral LPL at pH 7.0 and alkaline LPL at pH 8.5 (132,134). Additionally, an intramuscular HSL has been identified and appears to act similarly to the HSL in adipose tissue with an added pH sensitivity (35,133,185). Originally it was proposed that the alkaline pH LPL was responsible for hydrolyzing the intramuscular TAG pool, but experimental evidence for this is lacking (132,185). Currently, a model for the hydrolysis/uptake of TAG in muscle has been proposed as follows (Fig. 5). Catecholamines and/or glucagon bind to a receptor on the sarcolemmal surface which leads to an increase in the cAMP concentration. The increased cAMP leads to the activation of a neutral pH HSL, which is responsible for lipolysis of the intramuscular TAG pool into FFA for β-oxidation. The increased cAMP levels are also thought to stimulate the synthesis and/or transport of a cytoplasmic LPL to the lumenal surface of the capillary endothelium which functions to augment the availability of FFA from the circulation for intramuscular TAG pool replenishment (63,133,185). The contribution of intramuscular TAG hydrolysis during exercise has been estimated to be between 15 - 40% depending on the exercise protocol, intensity, duration, and


TAG
FFA
fabp
LPL
extracellular space
capillary endothelium
interstitial space
H
muscle membrane
fatp
cytosol
ATP
cAMP
LPL
FFA
protein kinase
HSL
TAG
FFA
β-OXIDATION

measurement technique (63,75,93,155,168,185). It is important to note that the total amount of FFA available for β-oxidation is represented by both the free and esterified portions within the muscle. Recent investigation by Dyck et al (47) utilizing a pulse-chase technique in an isolated rat muscle preparation during varying intensities of electrical stimulation have confirmed some previous indirect estimates of the contribution of intramuscular TAG during exercise in humans (87,108) and animals (75,168). Dyck et al (47) found that during contraction ~30% of the FFA made available to the muscle is esterified into the TAG pool. As exercise intensity increases, greater absolute amounts of FFA are esterified resulting in greater absolute rates of oxidation and esterification. A greater percentage of the FFA taken up by the muscle was also shunted toward oxidation, and a lower percentage toward storage. Overall, intramuscular TAG accounted for greater than 75% of the total muscle energy, while exogenous TAG accounted for ~3 % and CHO (endogenous and exogenous) accounted for ~20% across all exercise conditions. Together these studies illustrate that during exercise both the plasma FFA and the intramuscular TAG pool are important sources of fuel.

1.6.3 Effect of pH on Lipolysis

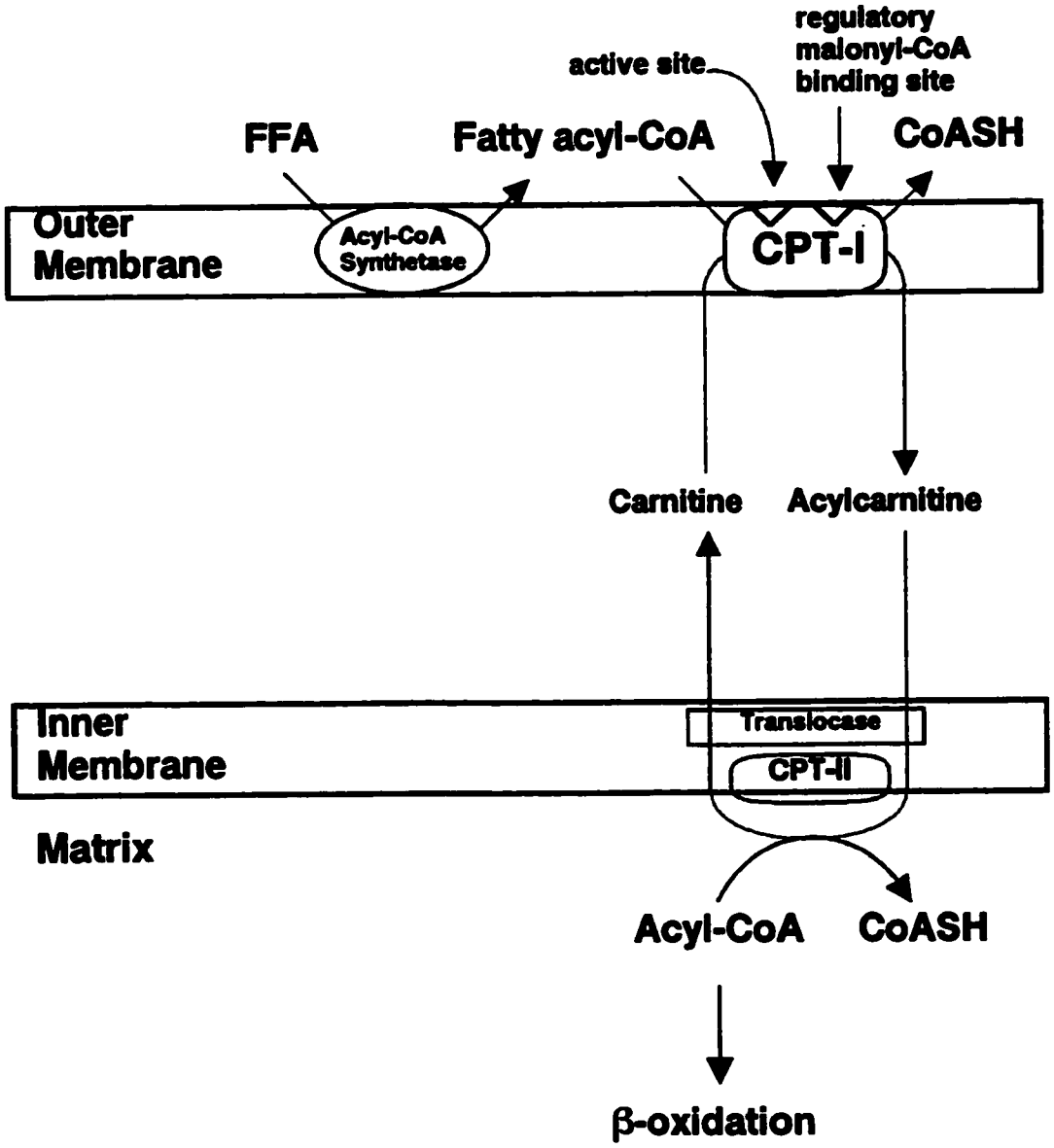
High intensity exercise (ie > 75% $VO_{2\,max}$) results in the production of lactate and H^+ and each has been shown to inhibit adipose tissue lipolysis. Early studies in dogs (83-86) demonstrated that lactate impairs adipose tissue lipolysis as evidenced by reduced FFA release during lactate infusion and exercise. The reduction in plasma FFA concentration also led to reduced uptake in the exercising

muscle and overall reduced oxidation of FFA. Investigation in isolated adipocytes provided an explanation for this phenomenon by demonstrating that decreases in pH of the perfusion medium markedly inhibited cAMP accumulation, thereby suggesting that the increased H^+ concentration interferes with the cAMP HSL activation cascade (55). Jones et al (95) found that induced acidosis, by NH_4Cl ingestion, significantly decreased the plasma glycerol and FFA concentrations compared to control conditions which suggested that adipose tissue lipolysis was impaired with acidosis. These authors felt the impairment was related to the alteration in the H^+ concentration and not lactate as the plasma lactate concentration was lower with acidosis.

Conversely, intramuscular TAG lipolysis has been shown to occur under conditions of increased H^+ concentration (93,117,168,171). A well designed study by Jones et al (93) examined TAG metabolism and FFA turnover during high intensity cycling in humans using radio-labeled palmitate. These researchers demonstrated that large increases in glycerol concentration occurred simultaneously with a decrease in the FFA turnover rate, suggesting that intramuscular TAG is used when lipolysis from adipose tissue is decreased. Recently, these results have been confirmed by Romijin et al (156). The overall results suggest that lipolysis does not cease with an increase in the H^+ concentration, rather the emphasis shifts to a greater reliance on intramuscular TAG stores.

1.6.4 FFA Entry into the Mitochondria

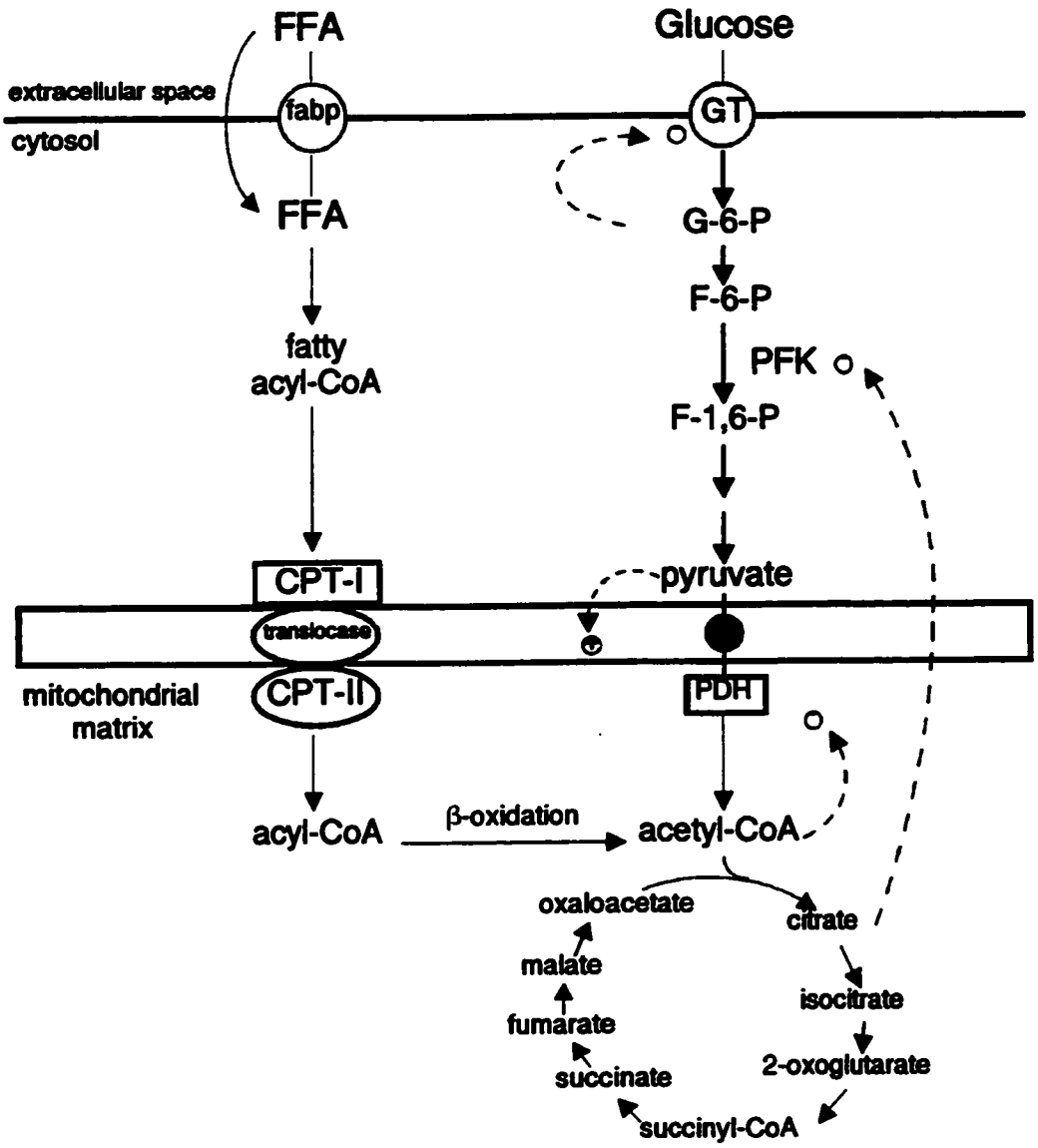
Once FFA is made available within the myoplasm it must gain access to the mitochondria for β-oxidation. The existence of a fatty acid binding protein (FABP) on the plasma membrane for the transport of FFA across the muscle membrane has been shown to exist. Movement of FFA within the muscle and between sites of storage and oxidation is accomplished by a transporter protein known as the fatty acid translocase protein (FATP) (10). In the rat heart the FATP has been shown to be pH sensitive such that a decrease in pH_i increases the dissociation of FFA from the carrier which would enhances FFA availability in conditions such as exercise when the demand for substrate is high (185). Once delivered to the cytoplasmic side of the outer mitochondrial membrane long chain FFA's are esterified with CoASH generating a fatty acyl-CoA molecule. Long chain fatty acids (LCFA) cannot freely diffuse across the membrane whereas medium chain and short chain FFA's can. The fatty acyl-CoA molecule then combines with carnitine in the inner membranous space catalyzed by CPT1 (Fig. 6). The specific transport of the acyl-carnitine molecule across the inter-membranous space is the function of the acyl-carnitine translocase protein. Once in the matrix side of the inner membrane, another protein CPT2 exchanges the carnitine residue of the acyl-carnitine molecule with CoA which yields an intramitochondrial fatty acyl-CoA molecule and regenerates carnitine. Carnitine then diffuses back to the outer membrane (121,185). Once inside the matrix the fatty acyl-CoA molecule undergoes


regulatory
malonyl-CoA
binding site
active site
FFA
Fatty acyl-CoA
CoASH
Outer
Membrane
Acyl-CoA
Synthetase
CPT-I
Carnitine
Acylcarnitine
Inner
Membrane
Translocase
CPT-II
Matrix
Acyl-CoA
CoASH
β-oxidation

step wise degradation via β-oxidation. The importance of CPT1 activity on FFA utilization will be highlighted in the following sections.

1.7 Significance of Pyruvate Dehydrogenase in Fuel Selection

The PDH_c occupies a unique position in skeletal muscle as it impacts fuel utilization and lactate production. PDH_c has long been thought to play a pivotal role in fuel selection as postulated in the classical theory of metabolic regulation known as "the glucose-fatty acid cycle". First proposed by Randle et al (147) this theory proposed a mechanism to explain the reciprocal relationship between glucose and FFA utilization *in vitro*, in rat heart and diaphragm muscle (144,146). Briefly, this theory suggests that muscle and whole body glucose utilization is down regulated proportionately when FFA availability and utilization from either endogenous or exogenous sources is increased. Conversely, elevated blood glucose, increased uptake and utilization of CHO inhibits TAG utilization (144,146). The mechanisms proposed to be responsible for these observations are as follows, and form the basis of the "glucose-fatty acid cycle" (Fig. 7). Increased FFA oxidation increases the mitochondrial acetyl-CoA/CoASH and $NADH/NAD^+$ ratios which activate PDHK and inhibit PDHP leading to a reduction in PDH_a activity and therefore pyruvate oxidation. The increased entry of FFA derived acetyl-CoA into the TCA cycle leads to reciprocal elevations in the citrate concentration. Citrate, in turn crosses the mitochondrial membrane into the cytosol and inhibits PFK, resulting in increased G-6-P and G-1-P concentrations. The increased G-6-P concentration inhibits


FFA
Glucose
extracellular space
cytosol
fabp
GT
FFA
G-6-P
F-6-P
fatty
acyl-CoA
PFK
F-1,6-P
CPT-I
pyruvate
translocase
mitochondrial
matrix
CPT-II
PDH
acyl-CoA
β-oxidation
acetyl-CoA
oxaloacetate
citrate
malate
isocitrate
fumarate
2-oxoglutarate
succinate
succinyl-CoA

hexokinase resulting in reduced glucose uptake. The increased G-1-P concentration results in end-product inhibition of Phos . The result of this series of events is the reduction in intramuscular glycogen degradation, glucose uptake, pyruvate production from glycolysis and reduced pyruvate oxidation via PDH_a. Since these early studies there has been continued controversy regarding the existence and/or regulation of the "glucose-fatty acid cycle" in human skeletal muscle during rest and exercise (167). Studies in humans utilizing fat feeding and fat emulsion infusions to elevate FFA availability have found that glucose uptake (52,66) is inhibited and muscle glycogen is spared (156,186,195) which supports the existence of the glucose-fatty acid cycle as classically proposed. However, these studies have not measured PDH_a, the citrate or G-6-P concentrations. Other studies have found evidence that contradicts the mechanisms suggested in the original glucose fatty-acid cycle. Constantin-Teodosiu et al (31) demonstrated that increases in the acetyl-CoA/CoASH ratio with high intensity exercise did not inhibit PDH_a transformation or flux. Dyck and associates (48) found that intralipid infusion at rest and during exercise did spare muscle glycogen but without significant changes in either the intramuscular acetyl-CoA or citrate concentrations. Peters et al (137) examined the regulatory role of citrate on PFK catalytic activity *in vitro* using purified rabbit muscle and demonstrated that citrate has little effect on PFK activity under simulated exercise conditions. Acute increases in FFA concentration at rest and during low intensity exercise, although successful in increasing the acetyl-CoA concentration did not result in any differences in glucose uptake, but PDH_a was

down-regulated (130). Finally, Putman et al (142) used an acetate infusion during rest and exercise in humans to elevate the muscle acetyl-CoA, citrate, and acetylcarnitine concentrations and found that under resting conditions PDH_a was decreased but during exercise these elevated metabolites failed to inhibit PDH_a. The combined data suggests that the classically proposed mechanisms of the "glucose-fatty acid cycle" may not operate during exercise.

1.7.1 The Reverse Glucose-Fatty Acid Cycle

Recent work by Sidossis et al (162,163) has also challenged the classic "glucose-fatty acid cycle". The original theory states that the increased provision of TAG impairs CHO oxidation. These authors have proposed an alternate theory termed the " reverse glucose-fatty acid cycle" based on experimental evidence that contradicts the classic theory. Their research suggests that it is the intracellular metabolism of glucose that determines FFA oxidation. The mechanisms proposed to be responsible for this reversed cycle, is the inhibition of LCFA entry into the mitochondria mediated by a CHO derived increase in the malonyl-CoA concentration which inhibits CPT1. Supporting evidence comes from studies in humans under both resting an exercise conditions. In the resting state, radio labeled oleate, (a LCFA) and octanoate, (a medium chain fatty acid) were infused under basal conditions and during a hyperglycermic/hyperinsulin clamp. The plasma concentrations of glucose, insulin, labeled oleate and octanoate were measured before and after the clamp as were the intramuscular concentrations of the acyl-CoA and acyl-carnitine esters. Oxidation rates of glucose and FFA were

also measured. The results demonstrate that during the clamp the LCFA-acyl-carnitine (oleate) formation and oxidation was significantly decreased from basal conditions. In contrast, the medium chain fatty-acyl-carnitine (octanoate) formation and oxidation was unchanged from basal levels during the clamp. These results suggest that it is glucose and/or insulin that regulates FFA oxidation by controlling the rate of LCFA entry into the mitochondria under resting conditions (162,163). To test whether the provision of glucose/insulin inhibits LCFA entry during exercise the same protocol was used during both low (40% $VO_{2\ max}$) and high (80% $VO_{2\ max}$) intensity exercise during basal and clamp conditions (161). The results of this study also suggest that it is the availability of glucose (rather then FFA) that determines substrate oxidation in humans during exercise.

The exact mechanism responsible for the inhibition of CPT1 is not clear but is probably not related to increases in malonyl-CoA concentration, at least, in human skeletal muscle. Odland et al (131) examined the malonyl-CoA content in human skeletal muscle during exercise at three different power outputs and found that the malonyl-CoA content remains relatively constant despite varying power outputs and did not correlate with the FFA oxidation rate. Other researchers have studied the possible role of a decrease in pH on CPT1 activity and have demonstrated *in vitro* rat muscle preparations that CPT1 is inhibited with decreases in pH (175). Human skeletal muscle *in vitro* analysis has confirmed this pH sensitivity and shown that human muscle CPT1 is much more sensitive to decreases in pH, with complete inhibition occurring at pH_i ~6.8 (174). The

combined results of the above studies suggests that inhibition of LCFA entry into the mitochondria in humans is not related to the malonyl-CoA content but rather may be mediated by a direct effect of H^+ on the CPT1 transporter.

1.8 Lactate Metabolism

Lactate is formed from pyruvate by the action of the near-equilibrium enzyme lactate dehydrogenase (LDH) by the equation (37):

(9) $\text{pyruvate}^- + \text{NADH} + \text{H}^+ \rightleftarrows \text{lactate}^- + \text{NAD}^+$

The activity of LDH is high in all muscle with the equation favoring the formation of lactate. From this equation it is also clear that lactate formation is influenced by changes in the H^+ and NAD^+ concentrations (41,92).

1.8.1 Proposed Mechanisms of Lactate Production

Lactate production during exercise has been attributed to the development of tissue hypoxia which results in the acceleration of glycolysis. This "O_2 limitation" was first proposed in 1923 by Hill and Lupton (71) and still receives widespread support today (104,188). Briefly, the theory proposes that as exercise intensity increases oxygen delivery to the mitochondria is compromised which limits substrate supply at cytochrome oxidase and leads to an increase in both cytosolic and mitochondrial NADH and ADP concentrations. The increased ADP concentration in turn leads to increases in the cytosolic AMP and free P_i concentrations. The combined increase in the ADP, AMP, and free P_i

concentrations accelerate glycogenolysis by increasing the catalytic activity of Phos and accelerates glycolysis by stimulating PFK. This combination ultimately leads to a greatly enhanced pyruvate production that cannot be oxidized by the TCA cycle and ETC due to the lack of oxygen availability. The excess pyruvate in the presence of a high cytosolic NADH concentration is converted to lactate by LDH resulting in increased lactate production. Support for this theory comes from the observation that lactate production increases during hypoxia and decreases with hyperoxia (105). Additionally, the examination of NADH accumulation during normoxic, incremental exercise in humans was found to coincide with lactate accumulation (159), but the NADH/NAD^+ measurements were made on whole cell extracts which do not reflect the mitochondrial redox state (141). Various studies have been undertaken using a variety of measurement techniques such as myoglobin saturation (26), the glutamine dehydrogenase reaction (64,141), surface fluorometry (89), and measurements of the oxidation state of cytochrome oxidase (173) have all shown that the redox state increases with exercise, and the partial pressure of oxygen (PO_2) never drops below the critical PO_2 value of 0.1 - 0.3 Torr (17) when the lactate concentration is increasing. Recent investigation by Richardson et al (152) have confirmed this earlier finding. These authors examined lactate formation during normoxia and hypoxia during single leg exercise in conjunction with assessment of myoglobin saturation with ^{1}H-phosphorous magnetic resonance spectroscopy (MRS). The results demonstrated that net muscle lactate efflux is unrelated to intracellular PO_2. The contribution of the NADH concentration

cannot be accurately assessed until a reliable method to measure the tissue concentrations is developed.

1.8.2 Lactate Production by Mass Action

PDH_c plays a key role in intramuscular lactate accumulation as it determines the rate of glycolytically derived pyruvate entry into the TCA cycle. Studies in humans have shown that the maximal catalytic activity of PDH_a is ~3 - 5 $mmol \cdot kg^{-1} \cdot min^{-1}$ ww (29,31,76,140,141) which is significantly lower than maximal Phos flux which can increase up to greater than 200 $mmol \cdot kg^{-1} \cdot min^{-1}$ww with maximal sprint exercise (21,22,135,149-151). Recent studies comparing the rates of pyruvate production by glycolysis and pyruvate oxidation by PDH_a during both maximal intermittent exercise (135,141) and incremental cycling (76) have demonstrated that lactate accumulation during moderate and high intensity exercise results from the differences between the maximal rates of pyruvate production by glycogenolysis/glycolysis and pyruvate entry into the TCA cycle via PDH_a. Studies by Timmons et al (178,179) examining the rate of entry of substrate into the TCA cycle at the onset of exercise under ischemic conditions found that the increase in lactate was due to the differences in the catalytic activities of Phos and PDH_a. Increasing the PDH_a activity by administration of dichloroacetate (DCA) prior to exercise significantly reduced lactate accumulation. The results of these studies suggest that ATP regeneration by oxidative phosphorylation can be essentially limited by acetyl-CoA delivery to the TCA and therefore flux through PDH_a.

In conclusion, based on the available data it appears that intramuscular lactate production results from the difference between the rate of substrate supply by glycogenolysis and substrate entry into the oxidative pathways via PDH_a and not the result of reduced oxygen availability.

1.8.3 Lactate Transport

Intramuscular lactate accumulation reflects the rate of production by LDH and the rate of efflux from the muscle (100), while the blood lactate concentration represents the balance between efflux from the exercising muscle and uptake by inactive tissue (9). Extensive work over the last several years has led to the discovery and characterization of a lactate transporter protein. The lactate transporter protein has been identified as a monocarboxylate carrier that co-transports lactate and protons across the plasma membrane (14,100). Currently, eight isoforms of the transporter protein termed monocarboxylate transporter (MCT) have been cloned and sequenced. The tissue distribution of each isoform has also been found to be variable. In skeletal muscle two isoforms have been found to exist - MCT1 and MCT4 (11,139). Investigation in humans (139) and rats (194) have revealed that the MCT1 and MCT4 isoform expression is different in the various muscle fibre types. MCT4 is found in all fibre types but is least in the more oxidative fibers (139,194). MCT1 expression is highest in fibers with a high oxidative capacity and very low in white muscle that is almost entirely glycolytic (139,194). Currently, it is not clear whether each isoform preferentially is involved in lactate extrusion or uptake. It has been postulated that the MCT1 isoform may be involved primarily in

the uptake of lactate to provide it as an oxidative substrate either post-exercise or during exercise in inactive tissue, while MCT4 may be predominately involved in lactate efflux.

1.8.3.1 Transporter Kinetics

The majority of studies examining the kinetics of the lactate transporter have used isolated sarcolemmal vesicles from rat (98,99,119,120,157,158,189), mouse (97,102) and humans (101). Based on these studies the transporter has demonstrated the following characteristics: it is saturable, has a high affinity for L-lactate, is sensitive to changes in the lactate/H^+ concentration gradients, is bidirectional,co-transports lactate/H^+ in a 1:1 ratio, and is competitively inhibited by cinemates. The MCT transporter accounts for 70-90% of the lactate transport across the physiological lactate concentration range and as such is the rate limiting step in lactate efflux from exercising muscle (60,119,120). Diffusion of the undissociated lactic acid does occur in the direction of the transmembrane lactate and H^+ concentration gradient but accounts for less than 20% of the lactate efflux, although higher contributions are seen with increased lactate and H^+ concentrations (100).

Early work on intact muscle using pH-sensitive electrodes while varying the perfusion medium lactate concentration with various chemical inhibitors of anion exchange pointed to both a carrier mediated transport process and passive diffusion (115,160). Lactate efflux was also thought to be transported by an exchange mechanism involving bicarbonate (HCO_3^-) due to the observation that lactate efflux

was enhanced when the external HCO_3^- concentration was increased (72,113). In an effort to clarify the exact nature of the effects of lactate, H^+ and HCO_3^- transmembrane concentration gradients on lactate efflux the isolated sarcolemmal vesicle preparation has been used extensively. The advantage of this method is the ability to control and/or manipulate the intra and extra vesicular medium. These studies have revealed that the lactate transporter is extremely sensitive to changes in the H^+ and lactate concentration, with the movement of both being in the direction of the prevailing concentration gradient. The H^+ gradient is the dominant factor determining the magnitude and direction of lactate transport in skeletal muscle (9,97,99-101,119,157,189). Based on these further studies it is now generally accepted that the enhanced lactate efflux observed with an elevated HCO_3^- concentration is attributable to the increase in the extracellular buffer capacity and not a direct effect on the lactate transporter (97,100).

Based on the kinetic data a model of lactate/H^+ transport has been proposed as follows (100). The carrier is loaded first by H^+ followed by lactate which reorients the carrier effecting the off loading of lactate followed by H^+. This model has a number of important characteristics a) lactate and H^+ are co-transported in an electrically neutral manner, b) a loaded carrier will reorient faster than and unloaded carrier, c) H^+ binding is a prerequisite for lactate binding and d) the protonated intermediate form of the carrier reorients slowly or not all. Based on this model it is suggested that during exercise, the increased lactate and H^+ concentrations within the muscle facilitates the efflux of both metabolites thereby contributing to the

maintenance of pH_i and muscle function. This is supported by the observation that recovery in stimulated muscle was delayed when lactate release and pH_i recovery were delayed with various lactate/proton transport inhibitors. Conversely, inhibition of the Na^+/H^+ or HCO_3^-/H^+ exchanger had no effect on muscle fatigue (97).

Blood lactate concentration is a function of lactate efflux from the muscle and uptake by inactive tissue (9,59). The same principles apply in transmembrane flux in that both the H^+ and lactate concentration gradients dictate the rate of uptake of lactate into the inactive tissue. During exercise and recovery from exercise, the increased blood lactate and H^+ concentrations favor the clearance of lactate from the plasma (59,60,100).

1.9 Summary of pH Effects on Muscle Metabolism

Hydrogen ions have negative effects on the proteins of key flux-generating and regulatory enzymes involved in both CHO and TAG metabolism which provide substrate for the provision of ATP. The key enzymes involved in CHO metabolism, Phos, PFK and PDH_c have been shown to be sensitive to increases in the H^+ concentration; Phos and PFK are inhibited while PDH_a is stimulated. Additionally, the HSL which is the rate-limiting enzyme responsible for adipose tissue lipolysis has been shown to be inhibited by increases in H^+. The combined change in these enzyme activities also suggest that fuel selection and utilization may be affected by alterations in the H^+ concentration. The lactate transporters MCT1 and MCT4 are also extremely sensitive to changes in both the lactate and H^+ concentration

gradients and in skeletal muscle, the increase in these two metabolites appear to be the only factors that can augment lactate efflux from the muscle (9). The majority of the studies contributing to this body of knowledge have been done utilizing *in vitro* preparations with only a few expanding this knowledge to human tissue. Overall, the results illustrate that an alteration in the H^+ concentration has the potential to affect fuel selection, lactate production and lactate transport and thus influence the appearance of lactate in the blood.

Most metabolites that take part in the muscle biochemical reactions are charged molecules. They can be influenced by the ionic status of the myocyte as demonstrated by the presence of H^+ in equations 1 through 9 in the preceding sections. In this way the reactions may both lead to changes in muscle H^+ concentration (and therefore pH) and be influenced in their equilibrium state by changes in H^+. It therefore is exceedingly difficult to ascertain what is happening within the muscle by changing the extracellular pH and to know where the effects are localized given the number of reactions and sites where H^+ is involved.

1.10 Rationale for Current Research

It has been established that increases in the H^+ and lactate concentrations occur within the blood and muscle compartments during moderate to high intensity exercise. Furthermore, it has been established that changes in H^+ concentration have metabolic effects by influencing the function of enzymes involved in both metabolic and contractile processes. In the early 1900's investigations concerning

the influence of pH on muscle found that an alteration in the extracellular pH with exercise also had effects on muscle function.

Numerous studies have been done both *in vitro* and *in vivo* under conditions of both extracellular metabolic acidosis and alkalosis and have led to the following universally demonstrated phenomenon. Acidosis reduces the appearance of lactate in the blood and exercise performance while alkalosis has the opposite effect (13,40,58,67,92,95,111,116,122,169,171,176,191). However, the mechanisms responsible for these changes are not clear. It has been suggested that these observations may be related to alterations in the muscle lactate production rate and/or lactate efflux. Unfortunately, these suggestions have been based on blood lactate measurements only. Only three previous studies have measured the intramuscular lactate concentration or pH_i directly by either by needle biopsy or P-NMR techniques. These studies have demonstrated that acidosis decreases and alkalosis increases the intramuscular lactate concentration (13,80,176). In the study by Sutton et al (176) other intramuscular metabolites were measured and these authors inferred that acidosis inhibited glycolysis at the level of PFK and possibly Phos. Only one study has measured lactate efflux under conditions of an extracellular acidosis and alkalosis and found that it was inhibited/augmented respectively (74).

Compounding this problem is the fact that although it is clear that a change in the extracellular pH has effects on lactate appearance in the blood and exercise performance it is unclear to what extent the pH_i is actually affected or by what

mechanism. Only one study by Hultman et al (80) has examined the pH_i following induction of a metabolic acidosis with NH_4Cl in humans during exercise. These authors utilized the homogenate technique on muscle biopsy samples and found that the pH_i is reduced by approximately 0.2 pH units following induction of acidosis. This 0.2 pH unit decrease in pH_i, compared to control, was also maintained throughout exercise. Unfortunately, there is a lack of evidence regarding the effects of $NaHCO_3$ ingestion on the pH_i. Only one study by Hood et al (74) has measured the pH_i with P-NMR during forearm exercise following $NaHCO_3$ ingestion and found that the pH_i was not different between conditions.

Although it can be stated with certainty that extracellular acidosis/alkalosis has effects on exercise performance and the blood lactate appearance, it remains unknown as to whether an extracellular acidosis/alkalosis has any effect on the pH_i, the activity of key rate-limiting metabolic enzymes, lactate production or lactate efflux. However, no human *in vivo* studies have been carried out to examine the impact of extracellular pH manipulation on: the activity of the key rate-limiting enzymes Phos, PFK and PDH_a; fuel utilization; lactate production or lactate efflux. It is therefore difficult to delineate the mechanisms responsible for the commonly observed changes in blood lactate concentration or exercise performance.

In an effort to determine the mechanisms responsible for the universally observed difference in the appearance of lactate in the blood with an induced extracellular acidosis/alkalosis two human *in vivo* experiments were designed and executed. The exercise protocol utilized in the two studies (chapters 2, 3) was

steady state cycling at low, moderate and high intensity following the induction of either a metabolic acidosis with oral administration of NH_4Cl or a metabolic alkalosis with oral administration of $NaHCO_3$. The steady state exercise protocol was chosen because the majority of previous studies have utilized exercise protocols that have resulted in differences in total work, fatigue rate and therefore differences in the ATP turnover rate between conditions. This made it difficult to ascertain if the results were due to the extracellular pH manipulation or simply the exercise protocol. To prevent these problems in the interpretation of the data, the steady state exercise protocol was chosen to keep the ATP turnover rates the same between the placebo and acidosis or alkalosis trials. Additionally, the dosage and administration protocol used for NH_4Cl and $NaHCO_3$ ingestion in each study, were in accordance with the dosages previously reported to result in an alteration in the extracellular pH and blood lactate concentrations.

The primary focus of the two studies was to determine the mechanisms responsible for the altered blood lactate appearance under conditions of an extracellular acidosis/alkalosis. Given that many *in vitro* studies (reviewed in the previous sections) have demonstrated that increases in H^+ concentration have effects on the rate-limiting enzymes Phos, and PDH_a which are involved in CHO and TAG metabolism the measurement of these enzymes and their regulators was conducted using the muscle biopsy technique. In addition the accumulation of the key glycolytic intermediates was evaluated to ascertain PFK catalytic activity and therefore glycolytic flux. Lactate production and efflux were also measured for two

reasons: first, lactate production can be affected by changes in the activity of the enzymes that produce /oxidize pyruvate (Phos and PDH_a respectively) as pyruvate is the substrate for LDH. Second, the appearance of lactate in the blood is a function of both its production and efflux rates. Neither study was intended to investigate the impact on exercise performance. The results of the acidosis study are presented in chapter 2 and the results of the alkalosis study are presented in chapter 3.

The intention of the present investigations was to extend the current knowledge concerning the mechanisms responsible for the effects of an extracellular pH on the appearance of lactate in the blood in humans, *in vivo*, by assessment of the arterial, femoral venous and intramuscular compartments.

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CHAPTER 2
EFFECT OF INDUCED METABOLIC ACIDOSIS ON HUMAN SKELETAL MUSCLE METABOLISM DURING EXERCISE

(Accepted for publication in Am.J.Physiol. (Endocrinology and Metabolism))

2.1 ABSTRACT

The roles of pyruvate dehydrogenase (PDH), glycogen phosphorylase (Phos) and their regulators in lactate (Lac^-) metabolism were examined during incremental exercise following ingestion of 0.3 grams/kg of either NH_4Cl (metabolic acidosis = ACID) or $CaCO_3$ (control = CON). Subjects were studied at rest, rest post-ingestion and during continuous steady-state cycling at three stages (15 min each): 30, 60, 75% of $VO_{2\,max}$. Radial artery and femoral venous blood samples, leg blood flow and biopsies of the vastus lateralis were obtained during each power output. ACID resulted in significantly lower intramuscular [Lac^-] (ACID 40.8 vs CON 56.9 $mmol \cdot kg^{-1}$ dw), arterial whole blood [Lac^-] (ACID 4.7 vs CON 6.5 $mmol \cdot l^{-1}$) and leg Lac^- efflux (ACID 3.05 vs CON 6.98 $mmol \cdot l^{-1} \cdot min^{-1}$). The reduced intramuscular [Lac^-] resulted from decreases in pyruvate production due to inhibition of glycogenolysis at the level of Phos *a*, and phosphofructokinase (PFK), together with an increase in the amount of pyruvate oxidized relative to the total produced.

The reduction in Phos *a* activity was mediated through decreases in transformation, decreases in free inorganic phosphate [P_i], and decreases in the post-transformational allosteric regulator free AMP. Reduced PDH activity occurred with ACID and may have resulted from alterations in [acetyl-CoA], [ADP_i], [pyruvate], [NADH] and [H^+] leading to greater relative activity of the kinase. The results demonstrate that imposed metabolic acidosis in skeletal muscle results in decreased Lac^- production due to inhibition of glycogenolysis at the level of Phos and increased pyruvate oxidation at PDH.

2.2 Introduction

Systemic acidosis in humans decreases lactate concentration ([Lac^-]) in the blood during exercise (22,40,44,48). Data extrapolated from animal studies have suggested this to result from changes in the rates of glycogenolysis, glycolysis and Lac^- efflux; but the mechanisms have not be elucidated (30,47,63). The reduction in [Lac^-] has been attributed primarily to the influence of pH on glycolysis at the level of phosphofructokinase (PFK) (34,37,67); but the *in vivo* effects of acidosis on the regulatory enzymes glycogen phosphorylase (Phos) and pyruvate dehydrogenase (PDH) have not been established. Phos and PDH occupy key, flux generating control points for glycogenolysis and oxidative phosphorylation in the tricarboxylic acid cycle (TCA) respectively, and therefore ultimately influence lactate production. Lactate accumulation results from conversion of non-oxidized pyruvate to lactate by lactate dehydrogenase (LDH) and as such will be influenced by both pyruvate

production from glycogen and pyruvate oxidation by PDH (14,41).

In an effort to clarify the nature of the effects of acidosis on lactate production we chose an oral dose of ammonium chloride (NH_4Cl) previously shown to induce a sufficient metabolic acidosis to influence plasma [Lac^-] during exercise (40,66). Continuous, dynamic steady state exercise at low, moderate and high intensity was chosen to follow the metabolic effects and compare fuel utilization against previously described carbohydrate and fat contributions at these intensities (55,56). This is the first *in vivo* study in humans to examine the key regulatory enzymes and their controllers during continuous dynamic steady state exercise under acidotic conditions.

The aim of the present study was first , to determine the effect of metabolic acidosis on the key regulatory enzymes Phos and PDH and their respective controllers; second to measure the effect of metabolic acidosis on glycolytic intermediates and muscle pyruvate production, oxidation; third to measure muscle lactate accumulation, production and efflux; and finally, to determine if acidosis has any effects on glucose uptake and free fatty acid (FFA) utilization during exercise.

2.3 METHODS

2.3.1 Subjects

Eight healthy male volunteers participated in the study [age 27 $\pm$ 1.9 (SE) yr; height 185 $\pm$ 3.4 cm; weight 82.7 $\pm$ 3.7 kg]. Written consent was obtained from each subject after explanation of the purposes and associated risks of the study

protocol. The study was approved by the Ethics Committees of both McMaster University and McMaster University Medical Centre.

2.3.2 Pre-Experimental Protocol

All subjects completed an initial incremental maximal exercise test on a cycle ergometer to determine $VO_{2\ max}$ and maximal work capacity using a metabolic measurement system (Quinton Q-Plex 2, Quinton Instruments, Seattle Washington). Mean $VO_{2\ max}$ for the group was 3.6 $\pm$ 0.3 l/min^{-1}. None of the subjects was well trained but all participated in some form of regular activity. Each subject was instructed to refrain from caffeine, alcohol and exercise 24 hrs before each trial and studies were carried out at the same time of day.

2.3.3 Experimental Protocol

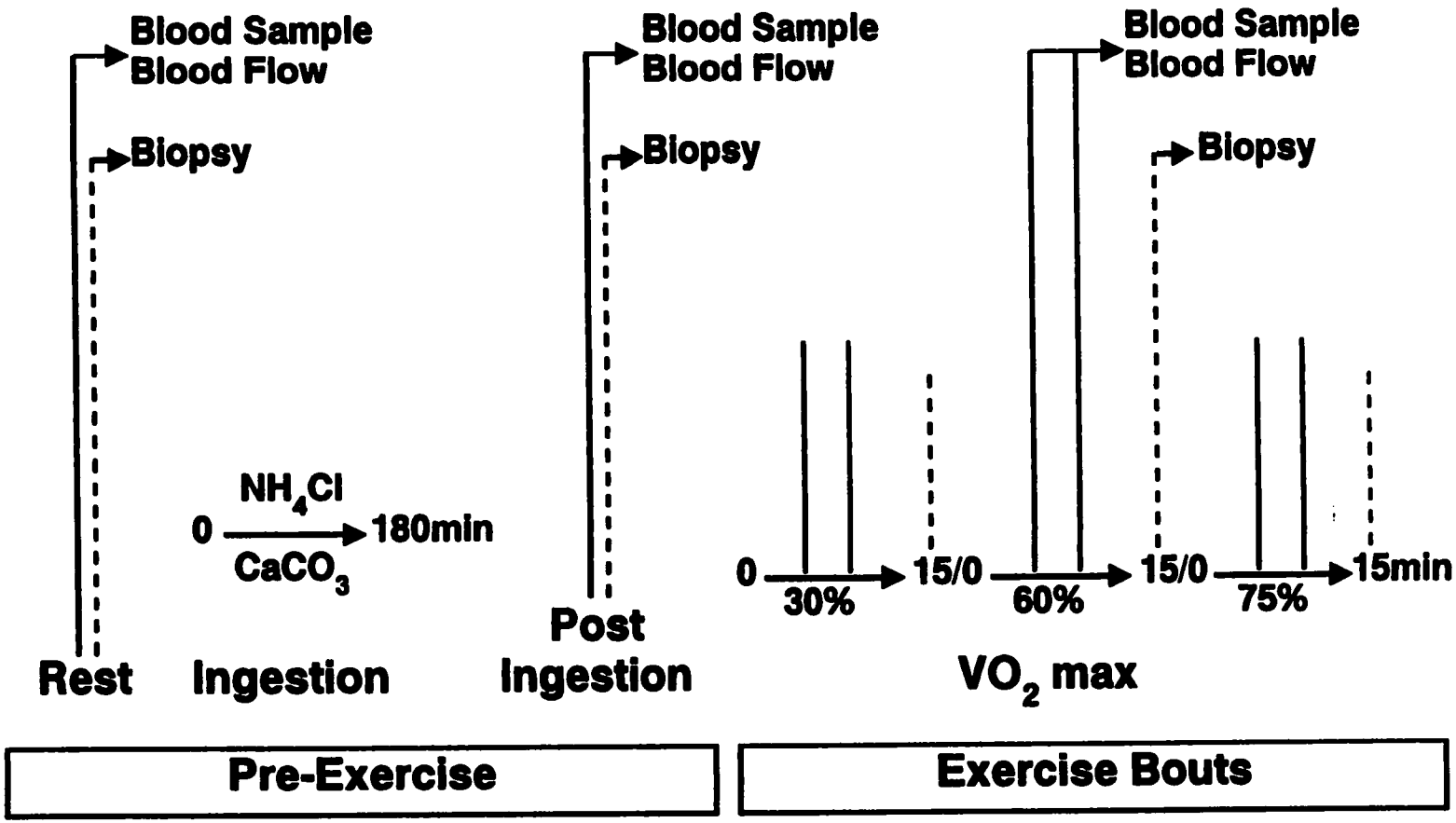
Each subject participated in two experimental trials separated by 2-3 weeks and were randomized to receive capsules with either 0.3 grams/kg of NH_4Cl (ACID) or 0.3 grams/kg of $CaCO_3$ (CON). On the morning of each trial the subjects reported to the laboratory following consumption of a standard light meal consisting primarily of carbohydrates. The exercise portion of the protocol consisted of three levels of continuous, steady-state exercise on a cycle ergometer at 30, 60 and 75% of VO_{2max} each maintained for 15 minutes, which began following insertion of arterial and femoral venous catheters, and ingestion of the required capsules.

A radial artery was catheterized with a Teflon catheter (20 gauge, 3.2 cm, Baxter, Irvine CA) percutaneously after anaesthetizing the area with 0.5 mls of 2% lidocaine without epinephrine (5). A femoral vein was catheterized

percutaneously for insertion of the thermodilution catheter (model # 93-135-6F, Baxter, Irvine, CA) using the Seldinger technique (5) following administration of 3-4 mls of lidocaine without epinephrine. Both the arterial and femoral venous catheters were maintained patent with sterile, non-heparinized, isotonic saline solution. Arterial and femoral venous blood samples were simultaneously taken at rest, post-ingestion and during each of the three exercise bouts at 7 and 11 minutes. Single leg blood flow measurements were collected following blood sampling at the same time points. Single leg blood flow was determined using the thermodilution technique as described by Andersen and Saltin (1): 10 mls of non-heparinized isotonic saline was injected and leg blood flow was calculated by a portable cardiac output monitor (Spacelab, Redmond, VA). At least three measurements were recorded at each sampling point and then averaged.

A total of five percutaneous needle biopsies of the vastus lateralis were taken, one at rest, one at rest post-ingestion and three during exercise at the end of each power output. The resting biopsies were obtained with the subject lying on a bed. The resting and exercise biopsies were obtained on opposite legs and then reversed for the second trial. Biopsy sites were prepared by making an incision through the deep fascia under local anaesthetic (2% lidocaine without epinephrine) as described by Bergström et al (4). Respiratory measurements expired volume (V_E), oxygen uptake (VO_2), carbon dioxide production (VCO_2) and respiratory exchange ratio (RER) were measured at 5 and 11 minutes of each exercise stage. (Figure 1).

STUDY PROTOCOL



2.3.4 Muscle Sampling and Analysis

Muscle samples were immediately frozen in liquid N_2. A small piece (10-35 mg) was chipped from each biopsy (under liquid N_2) for determination of the fraction of PDH in the active form (PDH_a) as previously described (15,56). The remainder of the sample was freeze-dried, dissected free of blood and connective tissue and powdered. One aliquot was analyzed for Phos activity according to the methods of Young et al (69). Briefly, a 3-4 mg sample of muscle was homogenized at -20°C in 0.2 mls of 100 mM of Tris/HCl (pH 7.5) containing glycerol, potassium fluoride and EDTA. Homogenates were then diluted with 0.8 mls of the same buffer without glycerol and homogenized further at 0°C. Total (*a* + *b*) Phos activity (measured in the presence of 3 mM AMP) and glycogen phosphorylase in the active *a* form (Phos *a*) (measured in the absence of added AMP) were measured at 30°C with a spectrophotometer. Maximum velocity (v_{max}) was derived from the equation described by Lineweaver and Burk (45), $1/V = (K_m/V_{max})(1/S) + (1/V_{max})$, where V is the initial reaction rate expressed as $mmol \cdot kg^{-1} \cdot min^{-1} dw$, S is the P_i concentration in $mmol \cdot l^{-1}$, and K_m is 26.2 $mmol \cdot l^{-1}$. The mole fraction of Phos *a* is presented as a percentage and calculated from $V_{max}\ a/V_{max}(a+b)$ X 100. Phos *a* measurements were made only on exercise samples for two reasons. First, resting samples must be kept at room temperature for ~ 30s before freezing for accuracy, which would have required two additional biopsies (59). Ethically this was not acceptable for the provision of one measurement. Second, the changes at rest and post-ingestion were not a main focus of this study. A second aliquot was used to determine muscle

glycogen fluorometrically using the enzymatic end-point method described by Bergemeyer (3). A third aliquot of dry muscle was extracted in 0.5M PCA and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$ and analysed for acetyl-CoA, free CoASH, total CoA, acetylcarnitine, free carnitine, total carnitine according to the methods of Cederblad et al (8). A fourth aliquot was used to determine ATP, pyruvate, lactate, phosphocreatine (PCr), creatine, glucose, glucose-6-phosphate (G-6-P), glucose-1-phosphate (G-1-P), fructose-6-phosphate (F-6-P) and glycerol-3-phosphate (G-3-P) concentrations using the methods described by Bergmeyer, and adapted for fluorometry (3). All muscle metabolites were normalized to the highest total creatine content for a given individual ($\bar{x}$ TCr = 133.4 $\pm$ 11.4 mmol kg^{-1} dw) to correct for non-muscle contamination. Free contents of ADP_f and AMP_f were calculated as described by Dudley et al (20), using the reactants and equilibrium constants of the near-equilibrium reactions catalysed by creatine kinase and adenylate kinase. ADP_f was estimated using the measured ATP, PCr and creatine contents and an estimated $[H^+]$ (calculated indirectly from muscle $[Lac^-]$ and [pyruvate] using the regression equation of Sahlin et al (61)). From this information the concentration of AMP_f was determined assuming a K_{obs} of 1.05 for the adenylate kinase reaction. Free P_i content was calculated from the sum of the estimated resting free $[P_i]$ of 10.8 mmol kg^{-1} dw (20) and the PCr - G-6-P - F-6-P - G-3-P between rest and each time point during exercise. For the purposes of ADP_f, AMP_f and free P_i calculations, no differences were observed between the rest and post-ingestion values and therefore the mean of the two was taken as the resting value.

2.3.5 Blood Sampling and Blood Analysis

Arterial and femoral venous blood samples (~10 mls) were collected into heparinized plastic syringes and placed on ice. One portion (1-2 mls) of each blood sample was analysed for blood gas determination (AVL 995 Automatic Blood Gas Analyser); O_2 and CO_2 content (Cameron Instrument CO., Port Arkansas, TX) and hemoglobin (OSM3 Hemoximeter, Radiometer, Copenhagen, Denmark). A second portion of each sample was deproteinized with 6% perchloric acid (PCA) and stored at -20°C until analysis for glucose, lactate, and glycerol according to the methods of Bergmeyer (3) adapted for fluorometry. The third portion of blood was immediately centrifuged at 15,900 *g* for 2 min and the plasma supernatant was frozen and later analysed for free fatty acids (FFA)(Wako, NEFA C test kit, Wako Chemical, Montreal, Canada). Hematocrit (Hct) was determined on blood samples using a heparinized micro capillary tube centrifuged for 5 min at 15,000 *g*.

2.3.6 Leg Uptake and Release of Metabolites, O_2, CO_2, and RQ

Uptake and release of metabolites (glucose, glycerol, lactate) were calculated from their whole blood measurements in arterial and femoral venous blood and leg blood flow according to the Fick equation. Since there were differences in the Hct over time within a condition and between matched arterial and femoral venous samples, venous samples were corrected for fluid shifts. Fluid shifts for the whole blood measurements were corrected using the differences in hemoglobin (Hb) to calculate a percent change in blood volume (%ΔBV) from the equation (assuming no change in intravascular hemoglobin) (29):

$$\%\Delta BV = [(Hb_{arterial}/Hb_{venous})-1] \times 100$$

This value was then multiplied by the measured venous value to yield a corrected value which was used in determining flux for that metabolite. Plasma FFA venous values were also corrected using changes in plasma [protein] to correct for changes in plasma water (29). The leg O_2 uptake and CO_2 production were calculated from their respective arterial and femoral venous content differences and blood flow.

Subjects exercised in steady state, and no significant differences occurred in blood flows or metabolite concentrations between the 7 and the 11 minute sampling points at each power output and therefore the two values were averaged to obtain one value for each power output. Reported values are for the single leg only.

2.3.7 Calculations

Flux through Phos was calculated from the differences in glycogen utilization divided by time. PDH_a flux was estimated from the PDH_a as measured in wet tissue and converted to dry tissue using the wet/dry ratio. Pyruvate production was calculated from the sum of the rates of glycogen breakdown and glucose uptake minus the sum of the rates of accumulation of muscle glucose, G-6-P, and F-6-P. Lactate production was calculated from the sum of the rates of muscle lactate accumulation and lactate release. Pyruvate oxidation was calculated as pyruvate production minus lactate production. All values are reported in $mmol \cdot kg^{-1} \cdot min^{-1}$ dw and are for single leg only. All values were calculated in three carbon units and assume a wet muscle mass of 5 kg.

Intramuscular pH (pH_i) was calculated from the [Lac^-] and [pyruvate] according to the methods of Harris et al (28). However, Hultman et al (35) found that calculated pH_i is 0.2 pH units more than measured pH_i with ingestion of the same dose of NH_4Cl that we used. Therefore, our estimated values from the [Lac^-] and [pyruvate] were calculated and then reduced by 0.2 pH units.

2.3.8 Statistical Analysis

Data was analyzed using two-way ANOVA with repeated measures (Time x Treatment) except were otherwise stated. When a significant *F* ratio was found the Newman-Keuls post hoc test was used to compare means. Data are presented as means ± SE. Significance was accepted at $p < 0.05$.

2.4 Results

2.4.1 Muscle Metabolism.

2.4.1.1 Glycogen Phosphorylase

Phos *a* decreased as exercise intensity increased in both conditions. At 75% $VO_{2\,max}$ Phos *a* was significantly lower during ACID (ACID 21.6 ± 4.5 vs CON 29.8 ± 5.9 mmol·kg^{-1}·min^{-1} dw)(Fig. 2).

2.4.1.2 Glycogen

Resting and post-ingestion muscle glycogen levels were not different between conditions (Table 1). Muscle glycogen content decreased with increasing power output but to a significantly greater degree with CON (Table 1). During the complete exercise study, total muscle glycogen utilization was

327 ± 22 mmol·kg^{-1}dw during CON compared to 229 ± 34 mmol·kg^{-1}dw with ACID. This corresponded to a 30% sparing of glycogen during ACID. Muscle glycogen utilization at each power output was similar between conditions at 30% $VO_{2\,max}$ but was significantly lower at both 60 (75 ± 20 vs 113 ± 7 mmol·kg^{-1}dw) and 75% $VO_{2\,max}$ (103 ± 16 vs 157 ± 18 mmol·kg^{-1} dw) during ACID compared to CON respectively (Fig. 3).

2.4.1.3 Glucose, G-6-P, F-6-P, G-1-P and G-3-P

Intramuscular accumulation of glucose increased with each power output similarly between conditions (Table 1). Intramuscular [G-6-P] and [F-6-P] increased with exercise and were significantly higher with ACID at all three power outputs (Table 1). Muscle [G-1-P] and [G-3-P] were similar between conditions, increasing with each power output (Table 1).

2.4.1.4 Lactate and Pyruvate

Muscle [Lac$^-$] increased with increasing power output but was significantly lower with ACID at both 60 (ACID 20.5 ±4.2 vs CON 34.6 ± 7.0 mmol·kg^{-1}dw) and 75% $VO_{2\,max}$ (ACID 40.8 ± 7.4 vs CON 56.9 ± 8.6 mmol·kg^{-1}dw) (Fig. 4). Similarly, muscle [pyruvate] was significantly lower with ACID during 75% $VO_{2\,max}$ (ACID 0.44 ± 0.05 vs CON 0.69 ± 0.09 mmol·kg^{-1}dw) compared to CON (Fig. 4).

2.4.1.5 Pyruvate Dehydrogenase Activity

Resting PDH_a (ACID 0.79 ± 0.13 vs CON 0.68 ± 0.19 mmol·kg^{-1}·min^{-1} ww) and post-ingestion (ACID 0.79 ± 0.12 vs CON 0.78 ± 0.24 mmol·kg^{-1}·min^{-1} ww) PDH_a levels were not different between conditions (Fig. 5). PDH_a levels increased

progressively with cycling but were significantly lower at 30 (2.35 ± 0.34 vs 2.94 ± 0.33), 60 (3.29 ± 0.27 vs 3.96 ± 0.34) and 75% VO_{2max} (3.91 ± 0.15 vs 4.77 ± 0.10) during ACID compared to CON respectively (Fig. 5).

2.4.1.6 CoA, Carnitine, and Acetylated Forms

Total muscle CoA was not different between conditions at rest or during exercise (Table 2). [Acetyl-CoA] increased with power output with ACID, these increases with exercise being significantly higher when compared to matched time points during CON (Table 2). In addition, the resting post-ingestion [acetyl-CoA] was significantly higher with acidosis (Table 2). Free CoASH declined equally between conditions with exercise intensity (Table 2). Acetylcarnitine followed a similar pattern to acetyl-CoA and did not differ between conditions (Table 2). Muscle total carnitine content increased significantly from rest to 75% VO_{2max} to the same degree in each condition while free carnitine decreased in a reciprocal manner with increasing power output. There were no differences between conditions for either total or free carnitine contents (Table 2).

2.4.1.7 ATP, ADP_f, AMP_f, Free P_i and Phosphocreatine

Muscle [ATP] was unaltered by exercise or as a result of acidosis. Muscle [ADP_f] and [AMP_f] increased with each power output but both were significantly lower with ACID at 75% VO_{2max} (Table 3). The free [P_i] increased with power output equally between conditions. PCr concentrations decreased with increasing power output similarly between conditions (Table 3).

2.4.1.8 Pyruvate production, oxidation and Lactate production and oxidation

Pyruvate production increased with each power output but was significantly lower at each power output during ACID. Pyruvate oxidation increased as power output increased but was significantly lower with ACID. Relative pyruvate oxidation expressed as the percentage of pyruvate produced that was oxidized was similar between conditions at 30% but was significantly higher at both 60 and 75% VO_{2max} during ACID (Table 4). Lactate production was also significantly lower with ACID at all exercise time points (Table 4).

2.4.2 Blood Metabolites, Blood Flow and Exchange Across the Leg

2.4.2.1 Blood pH, PCO_2 and HCO_3^-

Arterial pH, PCO_2, HCO_3^-, (Fig. 6) venous pH, and HCO_3^- (Table 5) were all significantly lower with ACID compared to CON at rest post-ingestion and each of the three power outputs.

2.4.2.2 Blood Lactate and Flux

Arterial [Lac^-] increased progressively with power output but was lower at both 60 and 75% $VO_{2\,max}$ with ACID (Table 5). Net release of Lac^- across the leg increased with power output but was significantly lower at both 60 and 75% $VO_{2\,max}$ with ACID (Fig. 7).

2.4.2.3 Blood FFA and Glycerol

Arterial plasma [FFA] declined progressively with power output was significantly lower during ACID compared to CON at post-ingestion, 30 and 60% $VO_{2\,max}$ (Table 5). FFA uptake occurred across the leg during CON and increased progressively with each power output. However, during ACID at 30 and 75% $VO_{2\,max}$ a net release occurred while at 60% $VO_{2\,max}$ a net uptake occurred which was significantly lower than CON (Fig. 8). Arterial [glycerol] increased with each power output but this increase was significantly lower with ACID (Table 5). During CON glycerol release occurred across the leg at 30% $VO_{2\,max}$ while at 60 and 75% $VO_{2\,max}$ and uptake across the leg was observed. However, during ACID a net release across the leg occurred at all three power output (Fig. 8).

2.4.2.4 Leg Blood Flow and Leg RQ

Leg blood flow increased progressively from rest to 60% $VO_{2\,max}$ similarly between conditions. However, at 75% VO_{2max} leg blood flow was significantly lower with ACID (Table 6). Leg O_2 uptake was not different between conditions, however, leg CO_2 production was significantly higher during all three power outputs with ACID (Table 6). Similarly, leg RQ was significantly higher during rest post-ingestion and all three power outputs with ACID compared to CON (Table 6).

2.4.2.5 Blood Glucose and Flux

Arterial [glucose] (Table 5) and leg glucose uptake (Fig. 7) were similar at all power outputs between conditions.

2.4.3 Respiratory Gas Exchange Variables

Whole body VO_2 increased similarly between conditions with each power output (Table 7). Whole body VCO_2 was significantly lower during 60 (2.47 ± 0.12 vs 2.57 ± 0.14 $l \cdot min^{-1}$) and 75% (3.29 ± 0.15 vs 3.40 ± 0.17 $l \cdot min^{-1}$) $VO_{2\,max}$ in ACID compared to CON, respectively. RER was also significantly lower during ACID at 75% $VO_{2\,max}$ (Table 7). V_E was significantly higher during ACID compared to CON at each exercise intensities (Table 7).

TABLE 1 - Muscle glycogen and glycolytic intermediate content in vastus lateralis at rest, post -ingestion and during cycle ergometry at 30%, 60% and 75% VO_{2max} after either CONTROL or ACID

MEASURE (mmol•kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Glycogen	CON	458.9 ± 36.0	479.2 ± 41.7	407.6 ± 41.2*	294.9 ± 41.6*	147.8 ± 26.7*
	ACID	499.8 ± 23.9	504.7 ± 20.3	460.0 ± 11.8	384.2 ± 19.9*†	260.8 ± 35.6*†
G-6-P	CON	0.83 ± 0.14	0.87 ± 0.17	2.22 ± 0.34*	2.82 ± 0.35*	2.92 ± 0.43*
	ACID	1.08 ± 0.11	0.92 ± 0.12	2.54 ± 0.40*†	3.50 ± 0.40*†	3.37 ± 0.31*†
G-1-P	CON	0.10 ± 0.02	0.14 ± 0.05	0.17 ± 0.04	0.25 ± 0.05*	0.22 ± 0.03*
	ACID	0.14 ± 0.03	0.09 ± 0.02	0.18 ± 0.04	0.23 ± 0.06*	0.30 ± 0.06*
F-6-P	CON	0.17 ± 0.01	0.18 ± 0.02	0.32 ± 0.05*	0.41 ± 0.06*	0.43 ± 0.04*
	ACID	0.20 ± 0.03	0.22 ± 0.05	0.41 ± 0.07*†	0.51 ± 0.07*†	0.54 ± 0.04*†
Glucose	CON	1.55 ± 0.27	1.89 ± 0.29	2.94 ± 0.55	6.00 ± 0.99*	10.52 ± 1.42*
	ACID	1.86 ± 0.66	2.95 ± 0.71	3.33 ± 0.41	7.25 ± 1.41*	10.34 ± 1.20*
G-3-P	CON	0.56 ± 0.08	0.68 ± 0.09	0.78 ± 0.09	2.85 ± 0.91*	4.31 ± 1.20*
	ACID	0.77 ± 0.10	0.59 ± 0.07	0.93 ± 0.18	3.00 ± 0.81*	4.29 ± 1.10*

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 2 - Muscle acetyl group content in vastus lateralis at rest, post -ingestion and during cycle ergometry at 30%, 60% and 75% VO_{2max} after either CONTROL or ACID

MEASURE (mmol•kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Free CoA, µmol/kg dw	CON	108.6 ± 14.2	103.3 ± 9.9	98.5 ± 11.3	88.5 ± 11.3*	93.2 ± 11.8*
	ACID	109.3 ± 11.0	96.9 ± 13.2	99.1 ± 7.0	98.1± 8.6*	95.0 ± 8.0*
Acetyl CoA	CON	6.7 ± 0.8	7.3 ± 0.8	11.3 ± 0.9*	17.9 ± 1.0*	23.7 ± 1.2*
	ACID	8.1 ± 0.4	9.0 ± 0.6†	12.2 ± 1.1*†	19.7 ± 2.3*†	24.3 ± 1.4*†
Total CoA, µmol/kg dw	CON	115.3 ± 14.7	110.5 ± 10.4	109.8 ± 11.6	106.4 ± 11.9	116.9 ± 12.0
	ACID	117.4 ± 10.7	105.9 ± 13.6	111.2 ± 7.1	117.84± 8.9	119.3 ± 9.1
Acetyl CoA:CoA	CON	0.06	0.07	0.11	0.20	0.25
	ACID	0.07	0.09	0.12	0.20	0.26
Acetlycarnitine	CON	1.63 ± 0.29	2.13 ± 0.46	7.13 ± 0.99*	15.0 ± 1.6*	19.4 ± 1.9*
	ACID	1.92 ± 0.54	2.85 ± 0.76	7.07 ± 1.23*	15.9 ± 2.3*	17.7 ± 1.4*
Total Carnitine	CON	23.2 ± 2.1	22.6 ± 1.5	24.8 ± 2.4	24.5 ± 2.5	25.7 ± 2.6*
	ACID	24.5 ± 2.1	24.2 ± 2.6	25.2 ± 2.3	25.5 ± 1.5	25.7± 1.9*
Free Carnitine	CON	20.5 ± 1.7	19.6 ± 1.1	16.5 ± 1.5*	8.2 ± 0.8*	5.7 ± 0.8*
	ACID	21.1 ± 1.2	20.2 ± 2.2	15.5 ± 0.8*	9.0 ± 1.1*	6.3 ± 0.7*

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 3 - Muscle high energy phosphate content in vastus lateralis at rest, post -ingestion and during cycle ergometry at 30%, 60% and 75% VO_{2max} after either CONTROL or ACID

MEASURE (mmol•kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
ATP	CON	25.3 ± 0.8	25.6 ± 0.5	26.0 ± 0.7	26.1 ± 0.7	24.5 ± 0.5
	ACID	24.9 ± 0.4	25.1 ± 0.7	25.7 ± 0.3	24.9 ± 0.9	25.1 ± 1.0
ADP_f (umol•kg^{-1} dw)	CON	142.6 ± 15.1	----	153.4 ± 18.1	272.2 ± 45.1*	543.0 ± 90.3*
	ACID	129.8 ± 15.7	----	152.1 ± 24.1	240.2 ± 50.4*	386.4 ± 60.1*†
AMP_f (umol•kg^{-1} dw)	CON	0.82 ± 0.17	----	0.95 ± 0.22	3.34 ± 1.38	13.44 ± 3.91*
	ACID	0.72 ± 0.16	----	0.98 ± 0.31	2.89 ± 0.98	6.64 ± 1.66*†
Free P_i	CON	10.8	----	23.0 ± 3.7	47.7 ± 5.5*	68.13 ± 7.37*
	ACID	10.8	----	23.2 ± 5.1*	49.8 ± 7.0*	66.6 ± 4.9*
Creatine Phosphate	CON	87.4 ± 4.5	85.5 ± 6.0	72.5 ± 7.0*	45.0 ± 5.2*	23.0 ± 5.5*
	ACID	83.7 ± 5.2	89.9 ± 4.2	72.4 ± 6.6*	42.6 ± 8.8*	24.8 ± 5.2*
pH_i	CON	7.21 ± 0.0	7.21 ± 0.0	7.04 ± 0.0*	6.91 ± 0.03*	6.82 ± 0.04*
	ACID	7.21 ± 0.0	7.21 ± 0.0	7.04 ± 0.0*	6.72 ± 0.03*	6.62 ± 0.04*†

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 4 - Muscle pyruvate production, oxidation, and lactate production at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ACID. Values are for single leg only.

MEASURE		$VO_{2\,MAX}$		
(mmol•kg^{-1} min^{-1} dw)	TRIAL	30 %	60 %	75%
Pyruvate production	CON	11.87 ± 1.91	19.88 ± 1.40**	26.10 ± 2.49**
	ACID	6.34 ± 1.06†	14.24 ± 2.55**†	19.58 ± 1.74**†
Lactate production	CON	1.71 ± 0.67	5.88 ± 1.19**	8.97 ± 1.75**
	ACID	0.71 ± 0.30†	3.12 ± 0.89**†	4.68 ± 0.88**†
Pyruvate oxidation	CON	10.12 ±1.89	13.99 ± 1.30**	18.99 ± 2.22**
	ACID	5.63 ± 1.21†	9.43 ± 1.64**†	14.90 ± 2.15**†
% Pyruvate Oxidized	CON	83 ± 0.07	71 ± 0.05	68 ± 0.04
	ACID	85 ± 0.08	85 ± 0.03†	80 ± 0.03†

Data are means ± SE; n= 8.

** Significantly different from first workload of same condition.

† Significantly different from matched times between conditions.

TABLE 5 - Arterial concentration of blood born substrates during rest, post-ingestion and cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ACID

MEASURE (mmol•l^{-1})	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Glucose	CON	5.46 ± 0.35	4.87 ± 0.11	5.08 ± 0.06	4.99 ± 0.18	4.71 ± 0.27*
	ACID	5.16 ± 0.08	4.95 ± 0.08	5.28 ± 0.21	5.18 ± 0.19	4.84 ± 0.28*
Lactate	CON	0.75 ± 0.06	0.57 ± 0.06	1.36 ± 0.28*	3.58 ± 0.47*	6.51 ± 0.60*
	ACID	0.87 ± 0.13	0.4 ± 0.06	0.83 ± 0.20	2.48 ± 0.48*†	4.74 ± 0.72*†
Glycerol, umol•l^{-1}	CON	24.0 ± 3.7	53.1 ± 11.3*	72.2 ± 16.6*	95.1 ± 18.8*	120.2 ± 20.4*
	ACID	26.7 ± 3.3	35.4 ± 3.9	48.8 ± 6.9*†	71. ± 11.7*†	90.3 ± 13.1*†
Plasma FFA	CON	0.35 ± 0.07	0.73 ± 0.13*	0.64 ± 0.13*	0.54 ± 0.06*	0.49 ± 0.06*
	ACID	0.32 ± 0.05	0.55 ± 0.07*†	0.44 ± 0.06†	0.41 ± 0.05†	0.39 ± 0.03
Venous pH	CON	7.37 ±0.01	7.37 ± 0.01	7.31 ± 0.01*	7.26 ± 0.01*	7.22 ± 0.01*
	ACID	7.37 ±0.01	7.21 ± 0.01*†	7.13 ± 0.01*†	7.10 ± 0.01*†	7.06 ± 0.02*†
Venous HCO_3^- (mEq/L)	CON	26.4 ± 0.5	26.1 ± 0.5	27.7 ± 0.5*	26.8 ± 0.6	24.4 ± 0.6*
	ACID	27.5 ± 0.7	15.7 ± 1.0*†	17.6 ± 0.6*†	17.9 ± 0.3*†	16.8 ± 0.3*†

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 6 - Leg blood flow, RQ, CO_2 Production, and O_2 Uptake at rest, post-ingestion and during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ACID

MEASURE (values are for single leg)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Blood Flow ($l \cdot min^{-1}$)	CON	0.37 ± 0.05	0.48 ± 0.06	3.37 ± 0.16*	4.44 ± 0.14*	5.09 ± 0.15*
	ACID	0.4 ± 0.04	0.41 ± 0.04	3.36 ± 0.14*	4.30 ± 0.18*	4.79 ± 0.17*†
RQ	CON	0.59 ± 0.09	0.63 ± 0.10	0.87 ± 0.06*	1.03 ± 0.05*	1.12 ± 0.04*
	ACID	0.61 ± 0.07	0.94 ± 0.19*†	1.10 ± 0.10*†	1.24 ± 0.07*†	1.37 ± 0.07*†
CO_2 Production ($ml \cdot l^{-1}$)	CON	33.6 ± 5.8	36.2 ± 5.6	92.1 ± 4.3*	134.2 ± 5.4*	153.2 ± 2.4*
	ACID	36.7 ± 3.5	48.2 ± 6.2†	111.2 ± 4.8*†	147.3 ± 3.8*†	169.8 ± 4.1*†
O_2 Uptake ($ml \cdot l^{-1}$)	CON	60.5 ± 6.6	58.8 ± 5.4	108.3 ± 7.8*	131.2 ± 7.1*	137.4 ± 4.2*
	ACID	62.4 ± 5.5	56.7 ± 7.1	104.5 ± 8.7*	120.2 ± 5.5*	125.6 ± 5.4*

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

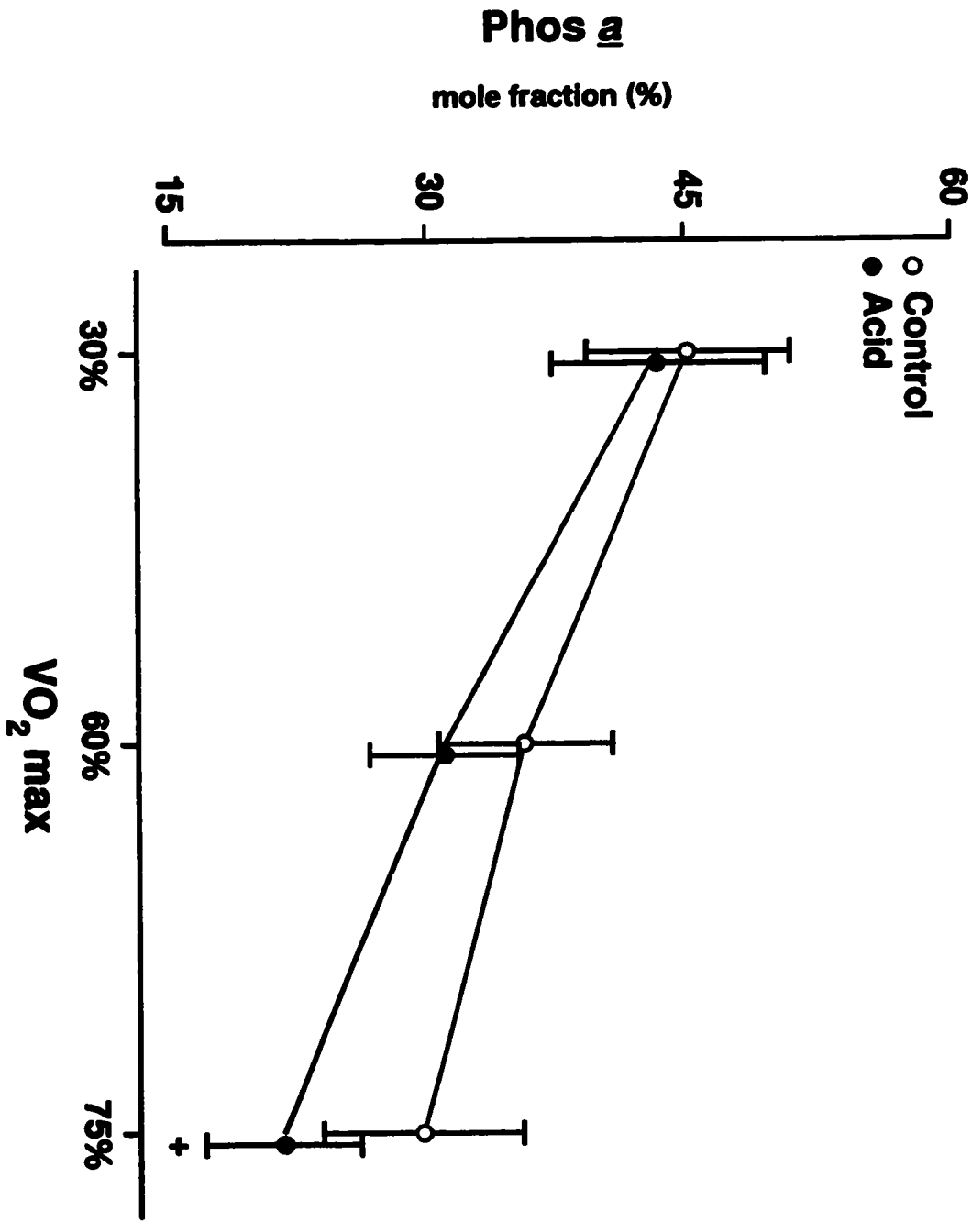
† Significantly different from matched times between conditions.

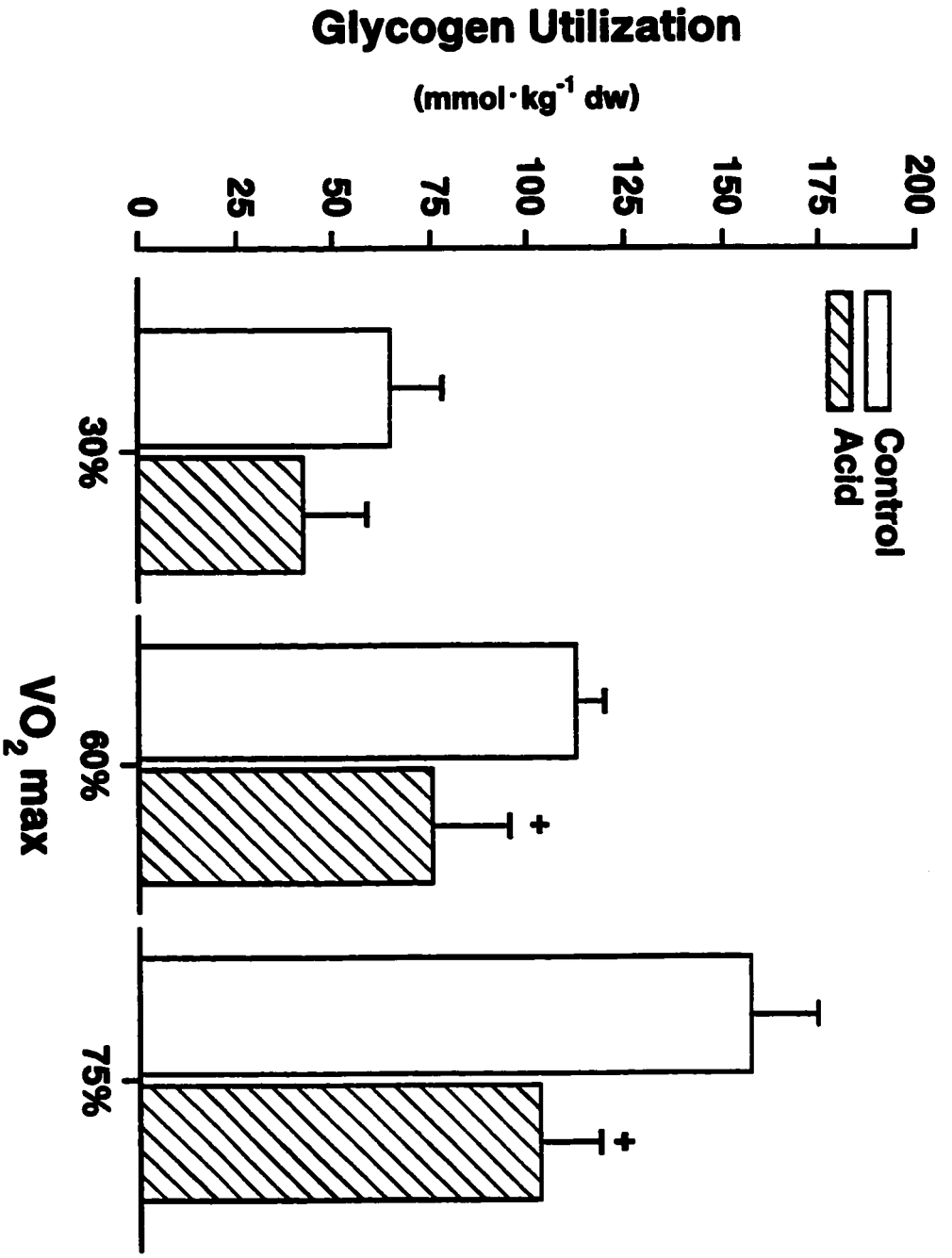
TABLE 7 - Respiratory variables during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ACID

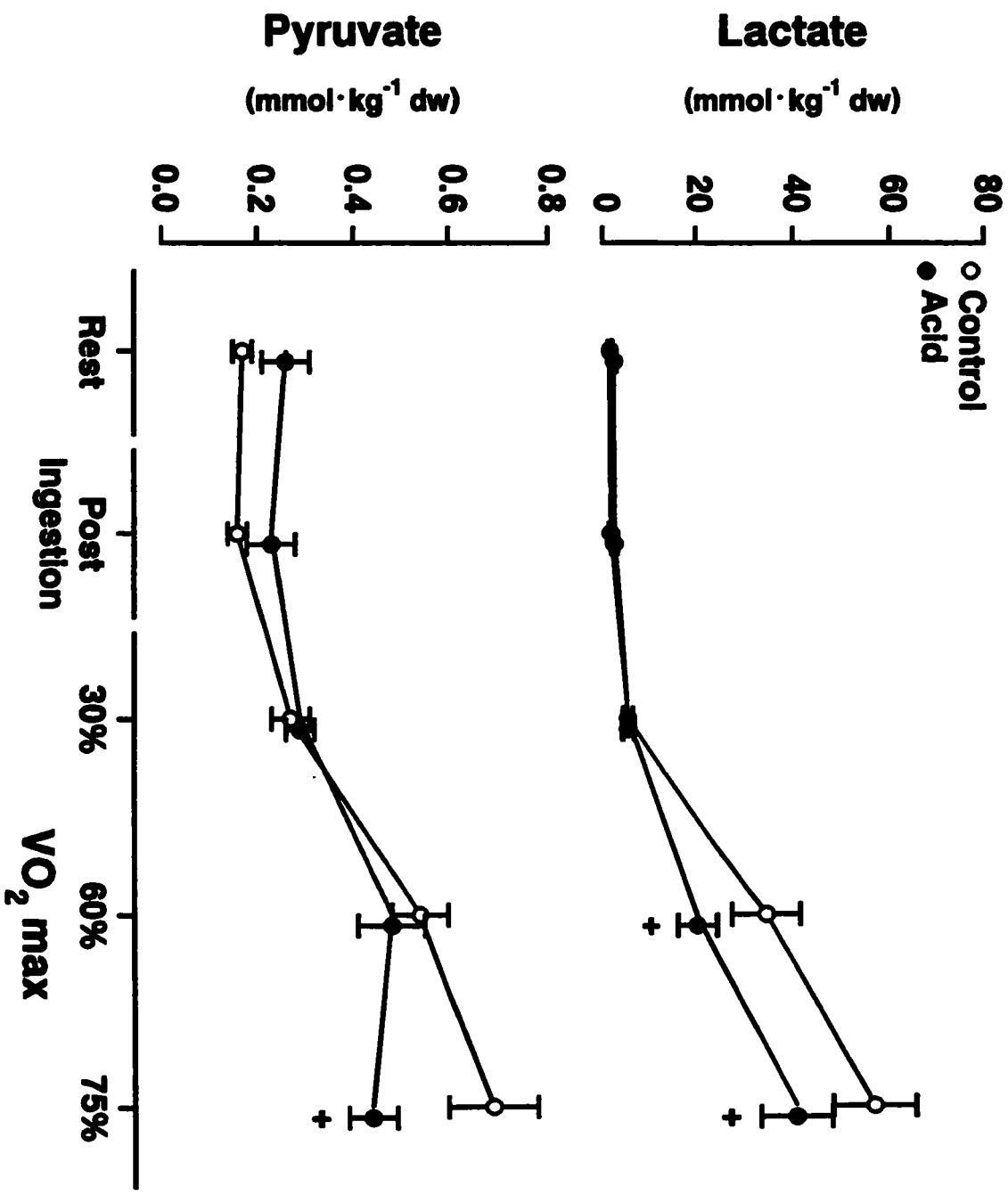
MEASURE ($l \cdot min^{-1}$)	TRIAL	$VO_{2\,MAX}$ 30 %	60%	75%
V_E	CON	36.44 ± 3.81	66.19 ± 5.39**	96.45 ± 8.63**
	ACID	43.73 ± 3.28†	77.47 ± 5.29**†	112.87 ± 7.51**†
RER	CON	0.98 ± 0.03	1.06 ± 0.02**	1.11 ± 0.02**
	ACID	0.98 ± 0.02	1.04 ± 0.03**	1.04 ± 0.03**†
VO_2	CON	1.47 ± 0.12	2.45 ± 0.15**	3.09 ± 0.17**
	ACID	1.43 ± 0.07	2.39 ± 0.14**	2.99 ± 0.29**
VCO_2	CON	1.42 ± 0.11	2.57 ± 0.14**	3.40 ± 0.17
	ACID	1.40 ± 0.06	2.47 ± 0.12**†	3.29 ± 0.15**†

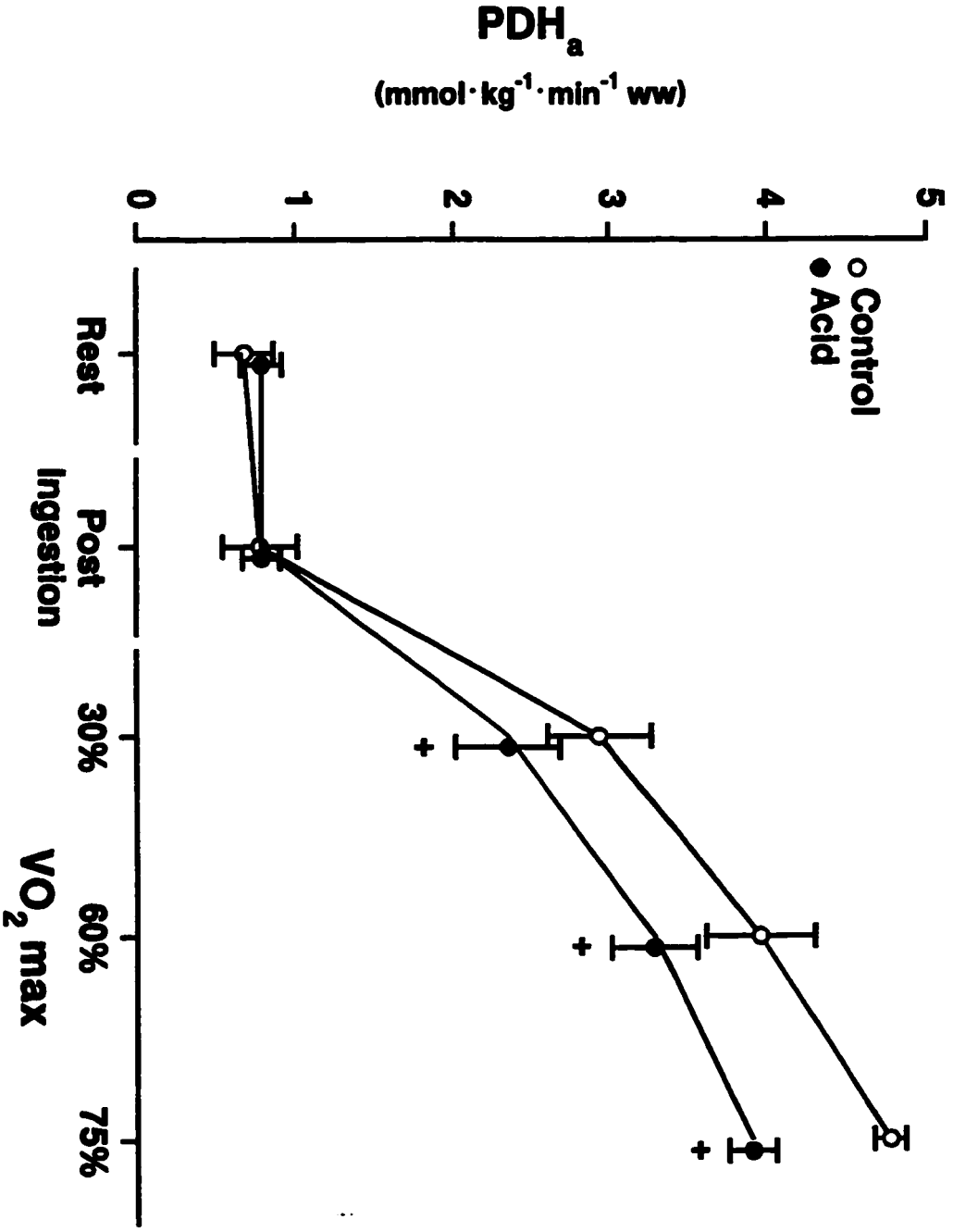
Data are means ± SE; n= 8. ** Significantly different from 30% of same condition.

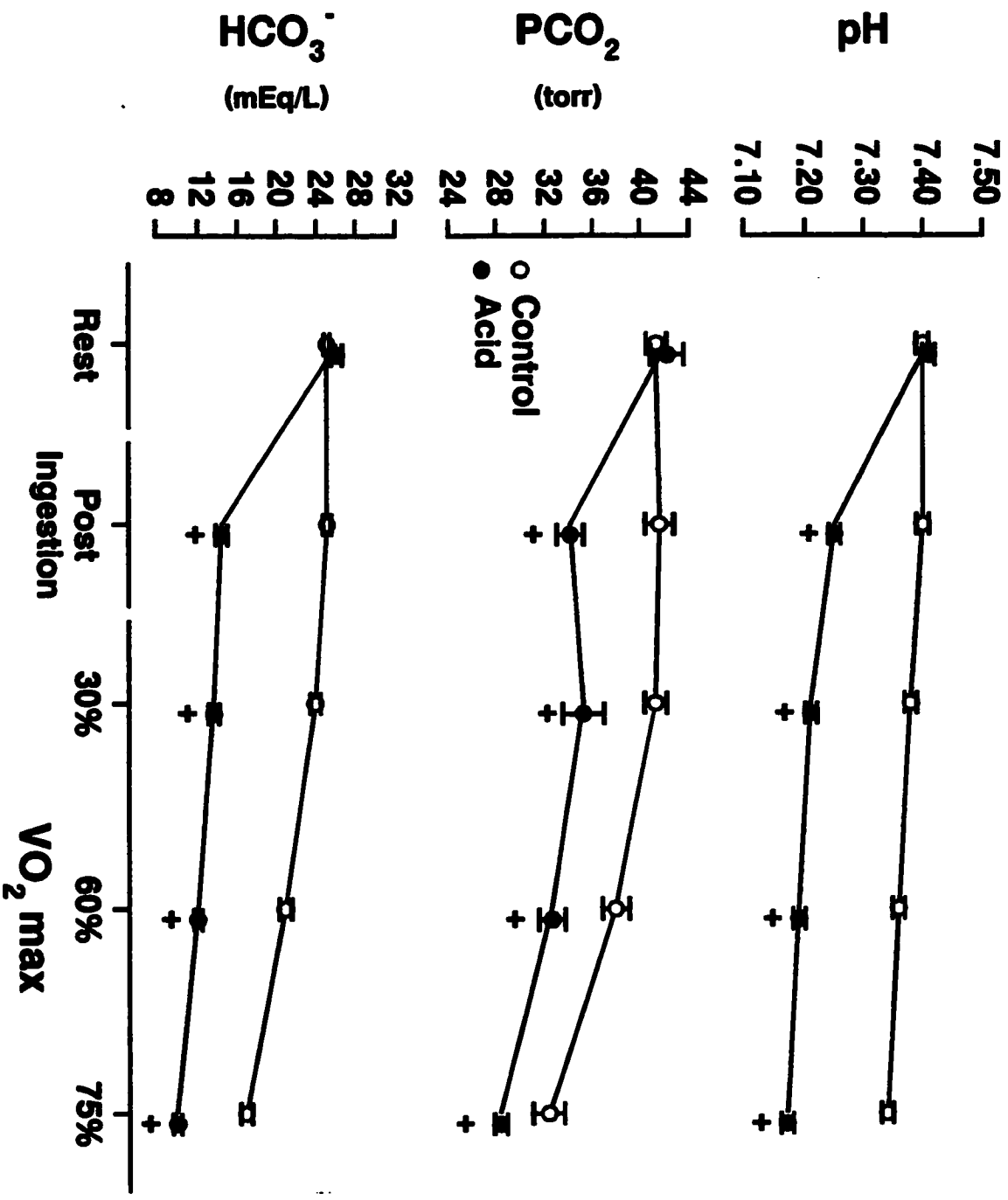
† Significantly different from matched times between conditions.

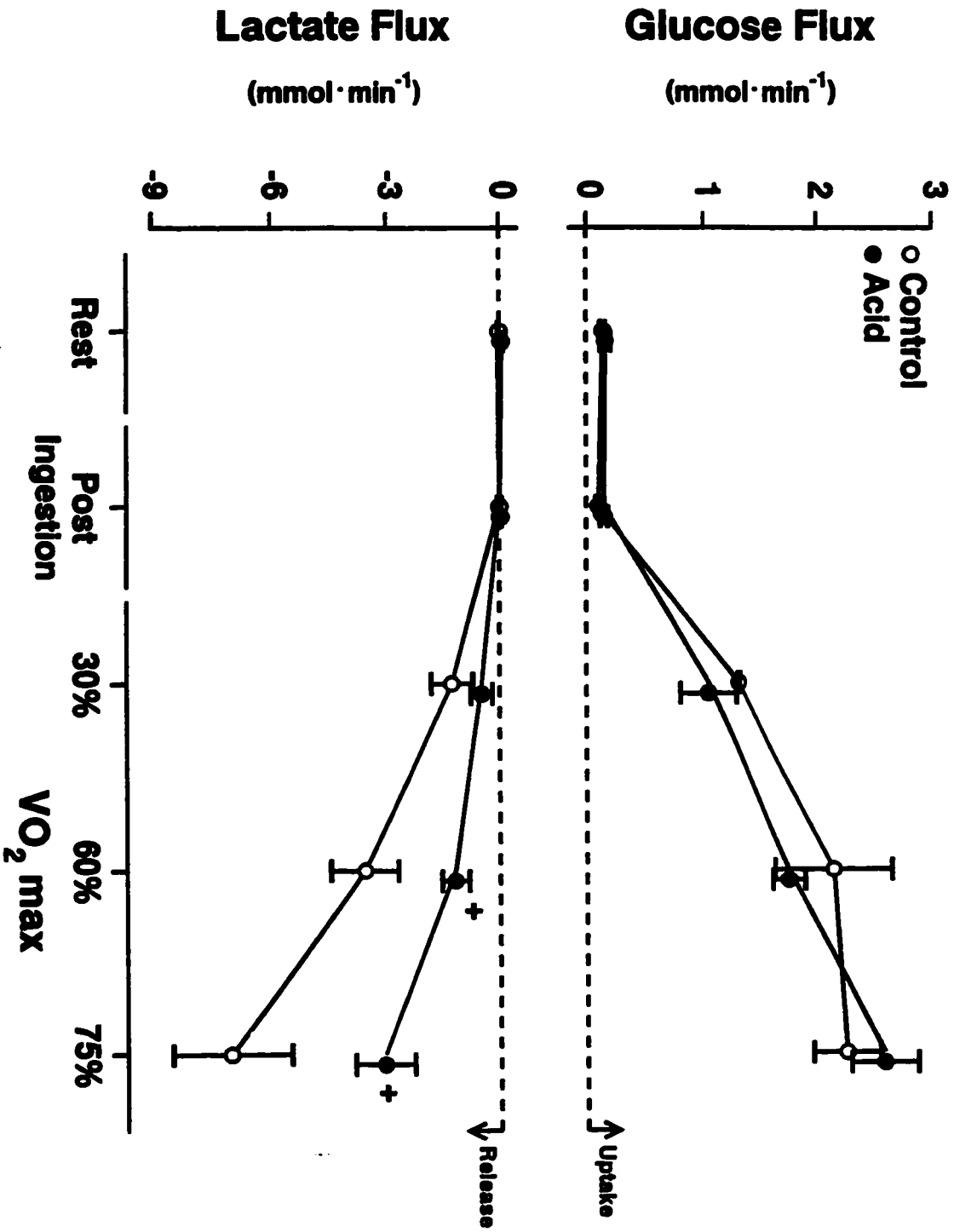

Phos a
mole fraction (%)
15
30
45
60
Control
Acid
30%
60%
75%
+
VO_2 max

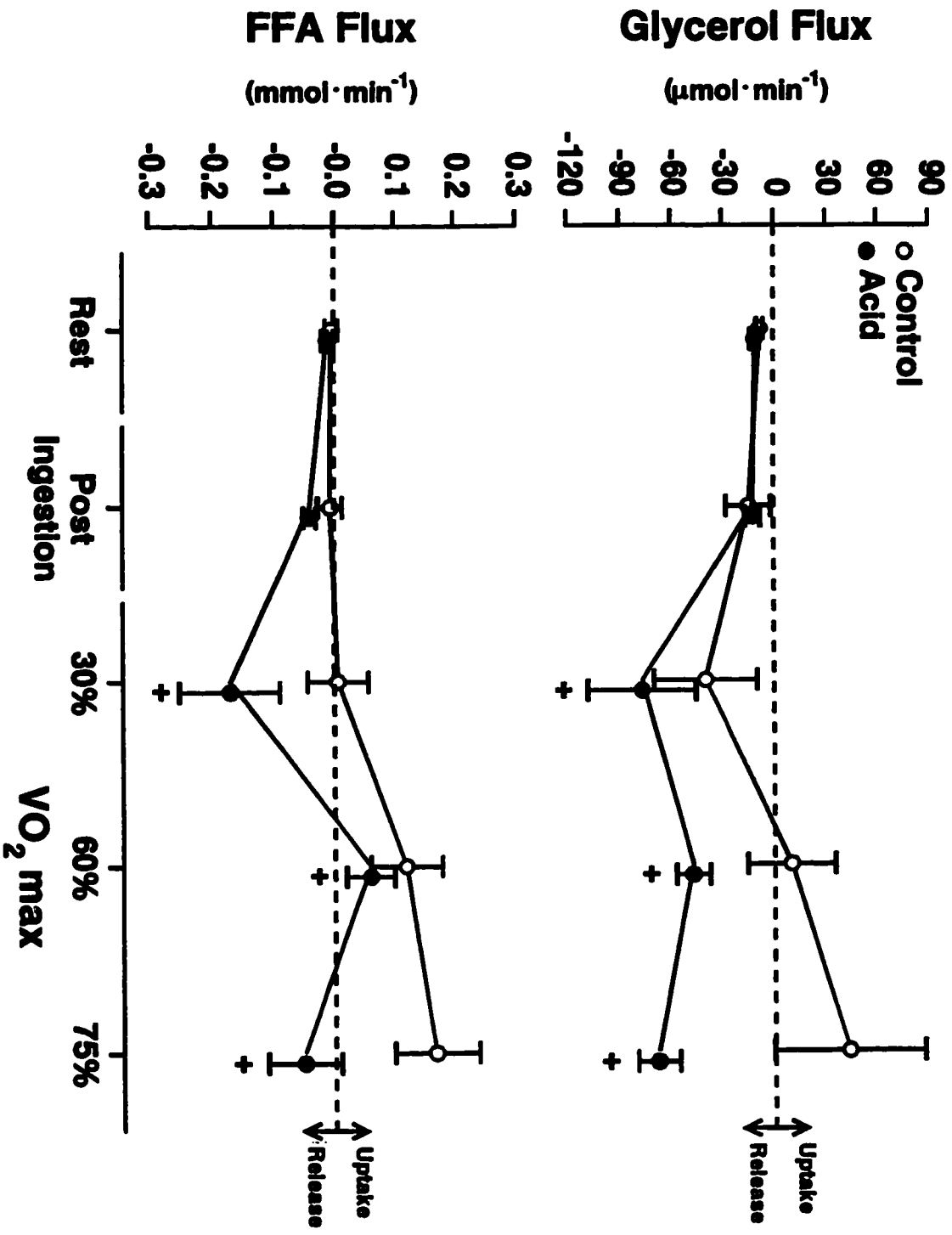

Glycogen Utilization
(mmol·kg-1 dw)
0
25
50
75
100
125
150
175
200
Control
Acid
VO2 max
30%
60%
75%
+
+


Pyruvate
(mmol·kg⁻¹ dw)
0.0
0.2
0.4
0.6
0.8
Lactate
(mmol·kg⁻¹ dw)
0
20
40
60
80
○ Control
● Acid
Rest
Post Ingestion
30%
60%
75%
VO2 max
+
+


PDH$_a$
(mmol·kg^{-1}·min^{-1} ww)
0
1
2
3
4
5
○ Control
● Acid
Rest
Post
Ingestion
30%
60%
75%
VO$_2$ max


HCO3-
(mEq/L)
8
12
16
20
24
28
32
PCO2
(torr)
24
28
32
36
40
44
pH
7.10
7.20
7.30
7.40
7.50
○ Control
● Acid
Rest
Post Ingestion
30%
60%
75%
VO2 max


Lactate Flux
(mmol·min⁻¹)
Glucose Flux
(mmol·min⁻¹)
-9
-6
-3
0
0
1
2
3
○ Control
● Acid
Rest
Post Ingestion
30%
60%
75%
VO2 max
+
+
Release
Uptake


Glycerol Flux
(μmol·min-1)
90
60
30
0
-30
-60
-90
-120
○ Control
● Acid
FFA Flux
(mmol·min-1)
0.3
0.2
0.1
-0.0
-0.1
-0.2
-0.3
Rest
Post
Ingestion
30%
60%
75%
VO2 max
Uptake
Release

2.5 Discussion

The present study examined the effects of acidosis on the metabolic responses in exercising muscle during continuous dynamic exercise for three power outputs - 30, 60, 75% $VO_{2\,max}$. The main effects of induced acidosis during exercise included: a reduction in intramuscular lactate accumulation due to reduced lactate production; reduced lactate efflux from the exercising leg; and a greater reduction in Phos *a* than PDH_a.

Lactate production within the muscle is dependent on the balance between the rates of pyruvate production and oxidation. Intramuscular lactate is formed from pyruvate by the action of the near equilibrium enzyme LDH (50). Pyruvate is the end product of glycolysis from glycogen degradation and glucose uptake. Pyruvate is oxidized via the TCA cycle and the electron transport chain after being converted to acetyl-CoA by PDH_a. Lactate production was reduced by 46% during the second power output and 48% in the third with ACID (Table 4). Not only was less Lac^- produced at both of these intensities, but the amount of pyruvate oxidized relative to that produced was significantly higher with ACID, resulting in a 14% increase during the second power output and 12% with the third (Table 4). These changes resulted from the effect of acidosis on the rate limiting enzymes Phos, PFK and PDH. The main control points for glycogenolysis/glycolysis involve Phos and PFK respectively, while entry into the oxidative pathways is controlled by PDH.

2.5.1 Regulation of Glycogen Phosphorylase

Phos is the flux generating enzyme responsible for glycogen degradation within skeletal muscle and is subject to both covalent and allosteric regulation. Phos *a* is considered the active form, active in the absence of AMP_f while Phos *b*, the less active form, requires AMP_f (10). Covalent Phos *b* to *a* transformation is mediated by phosphorylase kinase *a* which is activated by either an increase in cytosolic [Ca^{2+}] or epinephrine (60). Post-transformation allosteric regulation of Phos *b*, by the activators AMP, IMP, P_i and the inhibititors ATP and G-6-P, is also important (14).

ACID had a transformational effect, significantly reducing Phos *a* at the highest power output, resulting in a 34% reduction in muscle glycogen utilization. The reduction in Phos *a* transformation presumably resulted from the direct inhibition of phosphorylase kinase *a* by H^+, which has been previously demonstrated in intensely exercising muscle when the pH_i ~6.6, the same value estimated in the present study with ACID (9). In addition, the reduced availability of the substrate (HPO_4^{2-}) for Phos may have played a role (42), as the proportion of free P_i in the HPO_4^{2-} form falls by 30% as the pH fall from 7 to 6.5 (10).

AMP and IMP have been shown to stimulate Phos *a* (2,60) and Phos *b* (2,10) through post-transformational regulation . AMP acts on Phos *a* by reducing the K_m for P_i from 26.2 mM to 11.8 mM in the presence of 0.01 mM AMP (2,60). AMP act on Phos *b* in a similar manner but with a higher K_m. In the present study AMP accumulation was blunted with ACID, falling well below the 0.01 mM required for

activation. In support of the close relationship between Phos activity, glycogenolytic flux and [AMP_f], other studies utilizing caffeine ingestion (12) increased FFA availability (21,51) and short-term training (11) have demonstrated glycogen sparing during exercise, associated with blunted AMP_f accumulation. The reduction in [AMP_f] combined with the reduced availability of free P_i would prevent or reduce allosteric activation of Phos *a* and Phos *b*. IMP activates Phos b at a K_m of 1.2 mM (2). IMP was not measured in the present study but has been shown previously to increase with exercise and acidosis (10,19), which may have activated Phos *b*, thereby contributing to the maintenance of glycogenolytic flux.

G-6-P inhibits Phos *b* through end product inhibition, at concentrations of 0.3 mM and above (14). The [G-6-P] values with ACID, were above this value and therefore may have inhibited Phos at the higher power outputs. The G-6-P concentration was highest at the end of the second power output and then decreased by the end of the third power output. The elevated values at the end of the second power output may have inhibited Phos *b* thereby further reducing glycogenolytic flux and accounting for the lower [G-6-P] seen at the end of the third power output. This is confirmed by the simultaneous glycogen sparing that occurred. Previous investigation has alluded to this reduced glycolytic flux from inhibition before G-1-P with acidosis (66).

2.5.2 Regulation of Phosphofructokinase (PFK)

PFK is a non-equilibrium enzyme that converts F-6-P to fructose-1, 6-bisphosphate with consumption of ATP (50) with the relative activity being reflected

by changes in [F-6-P], and [G-6-P] with which it is in equilibrium. Increases in [H^+] have been shown to affect the kinetic and structural organization of PFK which ultimately results in increased affinity of the ATP binding site and reduced affinity of the F-6-P binding site (6,18,67). The magnitude of pH inhibition can be partially overcome by increases in the [F-6-P] only to a pH of 6.5; a value achieved with intense exercise (37). Previous human exhaustive exercise protocols have seen similar changes reflecting reductions in PFK activity with reduced pH_i (39,64). This increase in [F-6-P] although necessary to maintain partial enzyme activity, results in a reciprocal rise in the [G-6-P] which inhibits Phos and therefore reduces substrate supply. The results of the present study support this explanation. ACID resulted in a lower pH_i, higher [F-6-P], and higher [G-6-P] compared to CON (Table 1). This, together with the pattern of reduced [G-6-P] after the second power output with ACID reflects both a progressive increase in PFK inhibition, and the reciprocal inhibition of Phos flux and substrate supply that results when [F-6-P] increase to maintain flux through PFK. This reduced substrate supply and glycogenolytic flux is further evidenced by the glycogen sparing that occurred during the third power output.

The combined results demonstrate reduced glycogenolytic and glycolytic flux due to acidotic inhibition of both Phos and PFK, resulting in the observed decrease in pyruvate production (Table 4).

2.5.3 Regulation of Pyruvate Dehydrogenase

PDH_c is a mitochondrial enzyme that catalyses the decarboxylation of glycolytically derived pyruvate to acetyl-CoA for entry into the TCA cycle and therefore reflects the rate of entry of carbohydrate into the oxidative pathways. PDH transformation between the active PDH_a and inactive PDH_b forms is regulated by the balance between PDH kinase (PDHK) (deactivating) and PDH phosphate (PDHP) (activating) (58,68). The relative phosphatase/kinase activity is controlled by the mitochondrial acetyl-CoA/CoASH, ATP/ADP, NADH/NAD^+ ratios and the allosteric regulators Ca^{2+}, pyruvate and H^+ (56,58,68).

2.5.3.1 Acetyl-CoA/CoASH Ratio

ACID significantly elevated the [acetyl-CoA] at rest and throughout exercise, probably reflecting an increased utilization of intramuscular triacylglyerides (TAG) (Table 2, 5). It appears in the present study that intramuscular TAG was used rather than adipose tissue TAG as evidenced by the reduced arterial [FFA], [glycerol], and marked efflux from the exercising leg with increasing power output. Previous studies have demonstrated that acidosis impairs adipose tissue lipolysis by inhibiting the cAMP dependant activation of the hormone sensitive lipase (HSL)(23,36,40). Activation of the HSL is the rate limiting and flux generating step of TAG mobilization from adipose tissue. However, the rate of plasma FFA release is a poor estimate of lipolysis since the endothelial lining of the capillaries contains lipoprotein lipases; glycerol release provides a better estimate of lipolysis since adipose tissue lacks glycerol kinase (16). Precise regulation of intramuscular

lipolysis is not known but is thought to involve a HSL that is activated by a cAMP cascade and is sensitive to pH (25,52). Intramuscular lipolysis has been shown to occur during intense exercise in isolated muscle preparations (62,63) when the [H^+] is increased. Also, in exercising humans Jones et al (38) studied FFA turnover and found simultaneous increases in plasma [glycerol] with decreases in FFA turnover indicating intramuscular TAG utilization when FFA release from adipose tissue is significantly reduced. All of the above studies support our present findings of increased intramuscular TAG hydrolysis. Interestingly, the acetyl-CoA/CoASH ratio did not differ between conditions. Because acetyl-CoA stimulates PDHK while CoASH stimulates PDHP, with the net PDH_a resulting from both effects, it is possible that the increased [acetyl-CoA] has the dominating effect on PDH_a acting through increased kinase activity (68).

2.5.3.2 ATP/ADP Ratio

This ratio affects PDHK only since ATP is the substrate for the reaction and therefore competes with its product ADP which inhibits catalytic activity (68). The [ATP] did not change with exercise nor was it different between conditions. However, the [ADP_f] was significantly lower with ACID during 75% $VO_{2\ max}$ (Table 3). The ATP/ADP ratio declined with power output but to a lesser extent with ACID, which could have resulted in greater PDHK activity and therefore contributed to the lower PDH_a observed with ACID.

2.5.3.3 NADH/NAD$^+$ Ratio

The NADH/NAD$^+$ ratio was not measured in the present study. Previous studies utilizing indirect techniques have shown that the redox state and NADH/NAD$^+$ ratios decline with high intensity exercise indicating a more oxidized state (13,27,65). Based on these studies, the NADH/NAD$^+$ ratio should be declining, leading to an increase in PDH_a. However, in the present study, PDH_a was lower with ACID. It seems likely that changes in the NADH/NAD$^+$ ratio may have played only a minor role, compared to the effects of the other regulators, including Ca^{2+}, and pyruvate as previously suggested during high intensity exercise in humans (56). In addition, the increased utilization of intramuscular TAG with ACID, may have sufficiently increased the [NADH] resulting in greater activation of the kinase and subsequently a lower PDH_a (55).

2.5.3.4 Pyruvate

The [pyruvate] increases with exercise were lower with ACID; and although the [pyruvate] increased up to 60% $VO_{2\ max}$, it then declined by the end of the third power output (Fig. 4). Pyruvate acts directly on the kinase to inhibit activity and thus maintain PDH_a activity. In the present study, with ACID, the [pyruvate] was well below the K_i (0.5-2.0 mM) for PDHK and could have contributed to the reduced PDH_a (46). The importance of [pyruvate] on PDH_a has been demonstrated in human, diet manipulation studies (54,57). Total pyruvate production was lower with ACID compared to CON and resulted from decreases in glycogenolysis and glycolysis. The total amount of pyruvate oxidized was also lower with ACID but

exactly matched the ~4 mmol·kg^{-1}·min^{-1}·dw reduction in PDH_a compared to CON (Table 4, Fig 5). Despite lower PDH_a, the proportion of pyruvate oxidized was higher with ACID during both 60 and 75% $VO_{2\ max}$ compared to CON (Table 4). The reduction in intramuscular [pyruvate] with ACID from the second to third power output likely resulted from a marked inhibition in glycogenolysis/glycolysis with decreased pyruvate production due to the severe acidosis and an increase in pyruvate oxidation as PDH_a increased with the power output. These combined results suggest a better match between glycogenolytic flux and flux through PDH_a with subsequently lower pyruvate accumulation as also shown in previous studies (32,56). Thus acidosis results in relatively lower PDH inhibition (18%, Fig. 5) than Phos inhibition (27%, Fig. 2) at 75% $VO_{2\ max}$.

2.5.3.5 Hydrogen Ions and Calcium

Increases in the [Ca^{2+}] have been shown to increase PDH_a transformation by activating PDHP and inhibiting PDHK. Mitochondrial estimates of [Ca^{+2}] have been confirmed to be within the physiological range of calcium to cause half maximal activation (0.2-0.3 *u*M) (17). The [Ca^{2+}] should have been similar between conditions since subjects exercised at the same intensities in both trials.

Hydrogen ion has been shown to activate PDH_a in acidotic perfused rat hearts (53) and has been attributed to the differences in pH optimum of PDHK and PDHP. PDHK has a pH optimum of 7.0 - 7.2, with increased inhibition as pH falls while PDHP has a pH optimum of 6.7 - 7.1 (33). Based on these data our original hypothesis was that, like the heart, metabolic acidosis would increase PDH_a.

However, the results of the present study indicate that PDH_a is lower during ACID with each of the three power outputs. This can be explained by the influence of acetyl-CoA, ADP, pyruvate and possibly NADH that cumulatively resulted in greater activation of PDHK leading to a reduced PDH_a transformation. The $[H^+]$ may have had an effect but was overridden or masked by the other covalent modifiers.

2.5.4 Lactate Metabolism

Intramuscular lactate accumulation reflects the balance between the rates of lactate production and efflux from the muscle (24,41). The results of previous studies employing both metabolic acidosis (31,40,48,63,66) and respiratory acidosis (22,26,63) were similar to the present data, in showing elevated [F-6-P], inferring PFK inhibition; glycogen sparing; and reductions in both intramuscular and plasma $[Lac^-]$ when compared to control conditions. Respiratory acidosis has less of an overall effect on these parameters compared to metabolic acidosis possibly due to the enhanced catecholamine release accompanying hypercapnia (22). However, none of these studies was able to elucidate the mechanisms responsible for these universally observed changes accompanying acidosis.

The mechanisms responsible for lactate production by muscle have been controversial; conventionally lactate production has been attributed to O_2 limitation at the mitochondria (24,43). However, in the present study, the differences in lactate accumulation between conditions clearly did not result from an O_2 limitation at the mitochondria since leg O_2 uptake was similar, despite a reduction in blood flow with ACID during the highest power output (Table 6). Oxygen delivery was

maintained with ACID due to a pH mediated rightward shift of the oxyhaemoglobin dissociation curve, augmenting oxygen availability to the working muscle (34).

2.5.5 Lactate Transport

Intramuscular lactate accumulation is also a function of the rate of efflux from the muscle (41). ACID significantly reduced both arterial whole blood [Lac^-] and efflux from the leg during 60 and 75% $VO_{2\ max}$. Blood [Lac^-] represents the balance of lactate entry from muscle and uptake by inactive tissue (7). The present results during ACID may be explained by both an impairment of the lactate transport out of the exercising leg and enhanced uptake by non-exercising tissue.

Lactate transport across the sarcolemma occurs via a monocarboxylate lactate-proton co-transport protein, and as such is the rate limiting step in lactate efflux. Diffusion of lactate in the undissociated form of lactic acid in the direction of the trans-membrane [Lac^-] and [H^+] occurs, but accounts for only 20% of lactate efflux, although higher contributions are seen with higher lactate and H^+ concentrations. The monocarboxylate carrier accounts for 70-90% of lactate transport across the physiological range of [Lac^-] (41,49). Kinetic studies of the transporter using sarcolemmal vesicles have shown it to have a high affinity for L-lactate, to be sensitive to changes in [Lac^-], and [H^+] gradient, and to co-transport lactate and H^+ in a 1:1 ratio (41). In the present study with ACID there was a decrease in the intramuscular [Lac^-] and a decrease in the femoral venous [Lac^-] suggesting that the lower lactate efflux resulted from the reduced [Lac^-] gradient. However, the magnitude of the reduced lactate efflux was greater than the

reduction in intramuscular [Lac^-] (56% vs 28% respectively) suggesting a role for the transporter. The altered [H^+] gradient with ACID may have affected H^+ binding and off-loading and therefore transport (41). In addition, the elevated [H^+] within the muscle is expected to increase the proportion of HLactate and thereby reduce the availability of Lac^- to the transporter. ACID also induced a reduction in extracellular [HCO_3^-] which may have contributed to the reduced lactate efflux from the muscle. Investigators examining lactate efflux under varied external [HCO_3^-] in isolated muscle preparations have found reduced lactate efflux with low external [HCO_3^-] (30,47,63). In addition, studies comparing metabolic and respiratory acidosis in exercising humans (22,31,40,44) consistently demonstrate greater reductions in the appearance of plasma lactate, when acidosis is accompanied by reductions in [HCO_3^-]. During ACID in the present study, extracellular [HCO_3^-] was significantly reduced in both arterial and femoral venous blood at rest post-ingestion, and with each of the three power outputs, which is in agreement with the above evidence (Table 5). Additionally, enhanced lactate uptake by inactive tissue may have contributed to the lower arterial [Lac^-] with ACID. Uptake by inactive tissue has been shown to occur during exercise (24). Normally, with exercise increases in blood [H^+] and blood [Lac^-] create an inwardly directed [H^+] and [Lac^-] gradient which would facilitate uptake. Acidosis enhances the [H^+] gradient and therefore may enhance uptake compared to control conditions.

2.5.6 Summary and Conclusions

Imposition of a metabolic acidosis results in a complex series of metabolic effects during exercise reflecting changes in the activity of key regulatory enzymes and fuel utilization. The main findings of the study demonstrate that the reduced intramuscular [Lac^-] seen with acidosis result from a combined reduction in pyruvate production, and an enhanced percentage of pyruvate oxidized, due to a better match between the rate of glycogenolysis and the rate of flux through PDH_a. The reduced pyruvate production results from decreased glycogenolytic flux from a direct acidotic depression of Phos *a* transformation and reduced availability of substrate due to greater prevalence of $H_2PO_4^-$. Glycogenolytic flux via Phos *b* was also reduced due to the acidotic reduction in the allosteric modulators ADP_f and AMP_f. Acidotic inhibition of PFK resulted in elevations in the [F-6-P] and consequently the [G-6-P], which also contributed to the reduced glycogenolytic flux via end product inhibition. The combined results of reduced glycogenolytic and glycolytic flux resulted in glycogen sparing and reduced pyruvate production particularly at 75% $VO_{2\,max}$ when pH_i was the lowest. The reduction in carbohydrate utilization was accompanied by increased intramuscular TAG utilization and occurred due to the acidotic inhibition of adipose tissue lipolysis. This increased use of FFA was associated with elevations in the [acetyl-CoA]. The increased [acetyl-CoA], decreased [ADP_f] and decreased [pyruvate] collectively contributed to the reduced PDH_a transformation.

The decreased blood [Lac^-] seen with acidosis may reflect inhibition of the

lactate transporter by an altered [H^+] gradient or the reduced extracellular [HCO_3^-] - and not due to changes in the [Lac^-] gradient. Inactive muscle may have contributed significantly to the reduced arterial [Lac^-], but, the contribution was not assessed in the present study. The present data demonstrate that the reduced intramuscular lactate accumulation during acidosis results from both decreased production and enhanced oxidation through modulation of the key regulatory enzymes Phos, PFK and PDH_a.

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CHAPTER 3

EFFECT OF INDUCED METABOLIC ALKALOSIS ON HUMAN SKELETAL MUSCLE METABOLISM DURING EXERCISE

((submitted for publication in Am.J.Physiol. (Endocrinology and Metabolism))

3.1 ABSTRACT

The purpose of the study was to examine the roles of pyruvate dehydrogenase (PDH_a), glycogen phosphorylase (Phos) and their regulators in lactate (Lac^-) metabolism during incremental exercise following ingestion of 0.3 grams/kg of either $NaHCO_3$ (metabolic alkalosis = ALK) or $CaCO_3$ (control = CON). Subjects (n=8) were studied at rest, rest post-ingestion and during constant rate cycling at three stages (15 min each): 30, 60, 75% of $VO_{2\,max}$. Radial artery and femoral venous blood samples, leg blood flow and biopsies of the vastus lateralis were obtained during each power output. ALK resulted in significantly ($P < 0.05$) higher intramuscular [Lac^-] (ALK 72.8 vs CON 65.2 mmol·kg^{-1} dw), arterial whole blood [Lac^-] (ALK 8.7 vs CON 7.0 mmol·l^{-1}) and leg Lac^- efflux (ALK 10.0 vs CON 4.2 mmol·l^{-1}·min^{-1}) at 75% $\dot{V}O_{2max}$. The increased intramuscular [Lac^-] resulted from increased pyruvate production due to stimulation of glycogenolysis at the level of

Phos *a*, and phosphofructokinase (PFK), due to allosteric regulation mediated by increased [ADP_f], [AMP_f] and free P_i. PDH_a increased with ALK at 60% $VO_{2\,max}$ but was similar to CON at 75% $VO_{2\,max}$. The increased PDH_a may have resulted from alterations in the [acetyl-CoA], [ADP_f], [pyruvate], [NADH] and [H^+] leading to lower relative activity of PDH kinase, while the similar values at 75% $VO_{2\,max}$ may have reflected maximal activation. The results demonstrate that imposed metabolic alkalosis in skeletal muscle results in acceleration of glycogenolysis at the level of Phos relative to maximal PDH activation resulting in a mismatch between the rates of pyruvate production and oxidation leading to an increase in Lac^- production.

3.2 Introduction

Induced metabolic alkalosis by sodium bicarbonate ($NaHCO_3$) ingestion in humans has previously been shown to increase blood lactate concentration ([Lac^-]) during exercise (10,33,37,46,63). Unfortunately, the majority of studies exploring the effects of metabolic alkalosis on lactate metabolism have focussed on the possible performance enhancing capabilities of "bicarbonate loading" (31). Ingestion of sodium bicarbonate is thought to enhance performance by buffering the lactic acid produced with exercise, thereby limiting the effects of the decreased intramuscular pH (pH_i) (31). Metabolic alkalosis through ingestion or infusion of $NaHCO_3$ has been shown to enhance performance for short-duration, high-intensity exercise but the mechanisms have not been elucidated. Different mechanisms have been postulated to explain this, including an increase in muscle lactate

production (10,63) and/or enhanced lactate efflux from the muscle (44). Lactate accumulation results from the conversion of non-oxidized pyruvate to Lac^- by lactate dehydrogenase (LDH) and as such will be influenced by both pyruvate production from glycogen and pyruvate oxidation by pyruvate dehydrogenase (PDH) (18,38).

In an effort to discern the possible mechanisms responsible for the increased blood [Lac^-] with metabolic alkalosis we chose an oral dose of $NaHCO_3$ previously shown to induce a significant metabolic alkalosis, to influence the plasma [Lac^-], and to enhance performance (37). Continuous, dynamic constant rate exercise at low, moderate and high power output was chosen to follow metabolic effects and compare fuel utilization with previously described carbohydrate and free fatty acid (FFA) contributions at these power outputs (50).

The aim of the present study was *not* to examine the performance effect of an induced metabolic alkalosis during exercise. Rather the aim was first, to determine the effect of metabolic alkalosis on the key regulatory enzymes glycogen phosphorylase (Phos) and PDH and their allosteric regulators; second, to measure the effect of metabolic alkalosis on glycolytic intermediates and muscle pyruvate production, oxidation; third, to measure muscle lactate accumulation, production and efflux; and finally, to determine if alkalosis has any effects on glucose uptake and FFA utilization during exercise. This is the first human *in vivo* study to examine all these parameters simultaneously via assessment of the arterial, femoral venous and intramuscular compartments.

3.3 METHODS

3.3.1 Subjects

Eight healthy male volunteers participated in the study [age 23 ± 1.8(SE) yr; height 173 ± 3.8 cm; weight 75.3 ± 4.4 kg]. Written consent was obtained from each subject after explanation of the purposes and associated risks of the study protocol. The study was approved by the Ethics Committees of both McMaster University and McMaster University Medical Centre.

3.3.2 Pre-Experimental Protocol

All subjects completed an initial incremental maximal exercise test on a cycle ergometer to determine VO_{2max} and maximal work capacity using a metabolic measurement system (Quinton Q-Plex 2, Quinton Instruments, Seattle Washington). Mean $VO_{2\ max}$ for the group was 3.2 ± 0.2 l/min^{-1}. None of the subjects was well trained but all participated in some form of regular activity. Each subject was instructed to refrain from caffeine, alcohol and exercise for 24 hr before each trial and studies were carried out at the same time of day.

3.3.3 Experimental Protocol

Each subject participated in two experimental trials separated by 2-3 weeks and was randomized to receive capsules containing either 0.3 grams/kg of $NaHCO_3$ (ALK) or 0.3 grams/kg of $CaCO_3$ (CON). On the morning of each trial the subjects reported to the laboratory following consumption of a standard light meal consisting primarily of carbohydrates. The exercise portion of the protocol consisted of three levels of continuous, constant rate exercise on a cycle ergometer at 30, 60 and 75%

of VO_{2max} each maintained for 15 minutes, which began following insertion of arterial and femoral venous catheters, and ingestion of the required capsules.

A radial artery was catheterized with a Teflon catheter (20 gauge, 3.2 cm, Baxter, Irvine CA) percutaneously after anaesthetizing the area with 0.5 mls of 2% lidocaine without epinephrine (6). A femoral vein was catheterized percutaneously for insertion of the thermodilution catheter (model # 93-135-6F, Baxter, Irvine, CA) using the Seldinger technique (6) following administration of 3-4 mls of lidocaine without epinephrine. Both the arterial and femoral venous catheters were maintained patent with sterile, non-heparinized, isotonic saline solution. Arterial and femoral venous blood samples were simultaneously taken at rest, rest post-ingestion and during each of the three exercise bouts at 6 and 11 minutes. Single leg blood flow measurements were made following blood sampling at the same time points. Single leg blood flow was determined using the thermodilution technique as described by Andersen and Saltin (1): 10 mls of non-heparinized isotonic saline was injected and leg blood flow was calculated by a portable CO monitor (Spacelab, Redmond, VA). At least three measurements were recorded at each time point and then averaged.

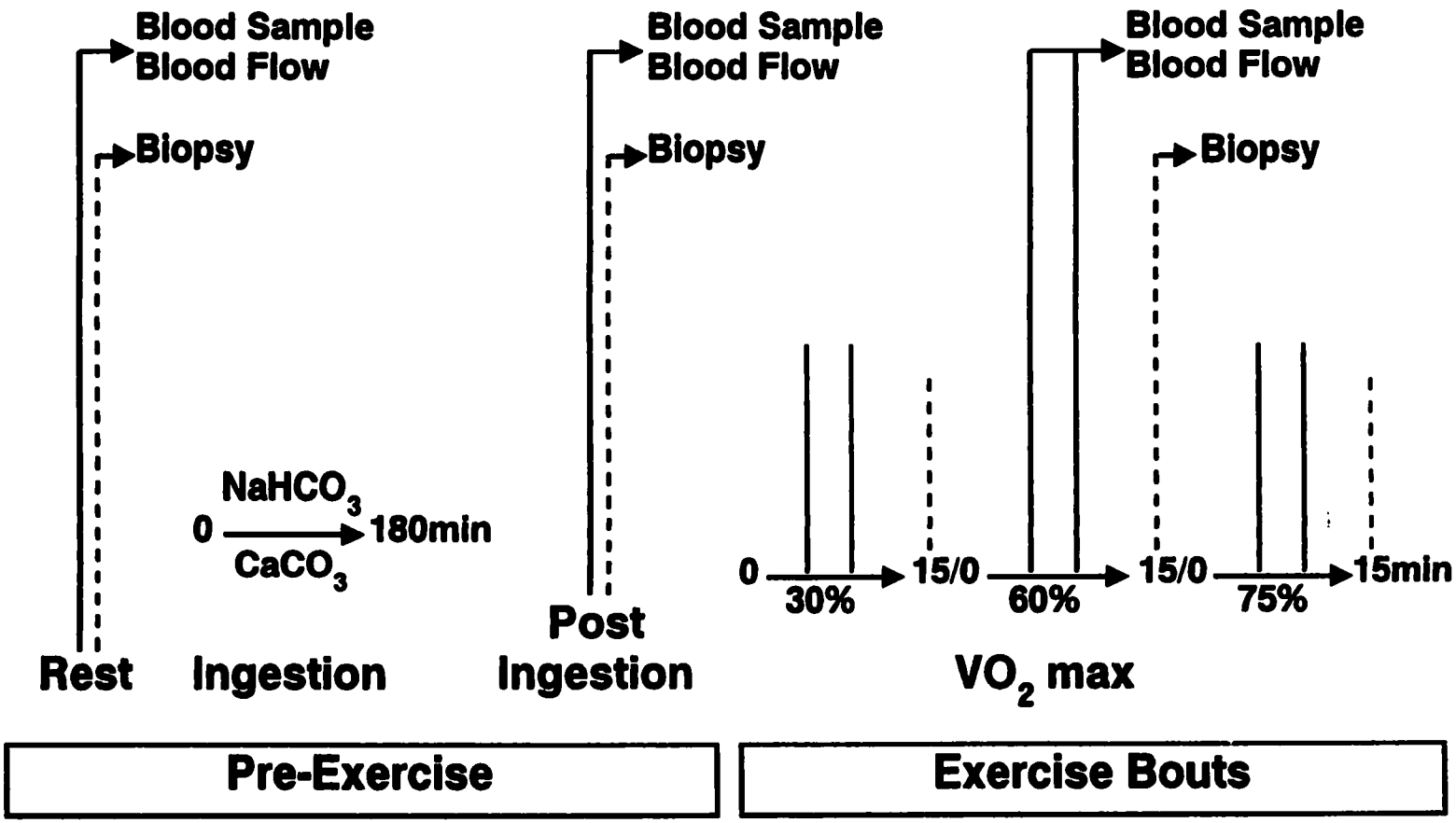
A total of five percutaneous needle biopsies of the vastus lateralis were taken, one at rest, one at rest post-ingestion and three during exercise at the end of each power output. The resting biopsies were obtained with the subject lying on a bed. The resting and exercise biopsies were obtained on opposite legs and then reversed for the second trial. Biopsy sites were prepared by making an incision

through the deep fascia under local anaesthetic (2% lidocaine without epinephrine) as described by Bergström et al (5). Respiratory measurements of V_E, VO_2, VCO_2 and respiratory exchange ratio (RER) were measured at 7 and 11 minutes of each exercise stage. (Fig. 1).

3.3.4 Muscle Sampling and Analysis

Muscle samples were immediately frozen in liquid N_2. A small piece (10-35 mg) was chipped from each biopsy (under liquid N_2) for determination of the fraction of PDH in the active form (PDH_a) as previously described (20,51). The remainder of the sample was freeze-dried, dissected free of blood and connective tissue and powdered. One aliquot was analysed for Phos activity according to the methods of Young et al (72). Briefly, a 3-4 mg sample of muscle was homogenized at -20°C in 0.2 ms of 100 mM of Tris/HCl (pH 7.5) containing glycerol, potassium fluoride and EDTA. Homogenates were then diluted with 0.8 mls of the same buffer without glycerol and homogenized further at 0°C. Total (*a* + *b*) Phos activity (measured in the presence of 3 mM AMP) and glycogen phosphorylase in the active *a* form (Phos *a*) (measured in the absence of added AMP) were measured at 30°C with a spectrophotometer. Maximum velocity (v_{max}) was derived from the equation described by Lineweaver and Burk (41), $1/V = (K_m/V_{max})(1/S) + (1/V_{max})$, where V is the initial reaction rate expressed as $mmol \cdot kg^{-1} \cdot min^{-1}$ dw, S is the P_i concentration in $mmol \cdot l^{-1}$, and K_m is 26.2 $mmol \cdot l^{-1}$. The mole fraction of Phos *a* is presented as a percentage and calculated from $V_{max}\ a/V_{max}(a+b)$ X 100.

STUDY PROTOCOL



assuming a K_{obs} of 1.05 for the adenylate kinase reaction. Free P_i content was calculated from the sum of the estimated resting free [P_i] of 10.8 mmol·kg^{-1} dw (23) and the ΔPCr - ΔG-6-P - ΔF-6-P - ΔG-3-P between rest and each time point during exercise. For the purposes of ADP_f, AMP_f and free P_i calculations, no differences were observed between the rest and post-ingestion values and therefore the mean of the two values was taken as the resting value.

3.3.5 Blood Sampling and Analysis

Arterial and femoral venous blood samples (~10 mls) were collected into heparinized plastic syringes and placed on ice. One portion (1-2 mls) of each blood sample was analysed for blood gas determination (AVL 995 Automatic Blood Gas Analyser); O_2 and CO_2 content (Cameron Instrument CO., Port Arkansas, TX) and haemoglobin (OSM3 Hemoximeter, Radiometer, Copenhagen, Denmark).
A second portion of each sample was deproteinized with 6% perchloric acid (PCA) and stored at -20°C until analysis for glucose, lactate, and glycerol according to the methods of Bergmeyer (4) adapted for fluorometry. The third portion of blood was immediately centrifuged at 15,900 *g* for 2 min and the plasma supernatant was frozen and later analysed for free fatty acids (FFA) (Wako, NEFA C test kit, Wako Chemical, Montreal, Canada). Hematocrit (Hct) was determined on blood samples using a heparinized micro capillary tube centrifuged for 5 min at 15,000 *g*.

3.3.6 Leg Uptake and Release of Metabolites, O_2 Uptake, CO_2 Production

Uptake and release of metabolites (glucose, glycerol, lactate) were calculated from their whole blood measurements in arterial and femoral venous blood and leg blood flow according to the Fick equation. Since there were differences in the Hct over time within a condition and between matched arterial and femoral venous samples, venous samples were corrected for fluid shifts. Fluid shifts for the whole blood measurements were corrected using the differences in hemoglobin (Hb) to calculate a percent change in blood volume (%ΔBV) as calculated by the equation (assuming no change in intravascular hemoglobin) (30):

$$\%\Delta BV = [(Hb_{arterial}/Hb_{venous})-1] \times 100$$

This value was then multiplied by the measured venous value to yield a corrected value which was used in determining flux for that metabolite. Uptake and release of plasma FFA was determined as above but venous values were corrected using changes in plasma [protein] to correct for changes in plasma water (30). The leg O_2 uptake and CO_2 production were calculated from their respective arterial and femoral venous content differences and blood flow.

Subjects exercised at a constant rate, and since no significant differences occurred in blood flows or metabolite concentrations between the 6 and the 11 minute sampling points at each power output; the two values were averaged to obtain one value for each exercise level. Reported values are for the single leg only.

3.3.7 Calculations

Flux through Phos and therefore glycogenolysis was calculated from the differences in glycogen utilization divided by time. PDH_a flux was estimated from the PDH_a as measured in wet tissue and converted to dry tissue using the wet/dry ratio. Pyruvate production was calculated from the sum of the rates of glycogen breakdown and glucose uptake minus the sum of the rates of accumulation of muscle glucose, G-6-P, and F-6-P. Lactate production was calculated from the sum of the rates of muscle lactate accumulation and lactate release. Pyruvate oxidation was calculated as pyruvate production minus lactate production. All values are reported in $mmol \cdot kg^{-1} \cdot min^{-1}$ dw and are for single leg only. All values were calculated in three carbon units and assume a wet muscle mass of 5 kg.

3.3.7.1 Intramuscular pH

Intramuscular pH (pH_i) was calculated from the [Lac^-] and [pyruvate] according to the methods of Sahlin et al (55).

3.3.7.2 Lactate Gradient and [H^+] Gradient

The lactate gradient between the plasma and muscle for both trials was calculated for arterial and femoral venous blood at 75% $VO_{2\,max}$ only. The lactate gradient between the muscle and arterial plasma was calculated as the difference between the wet weight intramuscular [Lac^-] and arterial plasma [Lac^-]. The lactate gradient from muscle to the femoral venous plasma was calculated as above using the non-corrected venous values. The arterial to muscle and femoral venous to muscle [H^+] gradients were calculated as the difference between their respective

blood compartment [H^+] and the calculated intramuscular [H^+].

3.3.8 Statistical Analyses

Data was analysed using two-way ANOVA with repeated measures (Treatment x Time) except where otherwise stated. When a significant *F* ratio was found the Newman-Keuls post hoc test was used to compare means. The following data were analysed using a 2-tailed paired dependent-sample Student's *t*-test: Phos *a*, and glycogen utilization at each power output. Data are presented as means ± SE. Differences were considered significant at $P < 0.05$.

3.4 RESULTS

3.4.1 Muscle Metabolism.

3.4.1.1 Glycogen Phosphorylase

Phos *a* activity did not change with power output during CON. However, with ALK, Phos *a* progressively decreased with power output and was significantly lower at 75% $VO_{2\,max}$ with ALK compared to CON (ALK 37.9 ± 5.1 vs 48.8 ± 5.0 mmol·kg^{-1}·min^{-1} dw) (Fig. 2).

3.4.1.2 Glycogen

Resting and rest post-ingestion muscle glycogen levels were not different between conditions (Table 1). Muscle glycogen content decreased with increasing power output but to a greater degree with ALK (Table 1). During the complete exercise study , total muscle glycogen utilization was 25% greater with ALK compared to CON (305 ± 9 vs 229 ± 10 mmol·kg^{-1} dw). No differences in muscle

glycogen utilization were observed at 30% $VO_{2\,max}$ but muscle glycogen utilization was significantly higher at both 60 and 75% $VO_{2\,max}$ with ALK compared to CON (60% -132 ± 15 vs 75 ± 6 mmol·kg^{-1} dw; 75% - 133 ± 15 vs 113 ± 12 mmol·kg^{-1} dw) (Fig. 3).

3.4.1.3 Glucose, G-6-P, F-6-P, G-1-P, and G-3-P

Intramuscular accumulation of glucose increased with exercise similarly between conditions (Table 1). Intramuscular [G-6-P] and [F-6-P] increased with increasing power output for the first and second power outputs but each was significantly lower with ALK at 75% $VO_{2\,max}$ only (Table 1). Muscle G-1-P and G-3-P were similar between conditions, increasing with power output (Table 1).

3.4.1.4 Lactate and Pyruvate

Intramuscular [Lac$^-$] increased with each power output but was significantly higher with ALK at both 60 (ALK 40.9 ± 8.3 vs CON 26.2 ± 5.0 mmol·kg^{-1} dw) and 75% (ALK 72.8 ± 11.8 vs CON 65.2 ±10.1 mmol·kg^{-1} dw) $VO_{2\,max}$ (Fig. 4). Muscle [pyruvate] increased with each power output and was similar between conditions at 30 and 75% $VO_{2\,max}$ and significantly higher with ALK during the second power output (Fig. 4).

3.4.1.5 Pyruvate Dehydrogenase Activity

Resting (ALK 0.55 ± 0.01 vs CON 0.55 ± 0.08 mmol·kg^{-1}·min^{-1} ww) and post-ingestion (ALK 0.55 ± 0.11 vs CON 0.53 ± 0.05 mmol·kg^{-1}·min^{-1} ww) PDH_a were not different between conditions. Under both conditions, PDH_a increased progressively with each power output but was significantly higher at 60% $VO_{2\,max}$

(4.17 ± 0.23 vs 3.77 ± 0.27) with ALK compared to CON respectively (Fig. 5).

3.4.1.6 CoA, Carnitine, and Acetylated Forms

Total muscle CoA was not different between conditions at rest or during exercise (Table 2). The [Acetyl-CoA] increased with each power output similarly between conditions at 30% but during both 60 and 75% $VO_{2\,max}$ the [acetyl-CoA] was significantly lower with ALK (Table 2). Free CoASH declined equally between conditions with exercise (Table 2). The acetyl-CoA/CoASH ratio was also significantly lower at 75% $VO_{2\,max}$ with ALK (0.26 ± 0.02 vs 0.37 ± 0.06) (Table 2). Acetylcarnitine followed a similar pattern to acetyl-CoA increasing with each power output but was not different between conditions (Table 2). Muscle total carnitine content increased significantly from rest to 75% $VO_{2\,max}$ to the same degree in each condition while free carnitine decreased in a reciprocal manner with increasing power output. There were no differences in total carnitine between conditions but free carnitine was significantly lower at 60% $VO_{2\,max}$ during ALK compared to CON (Table 2).

3.4.1.7 ATP, ADP_f, AMP_f, Free P_i and PCr

Muscle [ATP] was unaltered by exercise or as a result of alkalosis. Muscle [ADP_f] and [AMP_f] increased with each power output but both were significantly higher with ALK at 60 and 75% $VO_{2\,max}$ (Table 3). Free P_i increased with power output but to a significantly greater degree with ALK (Fig. 6). The [PCr] decreased with increasing power output but were significantly more depleted at each power output during ALK compared to CON (Fig. 6).

3.4.1.8 Pyruvate production, oxidation and Lactate production and oxidation

Pyruvate production increased with exercise intensity but was significantly higher during both the second and third power outputs with ALK. Pyruvate oxidation increased with exercise similarly between conditions at both 30 and 75% $VO_{2\ max}$. At 60% $VO_{2\ max}$ pyruvate oxidation was significantly higher during ALK (Table 4). Relative pyruvate oxidation expressed as the percentage of pyruvate produced that was oxidized was similar between conditions both 30 and 75% $VO_{2\ max}$. However, relative pyruvate oxidation was significantly higher during 60% $VO_{2\ max}$ with ALK (Table 4). Lactate production was similar between conditions during the first two power outputs but was significantly higher at 75% $VO_{2\ max}$ during ALK (Table 4).

3.4.2 Blood Metabolites, Blood Flow and Exchange Across the Leg.

3.4.2.1 Blood pH, PCO_2 and HCO_3^-

Arterial pH, HCO_3^- (Fig. 7), venous pH and HCO_3^- (Table 5) were all significantly higher during ALK compared to CON at rest post-ingestion and each of the three power outputs.

3.4.2.2 Leg Blood Flow and Leg RQ

Leg blood flow increased progressively from rest with exercise similarly between conditions (Table 6). Leg O_2 uptake, CO_2 production and leg RQ were not different between conditions, increasing with each power output (Table 6).

3.4.2.3 Blood Lactate and Flux

Arterial [Lac^-] increased progressively with each power output but was significantly higher at both 60 and 75% $VO_{2\,max}$ with ALK (Table 5). Net release of Lac^- across the leg also increased with each power output but was significantly higher during 75% $VO_{2\,max}$ with ALK (Fig. 8).

3.4.2.4 Blood Free Fatty Acids and Glycerol

Arterial plasma [FFA] declined progressively with exercise intensity similarly between conditions (Table 5). FFA release across the leg occurred at rest post-ingestion and at each of the power outputs during CON. However, with ALK a significantly lower net release occurred at rest post-ingestion, while during exercise at each of the power outputs a net uptake occurred (Fig. 9). Arterial [glycerol] increased with each power output similarly between conditions (Table 5). Glycerol release across the leg occurred at rest post-ingestion and 30% $VO_{2\,max}$ similarly between conditions. During 60% $VO_{2\,max}$ a net release occurred with CON, while a net uptake occurred with ALK. At 75% $VO_{2\,max}$ a net uptake across the leg occurred for both conditions but was significantly lower with ALK (Fig. 9).

3.4.2.5 Blood Glucose and Flux

Arterial [glucose] (Table 5) and leg glucose uptake (Fig. 8) were similar at all power outputs between conditions.

3.4.2.6 Lactate Gradient, H^+ Gradient and pH_i.

The lactate gradient between both the arterial and femoral venous plasma and muscle were not different between conditions at 75% $VO_{2\,max}$ (Table 7). The $[H^+]$ gradient between the arterial blood and muscle and the femoral venous blood and muscle were both significantly elevated with ALK compared to CON during 75% $VO_{2\,max}$ (Table 7). Intramuscular $[H^+]$ was significantly elevated during both the second and third power outputs with ALK (Table 7).

3.4.3 Respiratory Gas Exchange Variables

Whole body VO_2 increased similarly between condition with each power output (Table 8). Whole body VCO_2 was significantly higher during 60 (2.39 ± 0.09 vs 2.28 ± 0.13) and 75% (3.18 ± 0.11 vs 3.01 ± 0.22) $VO_{2\,max}$ in ALK compared to CON, respectively. RER was also significantly higher during ALK at both 60 and 75% $VO_{2\,max}$ (Table 8). V_E increased similarly between conditions (Table 8).

TABLE 1 - Muscle glycoen and glycolytic intermediate contents in vastus lateralis at rest, post-ingestion and during cycle ergometry at 30%, 60% and 75% VO_{2max} after either CONTROL or ALK

MEASURE (mmol•kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Glycogen	CON	479.7 ± 41.7	485.3 ± 57.8	447.2 ± 41.7	371.1 ± 56.0*	258.4 ± 49.8*
	ALK	483.8 ± 50.8	464.7 ± 55.8	460.3 ± 50.5	328.7 ± 60.3*†	195.3 ± 61.96*†
G-1-P	CON	0.26 ± 0.03	0.23 ± 0.03	0.25 ± 0.04	0.34 ± 0.03*	0.38 ± 0.02 *
	ALK	0.25 ± 0.03	0.24 ± 0.02	0.33 ±0.02	0.35 ±0.03*	0.45 ±0.05*
G-6-P	CON	1.03 ± 0.15	1.1 ± 0.14	2.13 ± 0.28*	3.43 ± 0.42*	4.60 ± 0.43*
	ALK	1.00 ± 0.16	1.15 ± 0.14	2.27 ±0.23*	3.30 ±0.21*	3.93 ± 0.33*†
F-6-P	CON	0.22 ± 0.02	0.21 ± 0.03	0.32 ± 0.04	0.56 ± 0.08*	0.74 ± 0.06*
	ALK	0.19 ± 0.03	0.21 ± 0.03	0.4 ±0.12	0.54 ±0.11*	0.62 ±0.08*†
Glucose	CON	1.82 ± 0.14	1.96 ± 0.25	2.21 ± 0.20	3.36 ± 0.49*	6.79 ± 1.38*
	ALK	1.17 ± 0.21	1.37 ± 0.31	2.67 ±0.38	4.19 ± 0.70*	6.94 ± 1.33*
G-3-P	CON	0.91 ± 0.17	0.99 ± 0.14	0.88 ± 0.17	2.14 ± 0.39*	4.22 ± 0.97*
	ALK	0.65 ± 0.10	1.11 ± 0.14	1.09 ± 0.21	3.03 ± 0.50*	4.23 ± 1.10*

Data are means ± SE; n= 8.

* Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 2 - Muscle acetyl group content in vastus lateralis at rest, post -ingestion and during cycle ergometry at 30%, 60% and 75% VO_{2max} after either CONTROL or ALK

MEASURE (mmol·kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	VO_{2MAX} 30 %	60 %	75%
Free CoA, µmol/kg dw	CON	58.45 ± 5.4	57.6 ± 5.4	63.8 ± 3.8	61.6 ± 6.5	58.5 ± 6.2
	ALK	69.0 ± 6.3	65.3 ± 6.8	62.5 ± 4.9	60.3 ± 7.2	59.2 ± 6.9
Acetyl CoA	CON	4.6 ± 0.4	7.0 ± 1.1	8.1 ± 1.0	13.3 ± 1.3*	20.2 ± 2.1*
	ALK	5.6 ± 0.4	5.9 ± 0.7	8.0 ± 0.7	11.6 ± 0.9*†	16.4 ±1.9*†
Total CoA, µmol/kg dw	CON	62.99 ± 5.37	64.53 ± 5.68	71.9 ± 4.04	74.55 ± 7.64	78.75 ± 7.17
	ALK	74.54 ± 6.30	71.14 ± 6.99	70.39 ± 4.98	73.88 ± 7.11	75.6 ± 7.68
Acetyl CoA:CoA	CON	0.08 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.23 ±0.01	0.37 ±0.04
	ALK	0.08 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	0.23 ±0.03	0.26 ±0.02†
Acetlycarnitine	CON	1.6 ± 0.42	2.57 ± 0.80	3.9 ± 0.68*	10.02 ± 1.37*	13.55 ± 1.53*
	ALK	1.55 ± 0.35	2.56 ± 0.64	4.09 ± 0.58*	11.05 ± 1.33*	13.66 ± 1.30*
Total Carnitine	CON	18.04 ± 1.02	18.7 ± 1.15	19.68 ± 1.23	20.12 ± 1.28*	19.94 ± 1.41*
	ALK	19.17 ± 0.96	19.26 ± 0.87	18.95 ± 0.82	20.1 ± 1.09	21.29 ± 1.15*
Free Carnitine	CON	16.16 ± 0.85	15.57 ± 0.74	14.93 ± 0.89	10.17 ± 1.20*	5.39 ± 0.77*
	ALK	17.08 ± 0.88	16.41 ± 0.71	14.36 ± 0.83*	7.94 ± 1.23*†	6.42 ± 1.18*

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 3 - Muscle high energy phosphate content in vastus lateralis at rest, post -ingestion and during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK

MEASURE (mmol·kg^{-1} dw)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
ATP	CON	23.91 ± 0.64	24.75 ± 0.75	25.91 ± 1.00	25.68 ± 0.98	24.18 ± 0.65
	ALK	25.72 ± 0.75	25.52 ± 0.57	25.88 ± 0.66	24.67 ± 0.75	24.13 ± 0.59
ADP_f (umol·kg^{-1} dw)	CON	136.31 ±14.47	----	120.07 ± 15.36	193.06 ± 17.27	302.1 ± 46.25*
	ALK	137.76 ± 15.06	----	137 ± 13.04	295.76 ± 37.8*†	452.6 ± 55.2*†
AMP_f (umol·kg^{-1} dw)	CON	0.8 ± 0.17	----	0.56 ± 0.13	1.43 ± 0.23	4.00 ± 1.03*
	ALK	0.76 ± 0.16	----	0.72 ± 0.12	3.80 ± 1.08*†	9.03 ± 2.09*†
Intramuscular [H^+]	CON	63.6 ± 0.61	63.4 ± 0.57	92.0 ± 0.72	113.1 ± 5.5*	160.5 ± 13.2
	ALK	62.8 ± 0.30	63.5 ± 0.38	94.0 ± 0.98*	131.9 ± 10.9†	182.0 ± 20.2†

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 4 - Muscle pyruvate production, oxidation, and lactate production at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK. Values are for single leg only.

MEASURE (mmol·kg^{-1} min^{-1} dw)	TRIAL	$VO_{2\,MAX}$ 30 %	60 %	75%
Pyruvate production	CON	7.66 ± 1.32	15.17 ± 0.84**	21.53 ± 1.58**
	ALK	7.80 ± 0.88	21.99 ± 1.15**†	24.26 ± 1.87**†
Lactate production	CON	1.28 ± 0.22	5.20 ± 1.04**	8.35 ± 1.23**
	ALK	2.12 ± 0.39	4.86 ± 0.63**	13.71 ± 1.86**†
Pyruvate oxidation	CON	5.65 ±0.61	9.97 ± 0.70**	13.18 ± 1.57**
	ALK	5.37 ± 0.88	17.14 ± 1.45**†	11.68 ± 1.29**
% Pyruvate Oxidized	CON	70 ± 2	67 ± 6	66 ± 2
	ALK	70 ± 4	80 ± 3**†	58 ± 2

Data are means ± SE; n= 8.

** Significantly different from first workload of same condition.

† Significantly different from matched times between conditions.

TABLE 5 - Arterial concentration of blood born substrates during rest, post-ingestion and cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK

MEASURE ($mmol \cdot l^{-1}$)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Glucose	CON	5.86 ± 0.32	5.13 ± 0.13*	5.18 ± 0.21*	5.07 ± 0.21*	4.86 ± 0.26*
	ALK	5.82 ± 0.51	5.31 ± 0.54*	5.07 ± 0.22*	4.81 ± 0.29*	4.93 ± 0.14*
Whole Blood Lactate	CON	0.79 ± 0.18	0.5 ± 0.05	1.62 ± 0.21*	3.99 ± 0.44*	7.00 ± 0.39*
	ALK	0.94 ± 0.16	0.61 ± 0.07	1.94 ± 0.13*	4.8 ± 0.45*†	8.72 ± 0.57*†
Plasma Lactate	CON	0.82 ± 0.24	0.58 ± 0.10	2.56 ± 0.35*	5.58 ± 0.61*	9.87 ± 0.88*
	ALK	1.03 ± 0.20	0.66 ± 0.04	2.93 ± 0.28*	7.42 ± 0.74*†	12.10 ± 1.09*†
Glycerol, $umol \cdot l^{-1}$	CON	22.3 ± 3.1	52.5 ± 8.21*	75.9 ± 9.9*	97.4 ± 13.6*	130.9 ± 16.5*
	ALK	25.1 ± 3.1	52.0 ± 8.9*	84.7 ± 11.1*	112.9 ± 16.8*	136.8 ± 19.7*
Plasma FFA	CON	0.32 ± 0.03	0.82 ± 0.07*	0.63 ± 0.06*	0.55 ± 0.04*	0.53 ± 0.04 *
	ALK	0.36± 0.04	0.82 ± 0.12*	0.71 ±0.09*	0.60 ± 0.08*	0.50 ± 0.05*
Venous pH	CON	7.36 ±0.01	7.36 ± 0.01	7.29 ± 0.01*	7.24 ± 0.02*	7.19 ± 0.02*
	ALK	7.36 ±0.01	7.43 ± 0.01*†	7.34 ± 0.01†	7.28 ± 0.02*†	7.24 ± 0.02*†
Venous HCO_3^- (mEq/L)	CON	25.7 ± 0.8	25.8 ± 0.5	27.6 ± 0.5	26.3 ± 0.8	23.50 ± 0.7*
	ALK	26.8 ±0.5	31.00 ± 0.9*†	31.30 ± 1.1*†	30.20 ± 1.5*†	26.50 ± 1.1†

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 6 - Leg blood flow, RQ, CO_2 production and O_2 uptake at rest, post-ingestion and during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK

MEASURE (values are for single leg)	TRIAL	Rest	Post-Ingestion	$VO_{2\,MAX}$ 30 %	60 %	75%
Blood Flow ($l \cdot min^{-1}$)	CON	0.41 ± 0.03	0.46 ± 0.04	3.10 ± 0.13*	3.92 ± 0.13*	4.58 ± 0.20*
	ALK	0.41 ± 0.05	0.41 ± 0.03	3.25 ± 0.16*	4.00 ± 0.21*	4.54 ± 0.21*
RQ	CON	0.64 ±0.09	0.84 ± 0.10*	0.95 ± 0.06*	1.05 ± 0.05*	1.08 ± 0.07*
	ALK	0.74 ±0.06	0.93 ± 0.04*	0.94 ± 0.04*	1.00 ± 0.05*	1.06 ± 0.03*
CO_2 Production ($ml \cdot l^{-1}$)	CON	31.53 ± 3.41	38.22 ± 2.71	106.04 ± 6.48*	135.59 ± 7.21*	153.58 *
	ALK	42.89 ± 2.71	47.54 ± 3.36	100.72 ± 4.65*	133.00 ± 4.90*	157.05 ± 5.62*
O_2 Uptake ($ml \cdot l^{-1}$)	CON	51.15 ± 3.93	47.34 ± 3.02	111.96 ± 3.09*	129.66 ± 2.90*	143.84 ± 5.75*
	ALK	58.7 ± 2.15	51.29 ± 3.38	107.44 ± 1.56*	133.17 ± 4.46*	148.24 ± 4.25*

Data are means ± SE; n= 8. * Significantly different from rest of same condition.

† Significantly different from matched times between conditions.

TABLE 7 - Lactate and hydrogen ion gradients from arterial to muscle and femoral venous to muscle during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK

MEASURE	TRIAL	75% $VO_{2\,MAX}$	
		Arterial - Muscle	Femoral Venous- Muscle
Lactate Gradient	CON	5.14 ± 1.85	3.71 ± 1.52
	ALK	5.06 ± 1.93	2.54 ± 2.00
H^+- Gradient	CON	110.5 ± 12.5	95.2 ± 12.9
	ALK	147.9 ± 17.3†	128.8 ± 18.2†

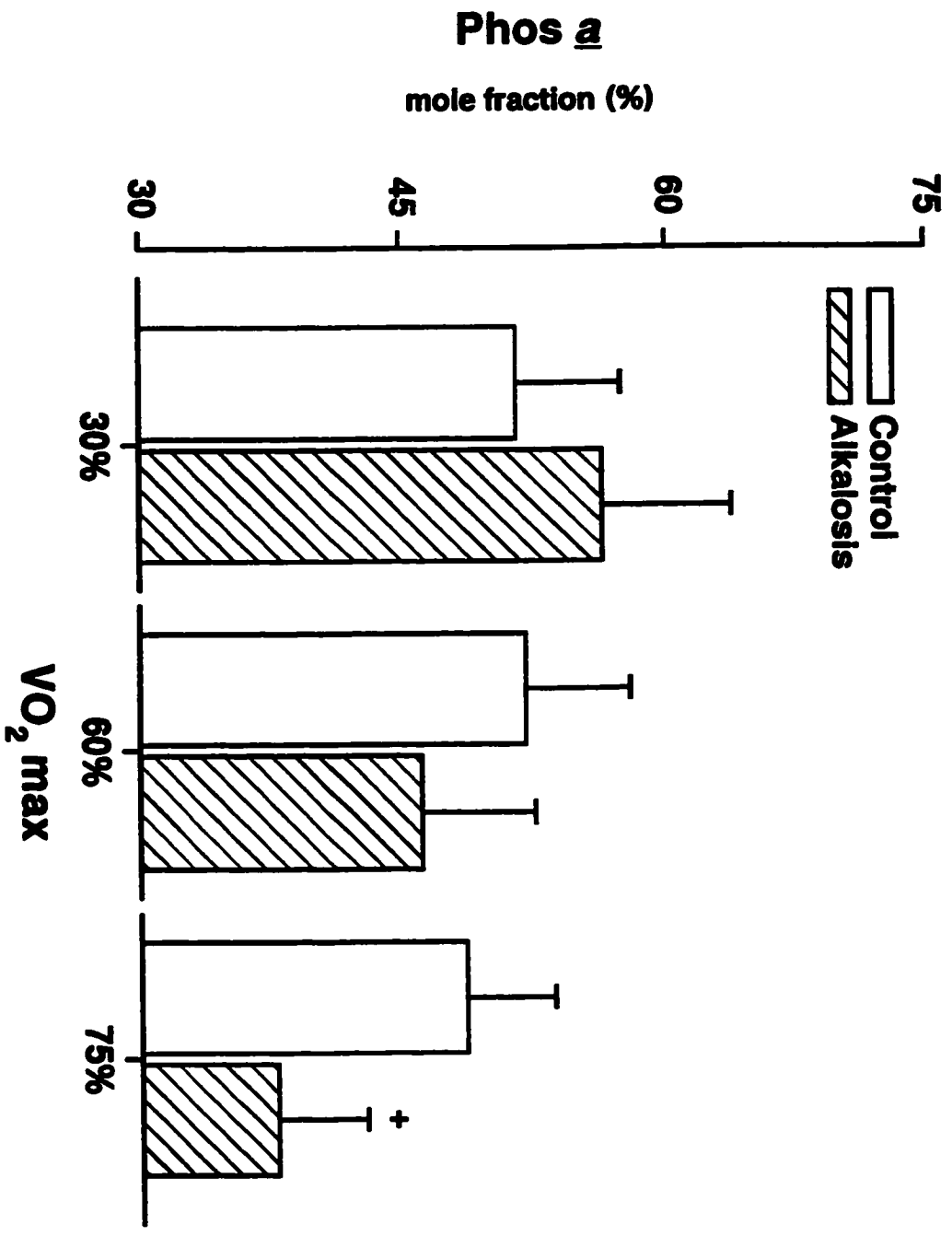
† Significantly different from matched times between conditions.

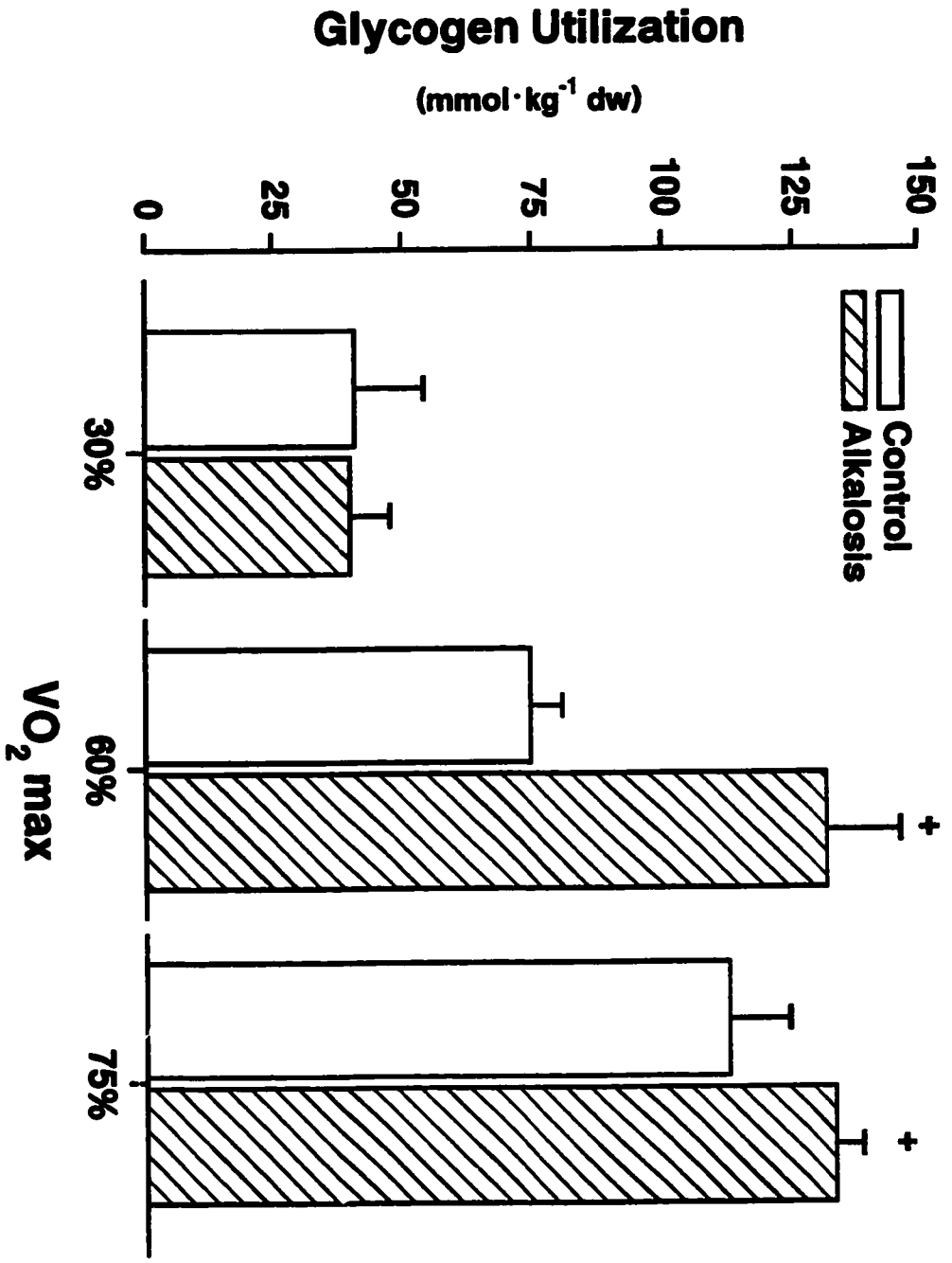
TABLE 8 - Respiratory variables during cycle ergometry at 30%, 60% and 75% $VO_{2\,max}$ after either CONTROL or ALK

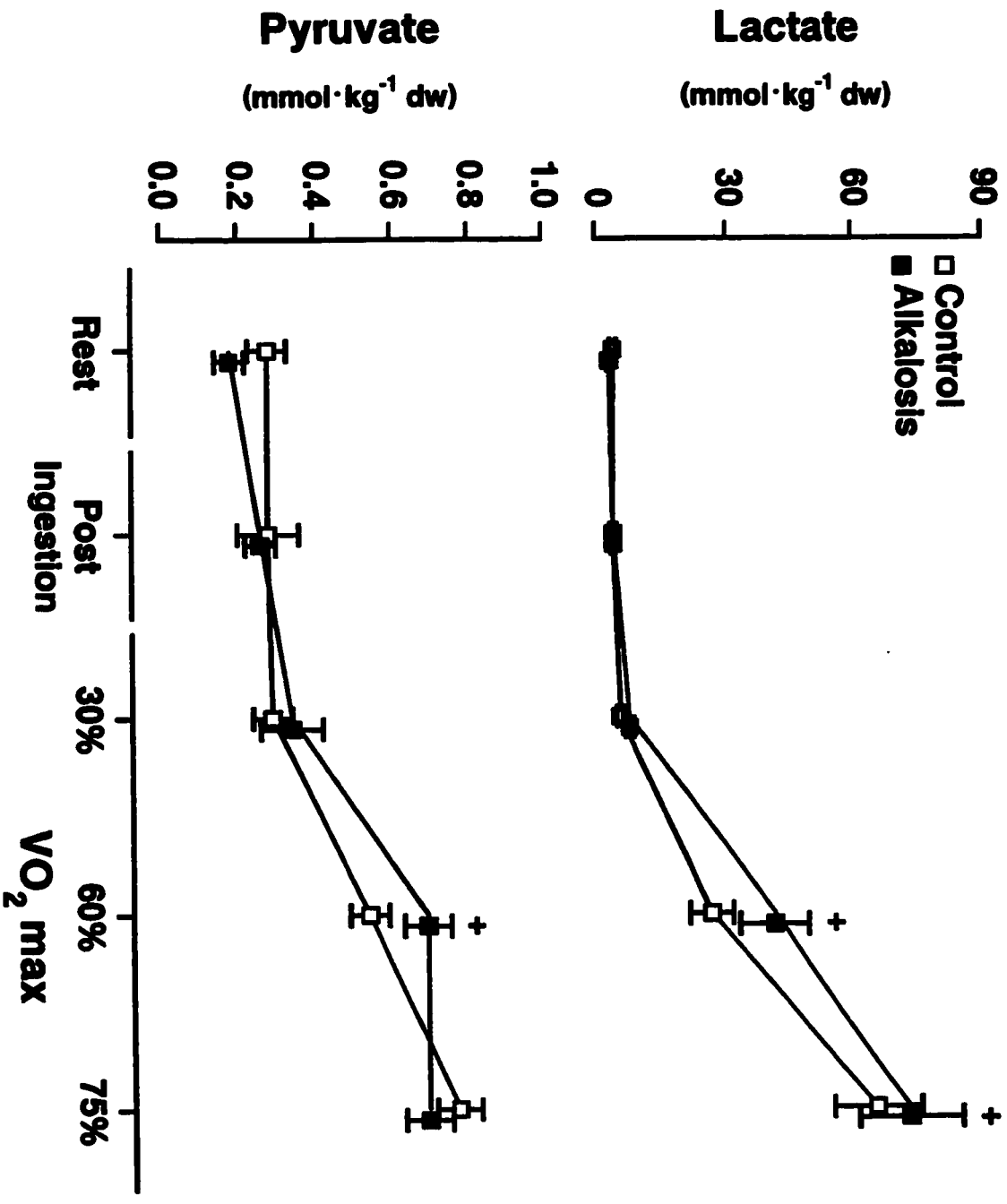
MEASURE ($l \cdot min^{-1}$)	TRIAL	$VO_{2\,MAX}$ 30 %	60%	75%
RER	CON	0.94 ±0.02	1.00 ± 0.03**	1.08 ± 0.04**
	ALK	0.96 ± 0.02	1.05 ± 0.02**†	1.13 ± 0.03**†
V_E	CON	36 ±1.1	64.1 ± 3.1**	94.5 ± 6.1**
	ALK	34.4 ± 1.7	61.8 ± 3.2**	90.3 ± 7.3**
VO_2	CON	1.39 ± 0.04	2.26 ± 0.07**	2.79 ± 0.14**
	ALK	1.41 ± 0.06	2.28 ± 0.09**	2.83 ± 0.16**
VCO_2	CON	1.31 ± 0.06	2.28 ± 0.13**	3.01 ± 0.22**
	ALK	1.36 ± 0.06	2.39 ± 0.09**†	3.18 ± 0.11**†

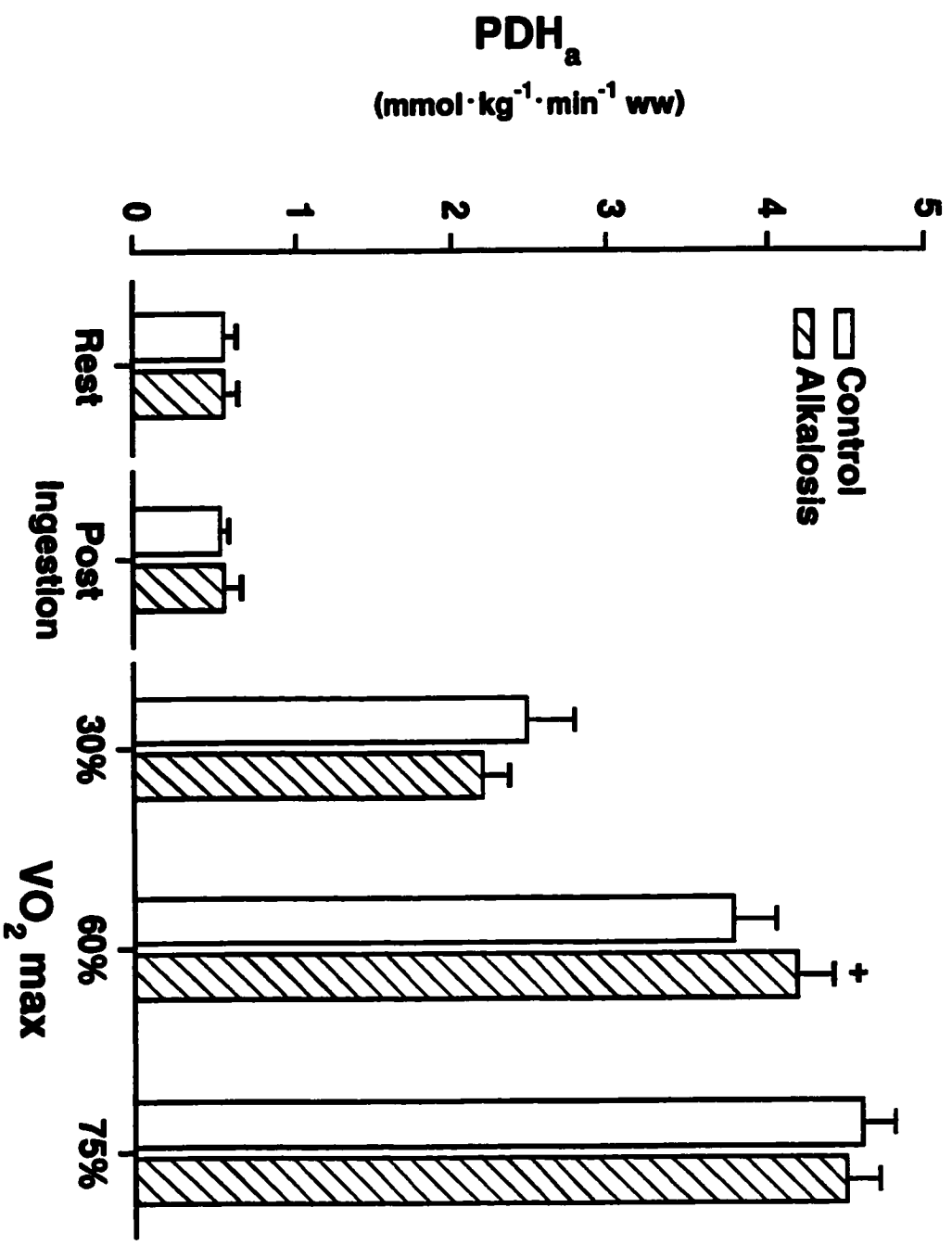
Data are means ± SE; n= 8. ** Significantly different from 30% of same condition.

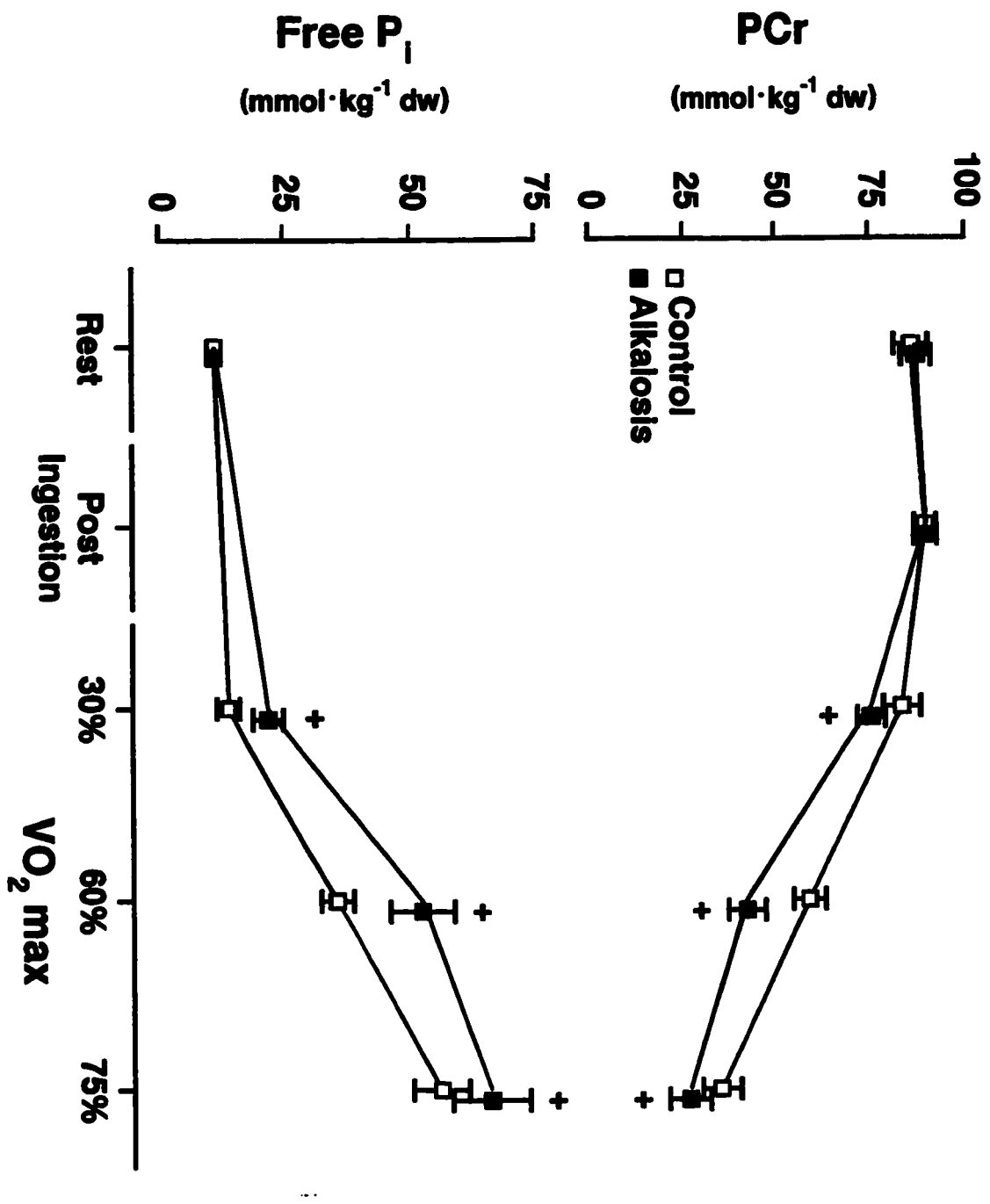
† Significantly different from matched times between conditions.

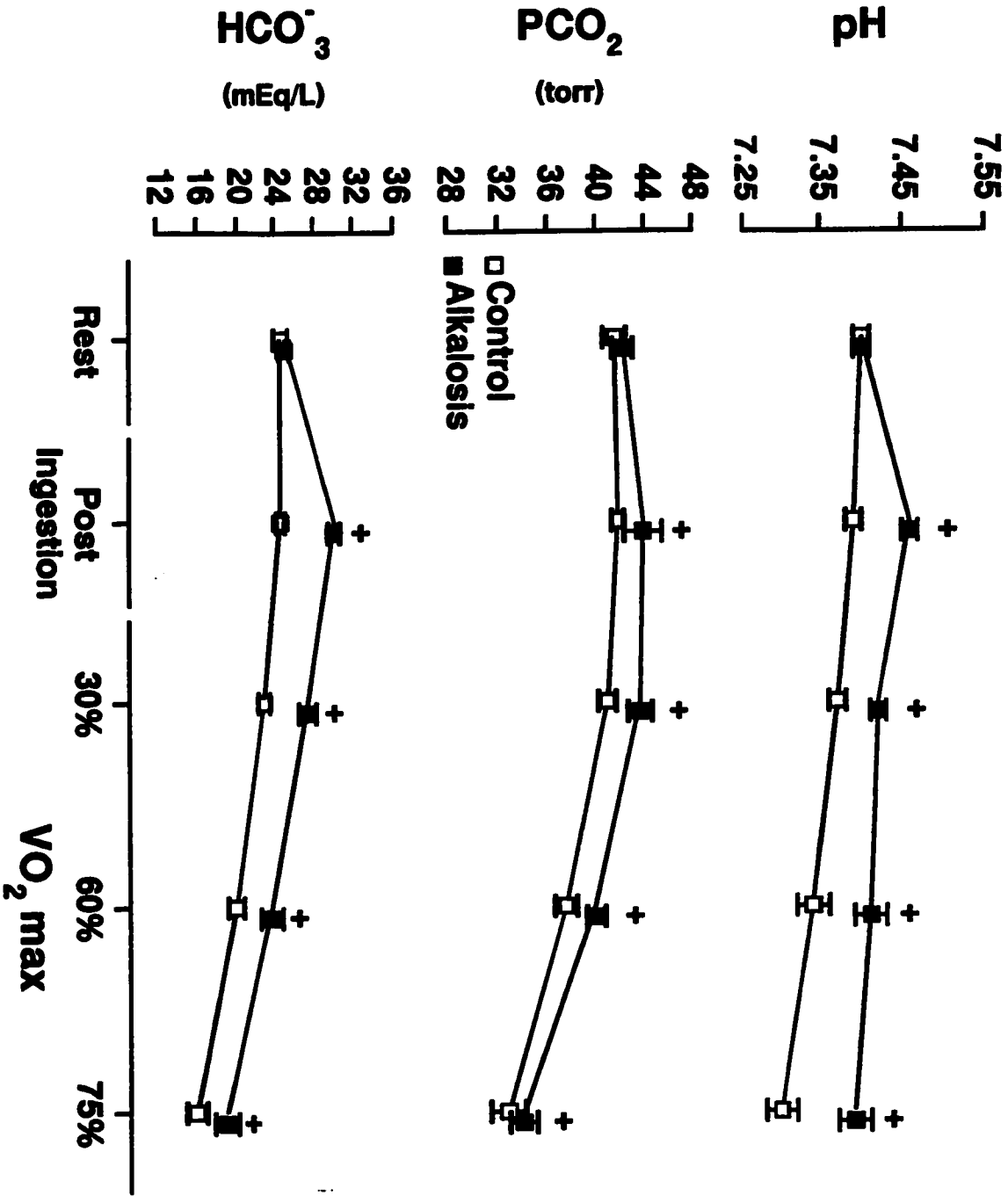

Phos a
mole fraction (%)
30
45
60
75
Control
Alkalosis
30%
60%
75%
+
VO2 max

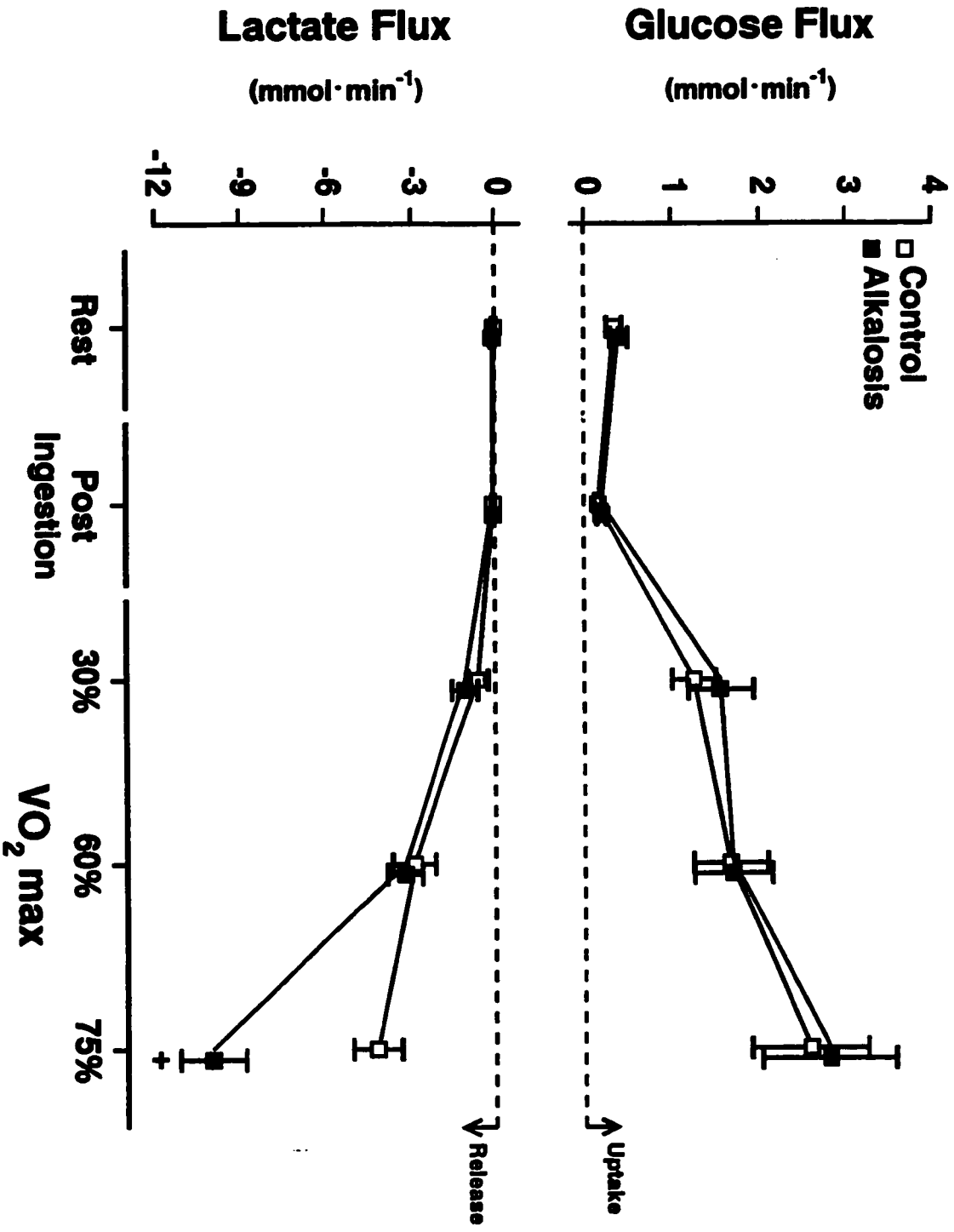

Glycogen Utilization
(mmol·kg⁻¹ dw)
0
25
50
75
100
125
150
Control
Alkalosis
30%
60%
75%
VO2 max
+
+

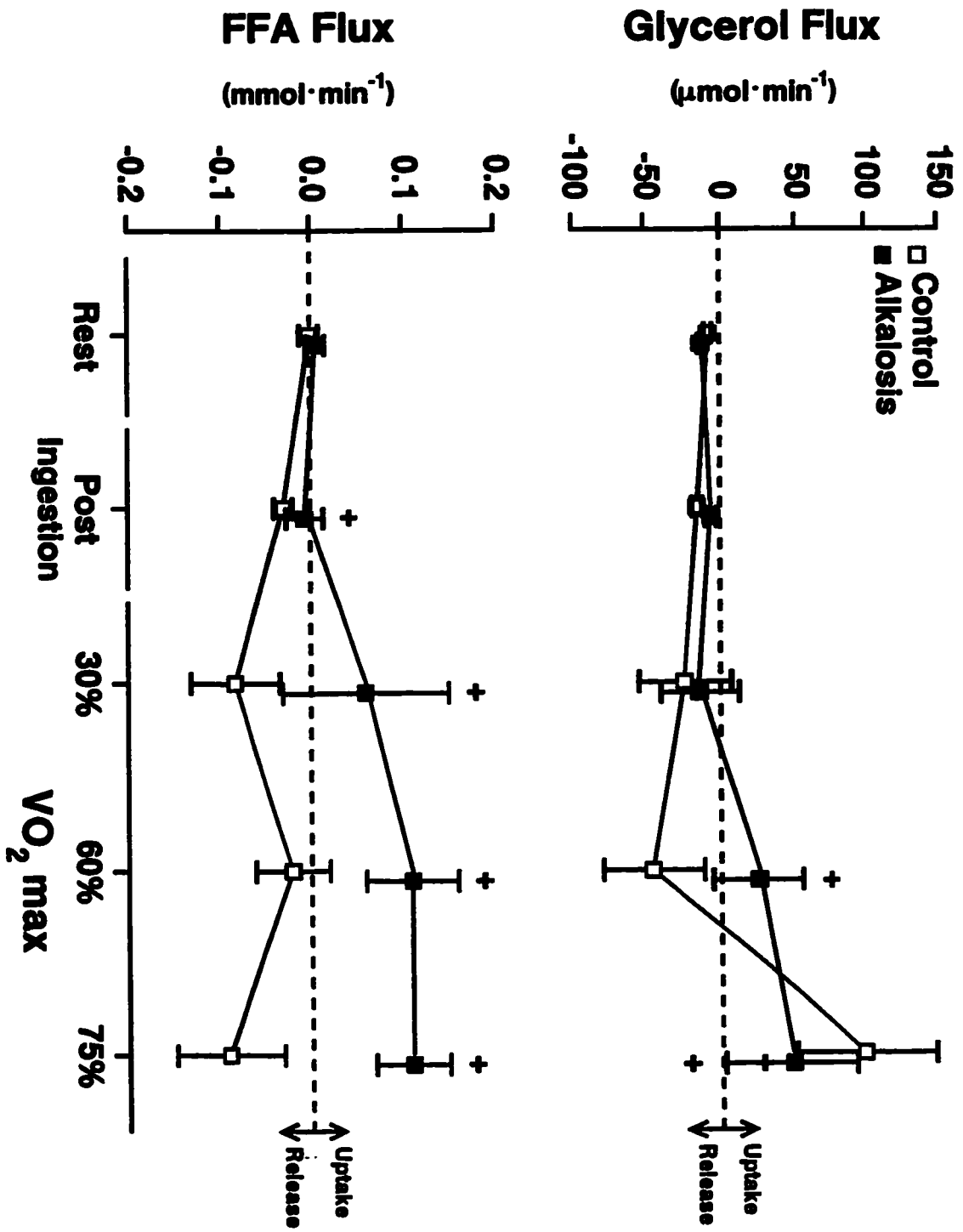

Pyruvate
(mmol·kg⁻¹ dw)
0.0
0.2
0.4
0.6
0.8
1.0
Lactate
(mmol·kg⁻¹ dw)
0
30
60
90
Control
Alkalosis
Rest
Post Ingestion
30%
60%
75%
VO2 max
+
+
+


PDH$_a$
(mmol·kg^{-1}·min^{-1} ww)
0
1
2
3
4
5
Control
Alkalosis
Rest
Post Ingestion
30%
60%
75%
VO$_2$ max
+


Free P_i
(mmol·kg^{-1} dw)
0
25
50
75
PCr
(mmol·kg^{-1} dw)
0
25
50
75
100
Control
Alkalosis
Rest
Post Ingestion
30%
60%
75%
VO_2 max


HCO3
(mEq/L)
12
16
20
24
28
32
36
PCO2
(torr)
28
32
36
40
44
48
pH
7.25
7.35
7.45
7.55
Control
Alkalosis
Rest
Post Ingestion
30%
60%
75%
VO2 max


Lactate Flux
(mmol·min-1)
Glucose Flux
(mmol·min-1)
-12
-9
-6
-3
0
0
1
2
3
4
Control
Alkalosis
Release
Uptake
Rest
Post Ingestion
30%
60%
75%
VO2 max


Glycerol Flux
(μmol·min-1)
-100
-50
0
50
100
150
Control
Alkalosis
Uptake
Release
FFA Flux
(mmol·min-1)
-0.2
-0.1
0.0
0.1
0.2
Uptake
Release
Rest
Post Ingestion
30%
60%
75%
VO2 max

3.5 DISCUSSION

The present study examined the effects of induced alkalosis on the metabolic responses in exercising muscle during continuous, dynamic, constant rate exercise at three power outputs - 30, 60, and 75% $VO_{2\ max}$. The main effects of alkalosis during this type of exercise occurred during the two higher power outputs and included: an enhanced glycogen utilization with concomitant increase in pyruvate production; increased intramuscular lactate accumulation; enhanced lactate efflux from the exercising leg and a greater relative activation of Phos than PDH_a.

Lactate production within the muscle is dependent on the balance between the rates of pyruvate production and oxidation. Intramuscular lactate is formed from pyruvate by the action of the near-equilibrium enzyme LDH (47). Although greater pyruvate production was observed at the two highest power outputs, lactate production was elevated over CON values only during 75% $VO_{2\ max}$ (Table 4). Lactate production at the highest power output resulted from a significant degree of mismatch between the rates of glycogenolysis/glycolysis and maximal PDH_a activation. At 60% VO_{2max} the absence of an increase in lactate production despite an increased pyruvate production resulted from an enhanced pyruvate oxidation relative to production due to greater PDH_a with ALK. These changes resulted from the effect of alkalosis on the rate limiting enzymes Phos, PFK and PDH. The main control points for glycogenolysis/glycolysis involve Phos and PFK respectively, while entry into the oxidative pathway is controlled by PDH (47).

3.5.1 Regulation of Glycogen Phosphorylase

Phos is the flux generating enzyme responsible for glycogenolysis within skeletal muscle and is subject to both covalent and allosteric regulation. Phos *a* is considered the active form, active in the absence of AMP_f, while Phos *b*, the less active form, requires AMP_f (12). Covalent *b* to *a* transformation is mediated by phosphorylase kinase *a* which is activated by either an increase in epinephrine or cytosolic [Ca^{2+}] via cAMP dependant and independent mechanisms respectively (54). Post-transformational allosteric activation of Phos *b* is mediated by AMP and IMP, while inhibition is mediated by ATP and G-6-P. Substrate regulation of both forms of Phos by the free P_i and glycogen concentrations is equally important (12,18)

Phos *a* was significantly reduced at the highest power output with ALK. This reduction presumably reflects the inhibition of phosphorylase kinase *a* by the increased [H^+] (Table 3). Previous studies have demonstrated this relationship between [H^+] and Phos *a* in intensely exercising muscle (13). However, the mole fraction of Phos in the *a* form is not the sole determinant of glycogenolytic flux, as previous studies examining the relationship between Phos *a* transformation and glycogenolysis have demonstrated (14,18,34,54) The present results of a higher glycogen utilization at 75% $VO_{2\ max}$ (133 $\pm$ 6 vs 113 $\pm$ 12) (Fig. 3) despite a lower Phos *a* (Fig. 2) as well as the observed greater glycogen utilization (132 $\pm$ 15 vs 75 $\pm$ 6) (Fig. 3) despite similar Phos *a* (Fig. 2) at 60% $VO_{2\ max}$ with ALK

are in agreement with these previous studies. Additionally, there may have been a hormonal effect throughout exercise due to a decrease in the circulating [epinephrine] with ALK which may have contributed to the lower observed Phos *a*. Bouissou and colleagues (10) observed a 34% reduction in the plasma [epinephrine] and higher intramuscular [Lac^-] with alkalosis in humans who cycled to exhaustion. The enhanced glycogenolysis despite similar or lower Phos *a* transformation with ALK likely resulted from post-transformational modulation by increases in the AMP_f and IMP concentrations and an increase in the concentration of its substrate free P_i.

AMP and IMP have been shown to stimulate Phos *a* (2,54) and Phos *b* (2,15). AMP acts on Phos *a* by reducing the K_m for P_i from 26.2 mM to 11.8 in the presence of as little as 0.01 mM AMP (2,54). AMP acts in a similar manner on Phos *b* but with a higher K_m. Both the [AMP_f] and [P_i] were significantly greater with ALK during both the second and third power outputs and were well above the required concentrations for activation (Table 3) (Fig. 6). In support of the close relationship between Phos activity, glycogenolytic flux and the [AMP_f], other studies utilizing caffeine ingestion (17), increased FFA availability (24,48), and short-term training (16) have demonstrated glycogen sparing during exercise, associated with blunted AMP_f accumulation. The increased [AMP_f] may have augmented activation of both Phos *a* and Phos *b*. IMP activates Phos *b* with a K_m of 1.2 mM (2). The [IMP] was not measured in the present study, but has been shown previously to increase with exercise and when [ADP_f] increases (64). The [ADP_f] was significantly

elevated with ALK with may have led to an increase in the [IMP], which may have activated Phos *b*.

ATP and G-6-P are both inhibitors of Phos *b*. ATP has a K_i of ~ 2 mM and to G-6-P of ~ 0.3 mM (25). In the present study the [ATP] remained constant throughout exercise and between conditions at levels above the K_i. The [G-6-P] was significantly lower with ALK at the highest exercise intensity but remained above the K_i. These combined results should favor a reduction in Phos activity and glycogenolysis. However, it has been previously demonstrated that the inhibition of Phos *b* by both ATP and G-6-P can be overcome when the [AMP] increases sufficiently (18). In addition, previous studies have demonstrated that glycogenolytic flux is closely tied to the availability of its substrate free P_i and the allosteric regulator AMP_f, both of which were elevated with ALK (16,17).

In summary, Phos activity and therefore glycogenolytic flux results from the combination of covalent, allosteric and substrate regulation. During ALK, despite a lower transformation of Phos *a*, glycogenolytic flux was enhanced and glycogen utilization increased during the second and third power outputs due to the maintenance of flux through post-transformational allosteric activation of Phos *a* + *b* by an increased [AMP_f], and possibly [IMP], and an increase in the concentration of its substrate free P_i.

3.5.2 Regulation of Phosphofructokinase (PFK)

PFK plays a key role in the regulation of glycolysis and therefore pyruvate production. PFK catalyzes the conversion of F-6-P to fructose 1, 6 - bisphosphate

utilizing ATP (47), with the relative enzyme activity reflected by changes in the [F-6-P] and [G-6-P] with which it is in equilibrium. PFK is subject to regulation by a large number of metabolites that function to either inhibit or activate the enzyme complex. ATP, H^+ inhibit, while ADP, AMP, P_i , and F-6-P activate, with the net enzyme function resulting from the combination of these inputs (66). These are the most potent regulators, which reflect the energy state and fuel utilization within the cell and thereby provide feedback regulation to adjust glycolytic flux.

The [ATP] remained constant between trials and across power outputs which provided a small degree of inhibition. As well, at the highest exercise intensity the intramuscular [H^+] was significantly elevated for both trials (Table 3), which would provide inhibition as previous *in vitro* studies utilizing constant [ATP] with declining pH have demonstrated (22,65). Human exhaustive exercise protocols have found similar changes reflecting reductions in PFK activity with reduced intramuscular [H^+] (36,59). The magnitude of the pH inhibition can be modulated by increases in the [F-6-P] and the activators ADP, AMP and free P_i. Alkalosis led to increases in the [F-6-P] and [G-6-P] during the third power output but to a significantly lower magnitude than CON. At this power output, pH_i was significantly lower, compared to CON, but may have failed to inhibit PFK activity due to positive modulation by the significantly elevated [AMP_i], [ADP_i], and [P_i]. AMP acts by augmenting PFK's affinity for its substrate F-6-P (9). The increased [F-6-P] although lower than CON, was elevated above the K_m of 0.1-0.2 mM, which could have opposed the pH inhibition by decreasing the affinity of the ATP binding site (39). The accompanying

rise in the [G-6-P] although above the K_i for Phos, may have been overridden by the positive modulation of Phos by the increased [AMP_f] and [P_f]. Previous *in vitro* studies have also demonstrated substantial acceleration of glycolysis with the lowering of the ATP/ADP ratio, a situation present with alkalosis at both 60% (CON 132 vs ALK 87) and 75% $VO_{2\ max}$ (CON 80 vs ALK 55) due to the increase in the [ADP_f] (71). The stimulatory effect of an increase in [ADP_f] on PFK activity has been shown to substantially increase with as little as a 2 mM increase in P_i, which also occurred in the present study with ALK (71).

The combined results demonstrate enhanced glycogenolytic/glycolytic flux with alkalosis due to allosteric up regulation of both Phos and PFK activity leading to the increased glycogen utilization and pyruvate production at the two highest power outputs.

3.5.3 Regulation of Pyruvate Dehydrogenase (PDH_a)

PDH_c is a mitochondrial enzyme complex that catalyzes the decarboxylation of glycolytically derived pyruvate and therefore reflects the rate of carbohydrate entry into the TCA cycle. PDH_c transformation between the active, PDH_a and inactive, PDH_b forms is regulated by the balance between PDH kinase (PDHK) (deactivating) and PDH phosphatase (PDHP) (activating) (52,68). The relative phosphatase/kinase activity is controlled by the mitochondrial acetyl-CoA/CoASH, ATP/ATP, NADH/NAD^+ ratios and the allosteric regulators Ca^{2+}, pyruvate and H^+. Increases in the ratios decrease PDH_a transformation while decreases in the ratios have the opposite effect. Increases in [pyruvate] inhibit the kinase only, increases

in [Ca^{2+}] inhibit the kinase and activate the phosphatase, and increases in [H^+] activate the phosphatase only (51,52,68). Due to the complex interaction of regulators and the observed differences in PDH_a, changes at both 60 and 75% $VO_{2\,max}$ will be discussed separately.

3.5.3.1 Changes in PDH_a at 60% $VO_{2\,max}$

In the present study PDH_a increased with power output as a result of the contraction induced increase in the [Ca^{2+}](19,21,34). However, increases in the [Ca^{2+}] cannot be the sole mechanism responsible for the increased PDH_a with ALK at this intensity since the power outputs were identical between trials. The elevated PDH_a with ALK resulted from changes in the allosteric regulators acetyl-CoA, ADP_f, H^+, pyruvate and the NADH/NAD^+ ratio.

The [acetyl-CoA] was significantly lower during this power output with ALK (Table 2) and probably reflects reduced FFA utilization in the face of increased glycogen utilization and pyruvate production (Table 4). Since acetyl-CoA inhibits PDHP, the reduced concentration would serve to activate the phosphatase and therefore contribute to the greater PDH_a observed with ALK (68).

The ATP/ADP ratio was significantly reduced with ALK (87 vs. 132) at this power output, due to a significant elevation in the [ADP_f] without changes in the [ATP] (Table 3). This ratio effects PDHK only as ATP is the substrate for the reaction and therefore competes with its product ADP which inhibits catalytic activity (68). The lower ATP/ADP ratio observed with ALK could have resulted in lower PDHK activity and therefore contributed to the greater PDH_a observed.

Hydrogen ion has been shown to activate PDH_a in acidotic perfused rat hearts (49) and has been attributed to the differences in pH optimum of PDHK and PDHP. PDHK has a pH optimum of 7.0 ~ 7.2, with increased inhibition as pH falls, while PDHP has a pH optimum of 6.7 ~ 7.1 (35). In the present study the calculated intramuscular [H^+] was significantly elevated at this power output with ALK compared to CON (Table 3) which may have resulted in greater activation of the phosphatase, and contributed to the increased PDH_a observed.

The intramuscular [pyruvate] was also significantly elevated with ALK at this power output (Fig. 4). Pyruvate is a potent stimulator of PDH_a as it is both substrate and an inhibitor of PDHK with a K_i of 0.5 - 2.0 mM (42). In the present study, the intramuscular [pyruvate] measured at this power output with ALK is below the K_i. However, the [pyruvate] was determined from a biopsy taken at the end of the exercise bout and given that both glycogen utilization and pyruvate production were significantly elevated, it is possible that the [pyruvate] rose above the K_i during the initial stages of exercise (20,51). In addition, the lactate production remained similar to CON despite the elevation in pyruvate production suggesting that most of the pyruvate made available to PDH was oxidized and therefore may not be reflected by the intramuscular [pyruvate] in the sample taken at the end of exercise. This is further supported by the observation that the amount of pyruvate oxidized relative to that produced was also significantly elevated with ALK at this power output (Table 4).

Neither the [NADH] nor the $NADH/NAD^+$ ratio was measured in the present study. However, previous studies utilizing indirect techniques have shown that the [NADH] decreases therefore the $NADH/NAD^+$ ratio declines with high intensity exercise (29,60), which would favor an increase in PDH_a. In addition, the markedly increased glycogen utilization with ALK may have led to reduced FFA utilization functioning as the "glucose-fatty acid cycle reversed" as previously demonstrated by Sidossis et al (56,57). These researchers have demonstrated in exercising humans, that the intracellular availability of CHO (rather than FFA) determines the nature of substrate oxidation when both CHO and FFA are made available during exercise. The mechanism whereby enhanced CHO availability reduces FFA oxidation is not precisely known but findings from *in vitro* studies using human tissue (61) and human exercise studies (56) point to the inhibition of long-chain fatty acids (LCFA) entry into the mitochondria by inhibition of carnitine palmitoyl-transferase I (CPT-1). The mechanism responsible for CPT-1 inhibition is not clear but may be mediated by a pH effect as it has been shown in isolated rat muscle preparation that CPT-1 is inhibited by low pH (62). In humans, the pH inhibition has recently been shown to be more sensitive than rat , with inhibition at a pH_i of ~ 6.8 (61). The reduced FFA utilization would lead to reduced intra-mitochondrial [NADH]. Cytosolic [NADH] would also decrease as NAD^+ would be required for the maintenance of glycolytic flux and would be provided from the conversion of pyruvate to Lac^-. In support of this, is the observation that intramuscular [Lac^-] increased with ALK in the absence of an increase in Lac^- production (Fig. 4,

Table 4). Since the mitochondrial and cytosolic compartments are thought to be in equilibrium, the overall result would be a reduction in PDHK activity due to decreased [NADH] and an increase in PDHP due to increased [NAD^+] and therefore contribute to the elevated PDH_a seen with ALK at this power output. The absence of a difference in both the leg RQ and mouth RER reflecting a change in the relative CHO and FFA utilization is not surprising. Previous authors have demonstrated the lack of sensitivity of both measures in detecting small changes in fuel utilization during high intensity exercise (50).

In summary, the significant increase in PDH_a observed with ALK at 60% $VO_{2\,max}$ can be attributed to the combined inhibitory effects of a decrease in The [acetyl-CoA], an increase in the ADP_f, pyruvate, and Ca^{2+} concentrations on PDHK and the stimulatory effects of the elevated [H^+] on PDHP.

3.5.3.2 Changes in PDH_a at 75% $VO_{2\,max}$

At this power output PDH_a transformation was similar between conditions and reflects the attainment of maximal PDH_a (Fig. 5). Previous studies have shown maximal activation at this power output (34,50). The increased pyruvate production with ALK resulted from a slightly higher glycogen utilization. The absence of a difference in the relative pyruvate oxidation rates between conditions was due to the similar rates of PDH_a. The higher lactate production rate observed with ALK resulted from the higher glycogenolytic/glycolytic rate. However, at this power output with ALK the major fate of the lactate produced was efflux from the muscle and *not* intramuscular accumulation, which will be discussed later.

3.5.4 Cellular Energetics

The rate of mitochondrial ATP production is regulated by oxygen (O_2) availability and the [NADH]/[NAD^+] and [ATP]/ [ADP]x[P_i] ratios (70). Oxygen availability was not limiting in the present study in either trial as both the mouth VO_2 and O_2 uptake across the leg were not different (Table 6,8). However, differences in glycogen and FFA utilization were apparent. During the two higher power outputs with ALK there was a decrease in FFA utilization as evidenced by the decreased [acetyl-CoA] and markedly higher glycogen utilization. During CON the significantly lower glycogen utilization necessitated an increase in FFA utilization to match energy production to ATP demand. The reduced FFA utilization with ALK may have decreased the mitochondrial [NADH] which would necessitate a higher [ADP_f] and [P_i] to drive oxidative phosphorylation according to the equation (70):

$$(1) \quad NADH_i + 2c^{3+} + 2\ ADP_e + 2\ P_{ie} \rightarrow NAD_i^+ + 2c^{2+} + 2\ ATP_e + H^+$$

(Where c^{3+} and c^{2+} are the oxidized and reduced forms of mitochondrial cytochrome c respectively and the subscripts i and e refer to the intramitochondrial and extramitochondrial pools of reactants respectively)

This phenomenon of an obligatory increase in the ADP_f and P_i concentrations was observed with ALK at the higher power outputs (Table 3, Fig. 6). The creatine kinase (CK) reaction and PCr play key roles in the regulation of oxidative phosphorylation and other metabolic processes as an "energy buffer" and "energy transport" system between the sites of ATP production and ATP utilization (67). The CK/PCr system is very sensitive to changes in the intracellular [ADP_f] and

serves to keep this concentration low to prevent the inactivation of cellular ATPases and the net loss of cellular adenine nucleotides (67). In addition to functioning as a 'barometer' for intracellular [ADP_i] and therefore mitochondrial respiration the CK/PCr system acts as a proton buffer since the production of ATP consumes both ADP and H^+ which are both products of ATP hydrolysis:

(2) $MgADP^- + PCr^{2-} + H^+ \approx MGATP^{2-} + Cr$

The coupling of CK with the ATPases at the site of utilization prevents the local acidification at the initiation of exercise prior to activation of glycogenolysis. The hydrolysis of PCr also liberates free P_i at the onset of exercise which is essential for the activation of glycogenolysis and glycolysis (3,67). Th increased degradation of PCr usually reflects a lack of mitochondrial derived ATP from oxidative phosphorylation (70). In the present study the increased PCr breakdown observed during ALK (Fig. 6) may have resulted from either a reduced [NADH] that accompanied a reduction in FFA utilization (Equation 1) or may have resulted from a change in the [H^+] with ALK (Equation 2). Unfortunately, which occurred first or the exact mechanism is not clear from the present results. Regardless of the mechanism it is clear that the increased degradation of PCr led to elevation in the free [P_i] which contributed to the increased glycogenolysis observed with ALK and is in agreement with previous studies examining the effect of increased FFA availability in rats (26) and humans (24) have found that the concentrations of ADP, P_i, PCr and NADH have direct effects on TCA cycle activity, mitochondrial respiration and glycogen metabolism.

3.5.5 Lactate Metabolism and Transport

Intramuscular lactate accumulation reflects the balance between the rates of lactate production and efflux from the muscle (7,38). Previous studies in humans employing metabolic alkalosis have focused on the effects of alkalosis on blood [Lac^-] and have demonstrated similar results to the present study ie. an increase in blood [Lac^-] (10,27,33,37,46,69). Only one study has investigated the effects of alkalosis on intramuscular lactate accumulation and found, as in the present study an increase in the muscle [Lac^-] (63). Only one study has examined lactate efflux during alkalosis and found similar results to the present study ie. and in increase in lactate efflux (33). However, none of the studies was able to elucidate the mechanisms responsible for these observations during induced metabolic alkalosis.

Lactate production during alkalosis at 75% VO_{2max} was increased compared to CON and resulted from the mismatch between the rates of glycogenolysis and PDH_a flux as evidenced by enhanced pyruvate production and significantly higher glycogen utilization in the absence of a difference in PDH_a between trials (Fig. 3, Table 4). The similar PDH_a between trials reflects maximal activation and is supported by the similar rates of pyruvate oxidation at this power output (Table 4). Therefore the only difference between trials at this power output was the augmented CHO utilization and thus glycogenolytic flux which greatly exceeded the maximal PDH_a flux. Previous studies have demonstrated that a mismatch does exist between the maximal rates of Phos and PDH_a at higher power outputs resulting in a significant increase in lactate production (34,51).

Intramuscular lactate accumulation is also a function of the rate of efflux from the muscle (38). ALK significantly increased arterial whole blood [Lac^-],and plasma [Lac^-] (Table 5) during both the second and third power outputs and enhanced efflux from the exercising leg during the highest power output only (Fig. 8). Blood [Lac^-] represents the balance of lactate entry from muscle and uptake by inactive tissue (7). The present results can be explained by both enhanced lactate transport out of the exercising leg and possibly reduced uptake by non-exercising tissue.

Lactate transport across the sarcolemma occurs via a monocarboxylate lactate-proton co-transport protein, and as such is the rate limiting step in lactate efflux (38,45). Kinetic studies of the transporter using isolated sarcolemmal vesicle preparations have shown it to have a high affinity for L-lactate and to be sensitive to changes in both the Lac^- and H^+ concentration gradients (38).

In the present study with ALK there was an increase in the intramuscular [Lac^-] but the Lac^- concentration gradient between the muscle and both the arterial plasma and femoral venous plasma compartments was similar between conditions (Table 7). Therefore the enhanced lactate efflux observed with ALK was not a function of the increase in intramuscular the [Lac^-]. This means that some other factor likely has an effect on the (8). The most plausible effector is H^+ as the [H^+] gradient between muscle and both the arterial and femoral venous blood were significantly elevated with ALK (Table 7)(38,40). ALK also induced a significant elevation in the extracellular [HCO_3^-] which may have contributed to the enhanced lactate efflux from the muscle. The importance of external [HCO_3^-] on lactate efflux

has been demonstrated in isolated muscle preparations with low external [HCO_3^-] yielding reduced lactate efflux (32,43,58). In addition, studies employing metabolic alkalosis in exercising humans (10,27,33,37,46,63,69) have consistently demonstrated increases in the appearance of lactate in the plasma when accompanied by increases in [HCO_3^-]. During ALK in the present study, the extracellular [HCO_3^-] was significantly elevated in both arterial and femoral venous blood at rest post-ingestion, and remained elevated throughout each of the three power outputs, which is an agreement with the above evidence (Table 5). Additionally, decreased lactate uptake by inactive tissue may have contributed to the higher arterial [Lac^-] with ALK. Uptake by inactive tissue has been shown to occur during exercise (28). Normally with exercise , the increased blood [H^+] and [Lac^-] create an inwardly directed [H^+] and [Lac^-] gradient which facilitates uptake into inactive tissue. However, alkalosis decreases the [H^+] gradient and therefore may reduce uptake compared to control conditions.

3.5.6 Summary and Conclusions

Induction of a metabolic alkalosis results in a complex series of metabolic effects during exercise reflecting changes in the activity of key regulatory enzymes and fuel utilization. The main findings of the study demonstrate that alkalosis during 60% $VO_{2\,max}$ leads to increased glycogen utilization and pyruvate production as a result of post-transformational allosteric activation of Phos mediated by increases in the ADP_f, AMP_f and free P_i concentrations. Greater PDH_a transformation also occurs with alkalosis at this moderate intensity as a result of the combined inhibitory

effects of a decrease in [acetyl-CoA]; an increase in [ADP_f], [pyruvate] and [Ca^{2+}] on PDHK and the stimulatory effects of an increase in [H^+] on PDHP. The net result is an enhanced pyruvate oxidation and therefore a lack of an increase in Lac^- production. The increase in intramuscular [Lac^-] observed with ALK at this power output results from the necessary re-generation of cytosolic NAD^+ to maintain glycolytic flux in the face of markedly increased CHO utilization.

High intensity exercise (75% $VO_{2\ max}$) with metabolic alkalosis leads to significantly increased Lac^- production, intramuscular accumulation and efflux. The increased lactate production and increased intramuscular accumulation results from the absence of down-regulation of glycogenolysis and glycolysis that typically occurs as pH_i declines. Instead, the increased ADP_f, AMP_f and free P_i concentrations competed with and/or negated the pH effect, resulting in the maintenance of glycogenolysis and therefore pyruvate production. However, the glycogenolytic rate exceeded the maximal PDH_a rate, resulting in the increased lactate production.

The increased blood [Lac^-] accompanying alkalosis likely results from the effects of an altered [H^+] gradient on the transporter; and not due to changes in the [Lac^-] gradient. Reduced uptake of lactate by inactive tissue may also have contributed to the increased arterial [Lac^-] with alkalosis, but, this contribution was not assessed in the present study. The present data demonstrate that the increased blood lactate concentration commonly observed with metabolic alkalosis results from a complex series of events that modulate the activities of the key regulatory enzymes Phos, PFK and PDH_a.

3.6 References

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CHAPTER 4

GENERAL DISCUSSION

4.1 Overview

The preceding chapters have outlined the current knowledge regarding the effect of changes in extracellular pH on the appearance of lactate in the blood and the known effects of H^+ on the activity of the key flux-generating and regulatory enzymes of metabolism - Phos, PFK, and PDH_c. However, the majority of studies examining the effects of an extracellular acidosis or alkalosis on the appearance of lactate in the blood have measured only the blood lactate concentration and/or performance. In addition, many of the exercise protocols utilized in previous studies have resulted in differences in total work, time to fatigue and therefore differences in the ATP turnover rate between conditions. This has made it difficult to determine if the results were due to the changes in pH or simply the exercise protocol. The mechanisms responsible for the change in the appearance of lactate in the blood and exercise performance with acidosis or alkalosis have been suggested to result from a change in the intramuscular lactate production rate secondary to alterations in the catalytic activities of the rate-limiting enzymes of metabolism. However, this explanation has been inferred from blood measurements only. No human skeletal

muscle *in vivo* studies have been conducted to examine the mechanisms responsible for the changes in blood lactate concentration with acidosis or alkalosis. In addition, because H^+ is involved in a multitude of reactions and can effect the catalytic activity of key rate-limiting enzymes, the potential exists that fuel utilization may also be altered with acidosis and alkalosis.

The preceding two chapters presented new, original research examining the effects of an extracellular metabolic acidosis (chapter 2) and alkalosis (chapter 3) on :

1) the activity of Phos and PDH_a and their respective metabolic regulators,
2) the accumulation of glycolytic intermediates and the pyruvate production rate,
3) the intramuscular glycogen utilization and lactate accumulation, to derive lactate production and oxidation rates,
4) lactate flux from the exercising leg and
5) carbohydrate and fat utilization

Measurement of the arterial, femoral venous, and intramuscular compartments and their constituents allowed the examination of the mechanisms responsible for the alteration in the appearance of lactate in the blood under conditions of acidosis and alkalosis.

4.2 The Integration and Significance of Findings

The present research is in agreement with the numerous previous studies that have reported decreases in blood lactate concentration with acidosis (9,13,14,18,33) and increases with alkalosis (3,9,13,18,19,33,35). However, the two present studies extend the knowledge by delineating the mechanisms responsible for the changes in the blood lactate concentration. Previous authors (9,13,33) have speculated that alterations in the lactate production rates might play a role and that there may be a direct pH effect on the lactate transporter. The present research confirms that acidosis and alkalosis decrease and increase the lactate production rates respectively. The present data show that the intramuscular lactate concentration is lower with acidosis and higher with alkalosis which is consistent with the two previous human studies measuring this metabolite in muscle biopsy samples under these conditions (3,33). The intramuscular lactate concentration represents the difference between lactate production and lactate efflux. Lactate production and intramuscular lactate accumulation is in turn reflected by the difference between the rate of pyruvate production from glycolysis and pyruvate oxidation via PDH_a.

Pyruvate production, oxidation, Phos flux, glycolysis and PDH_a activity were all affected by a change in the extracellular pH. Acidosis and alkalosis both had effects on Phos *a* transformation and the concentration of its allosteric modulators resulting in changes in glycogen utilization. Phos *a* transformation was reduced at the higher exercise intensities under both conditions, but acidosis also resulted in

significantly lower accumulation of the activators AMP_f and free P_i. This combination led to a marked reduction in glycogenolysis with acidosis. This is in agreement with previous studies that have examined Phos *a* transformation under acidotic conditions (4). In contrast, although alkalosis resulted in a reduced Phos *a* transformation compared to control, glycogen utilization was markedly elevated. The results indicate that this occurred primarily by a post-transformational effect as alkalosis markedly elevated the concentrations of AMP_f and free P_i. This extends and supports the previous studies in humans that have observed similar correlation between glycogenolysis and the AMP_f and free P_i concentrations (5-7,21). The present study results also support the contention that Phos *a* transformation is not the sole determinant of Phos flux. Transformation is important in setting the initial rate of CHO availability for oxidative phosphorylation but thereafter Phos flux is subsequently modified based on the prevailing energy state of the cell as defined by the $[ATP]/[ADP]x[P_i]$ ratio (10,36). Table 1 lists the main effects of acidosis and alkalosis on metabolism observed in the two present studies.

As the flux generating enzyme of the glycolytic pathway Phos sets the initial rate of substrate availability in the form of glucosyl units. PFK then determines the rate of substrate progression to the pathway terminus which is reflected in the pyruvate production rate. PFK activity was not measured directly but the relative activity can be accurately inferred from the accumulation of its substrate F-6-P (20,28,30). Together with the measurement of its allosteric regulators ADP_f, AMP_f,

Table 1 - Summary of effects of metabolic acidosis and alkalosis on muscle metabolism relative to control conditions.

MEASUREMENT	ACIDOSIS	ALKALOSIS
Lactate Efflux	↓	↑
Intramuscular [lactate]	↓	↑
Pyruvate production	↓	↑
Pyruvate oxidation	↓	@ 60%↑ @ 75% ø
% Pyruvate oxidized/produced	↑	@ 60%↑ @ 75% ø
Glycogen utilization	↓	@ 60%↑ @ 75% ↑
Phos *a* transformation	↓	@ 60%↓ @ 75% ↓
PFK*	↓	↑
PDH_a	↓	@ 60%↑ @ 75% ø
ATP	ø	ø
ADP_f	↓	@ 60%↑ @ 75% ↑
AMP_f	↓	@ 60%↑ @ 75% ↑
PCr degradation	ø	@ 60%↑ @ 75% ↑
P_i	ø	@ 60%↑ @ 75% ↑
Adipose tissue lipolysis	↓	ø
Intramuscular TAG lipolysis+	↑	↓

* denotes as assessed by the [G-6-P], [G-1-P] and [F-6-P]; + denotes as assessed by glycerol and FFA flux and [acetyl-CoA]; ø denotes no difference between control and condition (acidosis or alkalosis); ↓ denotes decreased compared to control; ↑ denotes increased compared to control; @ 60% denotes second exercise intensity; @ 75% denotes highest exercise intensity

H^+ and free P_i an assessment of PFK activity was possible. The combined results of the acidosis and alkalosis studies demonstrate that increases in the H^+ concentration have an inhibitory effect on PFK which becomes much more significant if the accumulation of its positive modulators are suppressed. This phenomenon was observed particularly with acidosis when the lack of substrate supply occurred as a result of reduced Phos catalytic activity and therefore glycogenolytic flux. Alkalosis had the opposite effect. Despite increases in the intramuscular H^+ concentration the significant elevation in substrate supply due to enhanced glycogenolysis in combination with increases in the concentrations of the positive modulators ADP_f, AMP_f and free P_i were able to override the inhibitory effects of an increased intramuscular H^+ concentration and maintain glycolytic flux. The reduced glycogenolytic and glycolytic flux rates with acidosis were reflected in the observed decrease in the pyruvate production rate. Conversely, the augmented glycogenolytic and glycolytic flux rates with alkalosis were reflected in increased pyruvate production rates.

Intramuscular lactate accumulation and lactate efflux are both affected by pyruvate production and pyruvate oxidation rates. The pyruvate production rate was altered with acidosis and alkalosis by a change in the activities of both Phos and PFK as discussed above. In addition, the pyruvate oxidation rates were altered with acidosis and alkalosis. Pyruvate oxidation is determined by the rate of pyruvate decarboxylation to acetyl-CoA by PDH_a. PDH_a transformation and activity is in turn linked to the availability of substrate (pyruvate), the energy status of the

cell reflected in the ATP/ADP and NADH/NAD^+ ratios; and the degree of FFA availability/oxidation via the acetyl-CoA/CoASH ratio. PDH_a was altered by both acidosis and alkalosis. Acidosis resulted in a lower PDH_a resulting from a reduced availability of pyruvate from glycogenolysis/glycolysis and a reduced ADP_f concentration which may have led to greater relative activity of PDHK/PDHP. Paradoxically, despite the lower PDH_a the intramuscular lactate concentration and lactate production rate were lower and attributable to a closer match between the rates of pyruvate supply by glycogenolysis and pyruvate oxidation by PDH_a. In contrast, alkalosis resulted in greater activation of PDH_a at 60% $VO_{2\,max}$ and a continued increase in PDH_a to maximal levels at 75% $VO_{2\,max}$. The enhanced PDH_a with the second power output may have resulted from the combined effects of a greater pyruvate availability as the glycogenolytic rate was significantly enhanced, and the reduction in the ATP/ADP ratio due large increases in the ADP_f concentration. Together, this probably resulted in significant PDHK inhibition resulting in a greater relative activity of PDHP/PDHK and therefore PDH_a. At this intensity, the lactate production rate was similar between conditions despite the significantly greater pyruvate production rate and can be attributed to the greater pyruvate oxidation by PDH_a. Exercise at the highest intensity resulted in comparable levels of PDH_a transformation between conditions due to the attainment of maximal activation, consistent with previous research findings at this intensity (10,22). However, despite this maximal activation the lactate production rate was significantly elevated with alkalosis. Based on the present research findings this

can be attributed to the lack of a decrease in glycogenolytic flux that normally accompanies exercise at this intensity as the intramuscular H^+ concentration increases. The maintenance of a high glycogenolytic rate led to a high pyruvate production rate that was significantly greater than the maximal available PDH_a flux and led to greater lactate formation by mass action. Importantly, these two studies also illustrate that lactate production is not the result of an oxygen limitation as both the mouth oxygen uptake and the uptake across the leg were similar across all trials despite large differences in lactate production. This is consistent with recent research finding that have demonstrated that lactate efflux is unrelated to intracellular PO_2 (26).

The present research also illustrates that changes in extracellular pH can have effects on fuel selection/utilization. Acidosis and alkalosis resulted in very different fuel utilization patterns. Acidosis resulted in inhibition of adipose tissue lipolysis and glycogen degradation which shifted the emphasis of substrate supply to intramuscular TAG stores. These findings support and extend previous research regarding the inhibition of adipose tissue lipolysis with increased H^+ concentration and the concomitant shift to increased intramuscular TAG utilization in the face of reduced CHO availability and FFA availability from adipocytes (8,11-13). In contrast, alkalosis resulted in reduced FFA utilization as glycogen degradation was significantly increased. Of interest with alkalosis was the fact that the acetyl-CoA concentration did not increase despite a significantly elevated pyruvate production rate. This probably reflects an increase in the rate of oxidative

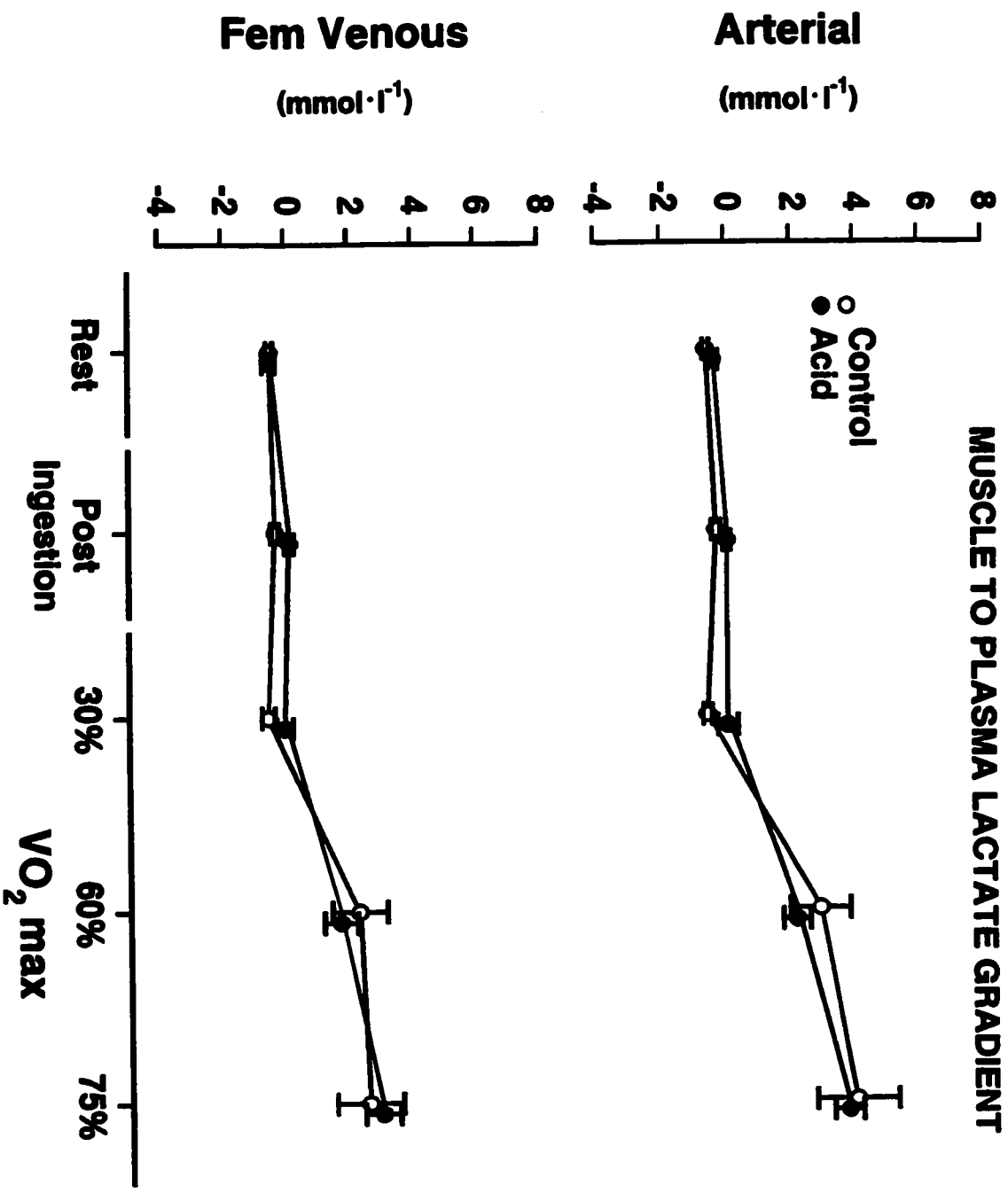
phosphorylation that may have been driven by the reduced intramuscular NADH concentration that accompanies reduced FFA utilization.

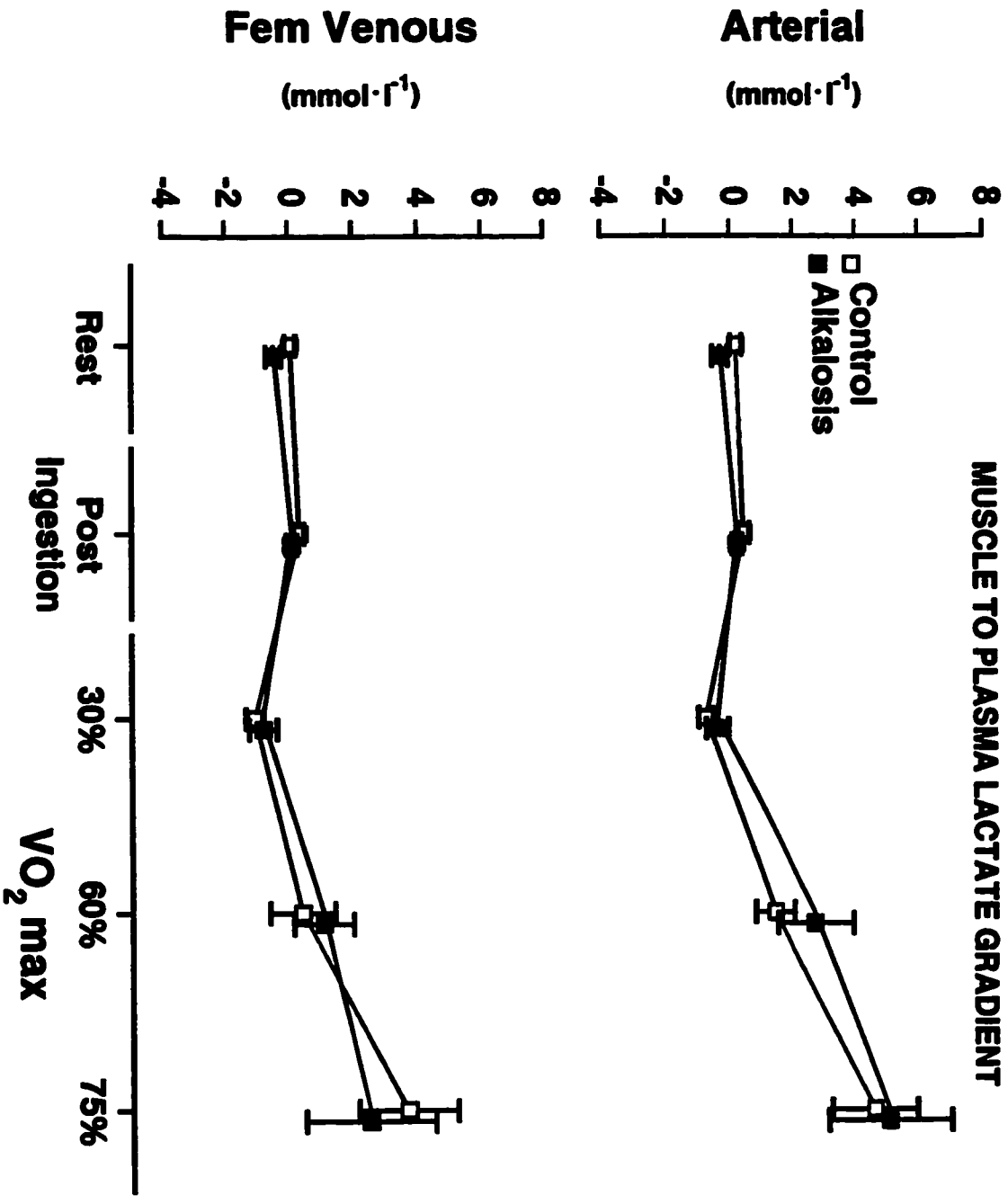
The results of the two present studies also support and extend the current knowledge concerning CHO and TAG interaction with respect to both the glucose-fatty-acid cycle during exercise as classically proposed and the reverse-glucose-fatty-acid cycle (23-25,27,29,38). Both of the present studies support the reverse-glucose-fatty-acid cycle which suggests that it is the availability of CHO that determines substrate utilization. This was observed with acidosis as the acidotic depression of CHO supply was met with augmented FFA utilization while alkalosis with the concomitant increased glycogen degradation resulted in reduced FFA utilization.

The appearance of lactate in the blood also represents the difference between lactate production rates and lactate efflux by the MCT transporter. In the present studies lactate efflux was measured under conditions of acidosis and alkalosis utilizing the Fick equation and found to be in agreement with the only other study to have measured lactate efflux directly (ie increased efflux with alkalosis and decreased efflux with acidosis) (9). However, the mechanisms responsible for this observation appear to be similar despite large differences in the extracellular pH and intramuscular lactate concentrations. The most important determinants of lactate efflux from skeletal muscle are the H^+ and lactate concentration gradients as these determine transporter activity (2). The present research sought to evaluate if the changes in blood lactate concentration and lactate efflux with

acidosis and alkalosis were merely a consequence of the altered lactate production rates. This question was answered by examining the relationship between the plasma to muscle lactate concentration gradients for both acidosis and alkalosis (Fig. 1,2). This analysis revealed that the changes in lactate efflux cannot be ascribed to result from simply a change in the lactate concentration gradient. Figures 1 and 2 illustrate that despite large differences in the intramuscular lactate concentration with acidosis and alkalosis compared to control the lactate concentration gradients are similar. This suggests that the MCT transporters were affected by other factors.

In summary, the studies presented in this thesis significantly contribute to our understanding of the mechanisms responsible for the differences in the appearance of lactate in the blood during exercise in the presence of an induced extracellular acidosis and alkalosis. The present studies clearly demonstrate that extracellular pH manipulation does have an effect on the activity of the key flux-generating enzymes Phos and PDH_c and the regulatory enzyme PFK, and therefore impacts lactate production, intramuscular lactate accumulation, and fuel utilization. The present research also illustrates the importance of the energy status of the cell as reflected by the phosphorylation potential in determining fuel selection, the rates of substrate supply and modulation of the catalytic activity of Phos, PFK, and PDH_a. The potential exists to use the present findings as a starting point for the development of studies to investigate clinical populations with diseases that result in an increased blood lactate concentration with exercise and poor exercise capacity.


Fem Venous
(mmol·l⁻¹)
Arterial
(mmol·l⁻¹)
-4
-2
0
2
4
6
8
Control
Acid
Rest
Post Ingestion
30%
60%
75%
VO2 max
MUSCLE TO PLASMA LACTATE GRADIENT


Fem Venous
(mmol·l⁻¹)
Arterial
(mmol·l⁻¹)
-4
-2
0
2
4
6
8
Control
Alkalosis
MUSCLE TO PLASMA LACTATE GRADIENT
Rest
Post Ingestion
30%
60%
75%
VO2 max

4.3 Future Directions

A few clinical conditions exist that result in poor exercise capacity and an accelerated appearance of lactate in blood at lower exercise intensities. Congestive heart failure (CHF) and chronic obstructive pulmonary disease (COPD) are two examples (15,16,34). Traditionally, both conditions have had their poor exercise capacity and accelerated lactate appearance in the blood attributed to decreases in the oxygen delivery to the working muscle during exercise. In CHF this has been thought to occur secondary to impaired cardiac function that results in poor peripheral blood flow (32,37). In COPD, the impaired oxygen delivery is secondary to lung dysfunction (15). However, recent investigation has revealed that the skeletal muscle in both conditions has a reduce oxidative capacity, which suggests that the skeletal muscle is the limiting factor during exercise (16,31). The majority of studies examining the mechanisms responsible for the accelerated appearance of lactate in the blood during exercise and the poor exercise tolerance have been descriptive in nature, examining only the blood lactate concentration. Only one study in CHF patient has used the muscle biopsy technique and found that some of the TCA cycle enzyme activities were reduced while glycogenolysis and glycolysis are acceierated relative to work rate (32). Additionally, non-invasive assessment of the skeletal muscle in CHF patients by P-NMR has shown that the PCr degradation is increased, PCr resynthesis is delayed and the intramuscular H^+ concentration is increased when compared to healthy controls (1,17,32). Examination of skeletal muscle function in COPD patients with P-NMR has similarly

demonstrated an accelerated PCr degradation rate and a lower pH_i when compared to controls (15). However, no studies have examined the activities of the key flux-generating and regulatory enzymes involved in CHO and TAG metabolism ie - Phos, PDH_a and PFK. As the studies presented in this thesis, so clearly demonstrate, the appearance of lactate in the plasma is directly attributable to the activity of these enzymes, and not related to the oxygen availability. Investigations with these patient populations should concentrate on examining at rest and during steady state exercise at intensities greater than 75% $VO_{2\,max}$ the activities of Phos, PFK and PDH_a and their respective regulators in order to discern the mechanisms responsible for the accelerated appearance of lactate in the blood. This is suggested on the basis of the results of the two present studies, as the majority of effects were observed at this intensity.

4.4 Conclusions

The present studies significantly contribute to the understanding of the mechanisms responsible for the universally observed increase in the appearance of blood lactate with an extracellular alkalosis and decrease with acidosis. The present research extends the finding of many *in vitro* studies on the effects of H^+ on the activity of Phos, PFK and PDH_a into humans, *in vivo*. The present acidosis study validates previous speculation that acidosis reduces glycolysis but extends the knowledge by illustrating that glycogenolysis and pyruvate entry into the TCA cycle are also affected by alterations in Phos and PDH_a catalytic activity which

ultimately resulted in reduced CHO utilization, lactate production and lactate appearance in the blood. The present alkalosis study clearly refutes the previously held notion that the enhanced appearance of lactate in the blood results from simply an increase in the blood buffering capacity. The results of the present research demonstrate that the enhanced blood lactate with alkalosis stems from an altered energy status of the cell that ultimately allowed greater CHO utilization at a lower intensity and the continuation of this despite increases in the H^+ at the highest work rate. This was accomplished by the acceleration of Phos flux and glycolysis by their positive modulators. The enhanced glycogenolysis was significantly higher than the available flux through PDH_a despite increases in its flux, resulting in increased lactate production and blood lactate appearance.

In addition, the significance of enzyme regulation by post-transformational modulation and its link to the energy status of the cell was shown to be very important for controlling both fuel selection and the rate of substrate supply. The combined results of both studies demonstrate that an alteration in the external environment of the muscle does have an impact on the intramuscular compartment.

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APPENDIX A:

SKELETAL MUSCLE PYRUVATE DEHYDROGENASE ACTIVITY DURING MAXIMAL EXERCISE IN HUMANS

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Skeletal muscle pyruvate dehydrogenase activity during maximal exercise in humans

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Putman, C. T., N. L. Jones, L. C. Lands, T. M. Bragg, M. G. Hollidge-Horvat, and G. J. F. Heigenhauser. Skeletal muscle pyruvate dehydrogenase activity during maximal exercise in humans. *Am. J. Physiol.* 269 (*Endocrinol. Metab.* 32): E458–E468, 1995.—The regulation of the active form of pyruvate dehydrogenase (PDH_a) and related metabolic events were examined in human skeletal muscle during repeated bouts of maximum exercise. Seven subjects completed three consecutive 30-s bouts of maximum isokinetic cycling, separated by 4 min of recovery. Biopsies of the vastus lateralis were taken before and immediately after each bout. PDH_a increased from 0.45 ± 0.15 to 2.96 ± 0.38, 1.10 ± 0.11 to 2.91 ± 0.11, and 1.28 ± 0.18 to 2.82 ± 0.32 mmol·min^{-1}·kg wet wt^{-1} during *bouts 1*, *2*, and *3*, respectively. Glycolytic flux was 13-fold greater than PDH_a in *bouts 1* and *2* and 4-fold greater during *bout 3*. This discrepancy between the rate of pyruvate production and oxidation resulted in substantial lactate accumulation to 89.5 ± 11.6 in *bout 1*, 130.8 ± 13.8 in *bout 2*, and 106.6 ± 10.1 mmol/kg dry wt in *bout 3*. These events coincided with an increase in the mitochondrial oxidation state, as reflected by a fall in mitochondrial NADH/NAD, indicating that muscle lactate production during exercise was not an O_2-dependent process in our subjects. During exercise the primary factor regulating PDH_a transformation was probably intracellular Ca^{2+}. In contrast, the primary regulatory factors causing greater PDH_a during recovery were lower ATP/ADP and NADH/NAD and increased concentrations of pyruvate and H^+. Greater PDH_a during recovery facilitated continued oxidation of the lactate load between exercise bouts.

acetyl-CoA/CoASH; ATP/ADP; exercise; hydrogen ion; lactate metabolism; phosphocreatine; pyruvate; redox state

SINCE THE STUDIES of Hill and Lupton in 1923 (19), muscle lactate production has been attributed to lack of O_2 supply. This theory still receives widespread support (see Ref. 23 for review) and is believed to operate as follows. With increasing intensity of contraction, tissue O_2 supply becomes limiting for aerobic metabolism resulting in the eventual cessation of aerobic ATP production. As mitochondrial ATP production decreases below ATP utilization by the muscle, cytosolic ATP levels decrease while ADP, AMP, and P_i increase, providing a stimulus to accelerate anaerobic glycolysis. Greater glycolytic rate leads to cytosolic pyruvate and NADH accumulation, which in turn react to form lactate, the end product of anaerobic glycolysis. Meanwhile, in the mitochondria, NADH and ADP also accumulate due to a substrate limitation of O_2 at cytochrome oxidase. This theory assumes that the cytosol and mitochondria are not separately regulated compartments and that NADH and ADP levels rapidly achieve equilibrium between compartments. As tissue O_2 decreases, the net effect of these events is an accelerated rate of anaerobic ATP production to augment or replace aerobic ATP production.

Considerable controversy still exists regarding the mechanism of lactate production in exercising muscle. Studies that have used direct measurement of whole tissue NADH and NAD report a reduction of the muscle redox state (33) and argue that lower tissue O_2 tension is responsible for both alterations in redox state and lactate accumulation in exercising muscle (7). In contrast, determination of mitochondrial redox state by determining the concentrations of reactants related to the mitochondrial enzyme, glutamate dehydrogenase, has yielded opposite results. Graham and Saltin (15) have shown that the mitochondrial redox state became more oxidized in human muscle during incremental exercise while lactate accumulation increased. This suggests that tissue O_2 levels are not compromised and that tissue hypoxia is not the cause of muscle lactate production during exercise. Similar studies examining stimulated dog muscle to simulate various exercise intensities also found that muscle lactate production was independent of tissue O_2 levels (8, 9, 21). Determination of mitochondrial redox state using surface fluorometry (21) has shown that increased mitochondrial oxidation occurs concurrently with lactate accumulation during exercise. Also, studies of myoglobin saturation (8, 9) indicate that tissue O_2 levels are maintained constant during work of increasing increments and are not limiting for optimal function of the electron transport chain.

Muscle lactate production may increase in the presence of adequate O_2 delivery if the rate of pyruvate production by glycolysis becomes greater than the rate of its oxidation by the pyruvate dehydrogenase complex (PDH_c). PDH_c is a multienzyme flux-generating complex that controls the rate of entry of pyruvate-derived acetyl-CoA into the tricarboxylic acid (TCA) cycle (37). During intense exercise, when the dependence on carbohydrate oxidation for energy production is maximized, the difference between glycolytic flux and flux through the active form of PDH_c (PDH_a) may become increasingly large. As the rate of pyruvate generation exceeds the capacity for oxidation by PDH_a, pyruvate should accumulate and react with glycolytically generated NADH to form lactate without a decrease in mitochondrial O_2. Conversion of pyruvate to lactate is catalyzed by the near-equilibrium enzyme, lactate dehydrogenase, which maintains lactate concentration an order of magnitude higher than pyruvate.

The purpose of the present investigation was to test the hypothesis that lactate accumulation in maximally contracting human skeletal muscle results from a greater rate of glycolytic pyruvate production than pyruvate

oxidation by PDH_a. We also studied the factors regulating PDH_a in human muscle during maximal exercise and measured the contribution of PDH_a flux to energy production during repeated bouts of maximal cycling exercise. This model was chosen because previous studies have shown a progressive fall in the work achieved during repeated 30-s bouts of maximal cycling, accompanied by a progressive reduction in lactate production (27, 34).

METHODS

Subjects

Seven male subjects (mean ± SE, age 22.4 ± 0.8 yr, height 178 ± 2 cm, and weight 73.8 ± 4.4 kg) participated in this study. Approval was obtained from the ethics committee of McMaster University. Written informed consent was obtained from each subject after an explanation of the attendant risks associated with the study protocol.

Experimental Protocol and Determination of Muscle Performance

Each subject completed three 30-s bouts of isokinetic cycling at 100 rpm separated by 4 min of rest-recovery as previously described (34). Biopsies of the vastus lateralis were taken immediately before and after each 30-s bout. The isokinetic cycle ergometer used in this study has previously been described (26). Briefly, the cycle was fitted with a 3-hp DC motor which set the upper limit of crank velocity at 100 rpm for this study. The forces exerted by the subjects were recorded by strain gauges attached to the pedal cranks. Electrical signals were transferred to a chart recorder and computer. Work was calculated for each pedal stroke as the product of impulse and angular velocity. Total work was calculated as the sum of 50 pedal strokes for two legs over each 30-s period.

Preexperimental Protocol

Because it was not possible to make all measurements on the same day, 1 wk before biopsy samples were collected, each subject completed the cycling protocol while expired gases were continuously sampled. O_2 uptake ($\dot{V}O_2$) and CO_2 output ($\dot{V}CO_2$) measurements were made from expired gases using a Quinton metabolic cart (Quinton Q-plex 1, Quinton Instrument, Seattle, WA). Respiratory exchange ratio (RER) was calculated as the quotient of $\dot{V}CO_2/\dot{V}O_2$. Data were averaged over 15-s intervals.

Muscle Sampling

Biopsy sites were prepared on each thigh superficial to the vastus lateralis. Under local anesthesia (2% lidocaine without epinephrine) incisions were made through the skin to the deep fascia. Biopsies of the vastus lateralis were obtained as described by Bergström (2). Biopsies were immediately frozen in liquid N_2 and removed from the needle while still frozen. Under liquid N_2, 10–30 mg of tissue were chipped from each biopsy and dissected free of blood and connective tissue. From this, two clean 5- to 15-mg portions were stored separately under liquid N_2, one for determination of PDH_a and the other for determination of total pyruvate dehydrogenase activity (PDH_t). The remaining muscle was freeze dried, dissected free of blood and connective tissue, and stored dry at −50°C until analyzed for metabolite concentrations.

PDH_a Analysis

PDH_a and PDH_t were measured as previously described (29, 30) using a radiometric method (11) that determines the rate of acetyl-CoA production as a function of time. Briefly, for the measurement of PDH_a the sample was gently homogenized on ice in a solution containing 50 mM tris(hydroxymethyl)aminomethane (Tris), 200 mM sucrose, 50 mM KCl, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 5 mM $MgCl_2$, 50 mM NaF, 5 mM dichloroacetic acid (DCA), 1 mM dithiothreitol (DTT), and 0.1% Triton X-100 (vol/vol) at pH 7.8. For the measurement of PDH_t, the sample was homogenized in a similar buffer with the following differences: exclusion of 50 mM NaF and inclusion of 10 mM $CaCl_2$, 25 mM $MgCl_2$, 4 U/ml hexokinase, 10 mM glucose, and 10 mM DCA.

The buffer used to assay PDH_a and PDH_t was modified somewhat from the original (11) as we have previously described (29, 30): it contained 100 mM Tris, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 3.0 mM NAD^+, 1.0 mM CoASH, 1.0 mM thiamine pyrophosphate, and 1 mM DTT, and the pH was adjusted to 7.8. The reaction was initiated by adding pyruvate to a final concentration of 1.0 mM. Aliquots (200 μl) of the reaction mixture were sampled at 1, 2, and 3 min, and the reaction was stopped by adding each aliquot to 40 μl of 0.5 M perchloric acid (PCA). After 5 min each aliquot was neutralized with 1.0 M K_2CO_3 and centrifuged for 3 min at 15,900 *g* (microcentrifuge E, Beckman Instrument, Mississauga, Ont., Canada). The resulting supernatant was stored at −50°C until analyzed for acetyl-CoA by the method of Cederblad et al. (5). Plots of acetyl-CoA as a function of time were used to determine the reaction rates. For analysis of PDH_t, each sample was incubated on ice for 1 h, which was found to be optimal for complete transformation to PDH_t. PDH_t was taken as the mean of the two highest measurements for each subject.

Metabolite Analysis

Just before metabolite analysis a portion of the dry powdered muscle was extracted in a solution of 0.5 M PCA and 1 mM EDTA and neutralized to pH 7.0 with 2.2 M $KHCO_3$. Three such extractions were completed. The first extraction was used for the determination of ATP, AMP, phosphocreatine (PCr), acetyl-CoA, CoASH, total CoA, acetylcarnitine, free carnitine, total carnitine, lactate, glutamate, and oxoglutarate. ATP and PCr were measured on the same day as extraction. A second portion of the dry muscle was extracted for analysis of muscle pyruvate and ADP and assayed on the same day. A third portion of dry muscle was extracted before NH_3 analysis. These samples were stored in liquid N_2 as a PCA extract and were neutralized just before the assay to prevent loss of NH_3 from the samples.

Neutralized PCA extracts were analyzed for ATP, ADP, AMP, PCr, lactate, oxoglutarate, glutamate, pyruvate, and NH_3 as described by Bergmeyer (1). CoASH, carnitine and their acetylated forms were analyzed as described by Cederblad et al. (5).

Calculations

The mitochondrial redox state (NADH/NAD) was calculated from the glutamate dehydrogenase reaction using measurements of glutamate, NH_3 and oxoglutarate as described by Graham and Saltin (15). The mitochondrial redox state was calculated as follows

$$[NADH]/[NAD] = (k_{eq} \times [glutamate])/([oxoglutarate] \times [NH_3])$$

where the equilibrium constant (k_{eq}) was 3.87×10^{-10} M, as calculated by Williamson et al. (38) at a $[H^+]$ of 100 nM (pH 7.0). The cytosolic redox state was calculated as NAD/NADH,

or the reciprocal of the mitochondrial redox state, as follows

$$[NAD]/[NADH] = ([pyruvate] \times [H^+])/(k_{eq} \times [lactate])$$

where k_{eq} was 1.11×10^{-11} M, also as calculated by Williamson et al. (38).

Intracellular pH was calculated from muscle lactate and pyruvate using the empirical relationship of Sahlin et al. (32) and validated by Sprietet al. (34) for the type of exercise used in the present study. For rest the following equation was used

$$pH = 7.22 - 0.00521 \times (lactate + pyruvate)$$

For exercise samples the following equation was used

$$pH = 7.06 - 0.00413 \times (lactate + pyruvate)$$

where the units of expression for lactate and pyruvate are millimoles per kilogram of dry weight.

Correlation and Multilinear Regression Analysis

Correlation and multilinear regression analyses were completed to determine which regulatory factors best predicted PDH_a at rest and during recovery and to characterize their relationship to PDH_a.

Statistical Analysis

Data were analyzed by a one-way analysis of variance with repeated measures over time. When a significant F ratio was found, the Newman-Keuls post hoc test was used to compare means for all data except NADH/NAD. When a significant F ratio was found for NADH/NAD, Fisher pairwise comparisons were used to identify differences. For $\dot{V}O_2$, $\dot{V}CO_2$, and RER data, analysis was completed only on those points demarcated by SE bars. Results were considered significant at $P < 0.05$.

RESULTS

Total Work

Total work performed during the first 30-s bout of isokinetic cycling was 18.7 ± 1.2 kJ. It significantly declined to 15.6 ± 1.2 kJ in *bout 2* and still further to 14.2 ± 1.3 in *bout 3*. Total work did not differ between the preexperimental and experimental trials. During the second trial total work also declined with each successive bout: from 19.3 ± 0.9 to 16.3 ± 1.0 and 14.2 ± 1.2 kJ for *bouts 1*, *2*, and *3*, respectively.

$\dot{V}O_2$, $\dot{V}CO_2$, and RER

Resting $\dot{V}O_2$ (Fig. 1) was 0.46 ± 0.04 l/min and reached a peak value of 2.84 ± 0.17 after 30 s of cycling. Peak $\dot{V}O_2$ increased from 0.83 ± 0.08 to 3.27 ± 0.12 and 0.84 ± 0.10 to 3.14 ± 0.11 l/min in *bouts 2* and *3*, respectively. During the rest periods $\dot{V}O_2$ partially recovered but remained elevated over basal resting levels. Before *bouts 2* and *3* $\dot{V}O_2$ was 0.83 ± 0.08 and 0.84 ± 0.10 l/min, respectively. Four minutes after *bout 3*, $\dot{V}O_2$ was 0.86 ± 0.11 l/min.

Resting $\dot{V}CO_2$ (Fig. 1) was 0.44 ± 0.06 l/min, increased to 3.31 ± 0.20 after 30 s of cycling, and reached a peak of 3.80 ± 0.11 15 s later. Peak $\dot{V}CO_2$ was reduced to 3.08 ± 0.08 in *bout 2* and 2.45 ± 0.12 in *bout 3*. During the 4-min rest periods $\dot{V}CO_2$ fell to 1.20 ± 0.11, 0.99 ± 0.11, and 0.86 ± 0.10 l/min.

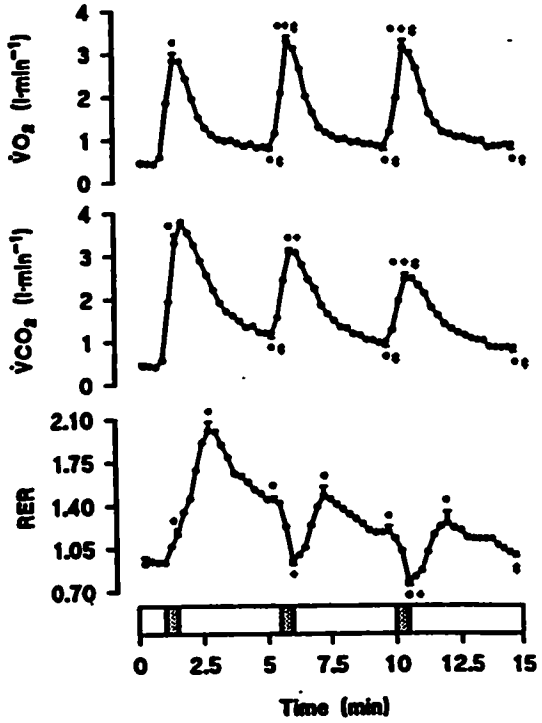

Fig. 1. O_2 uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$), and respiratory exchange ratio (RER) during 1 min of rest before commencement of exercise, rest-recovery (open area), and 30 s of maximal isokinetic cycling exercise (shaded squares). Data are means ± SE. *Significantly different from rest. †Postexercise different from preexercise of same bout. ‡Significantly different from post-*bout 1*.

With the onset of cycling in *bout 1* CO_2 evolution was greater and more rapid than $\dot{V}O_2$. This was followed by a slower rate of change in $\dot{V}CO_2$ during the ensuing rest period, which resulted in a rise in the RER (Fig. 1) that remained elevated throughout the first rest period. During *bouts 2* and *3* of cycling the magnitude of CO_2 evolution declined so that it was lower than $\dot{V}O_2$, resulting in a dramatic decline in the RER. The RER (Fig. 1) was 0.93 ± 0.05 at rest, rose to 1.17 ± 0.04 after 30 s of cycling, and reached a peak value of 2.01 ± 0.07 at 1.25 min into the first 4-min rest period. Thereafter the RER slowly declined to reach 1.45 ± 0.02 before *bout 2*, during which after another 30 s of maximal cycling it declined further to 0.95 ± 0.03. At 1.25 min into the second 4-min rest period the RER again reached a peak value of 1.48 ± 0.07. Before *bout 3* the RER recovered to 1.19 ± 0.04 and the ensuing 30 s of cycling again resulted in a rapid decrease to 0.78 ± 0.03. RER reached a peak value of 1.26 ± 0.09 at 1.5 min into the last recovery period and was 1.00 ± 0.02 at the end.

PDH_a and PDH_c Transformation

PDH_a (Fig. 2) was 0.45 ± 0.15 mmol·min^{-1}·kg wet wt^{-1} before commencement of exercise, which represented 14.4 ± 3.9% transformation of PDH_c to PDH_a. PDH_a rose to 2.96 ± 0.38 during *bout 1* of maximal cycling exercise, which represented complete transforma-

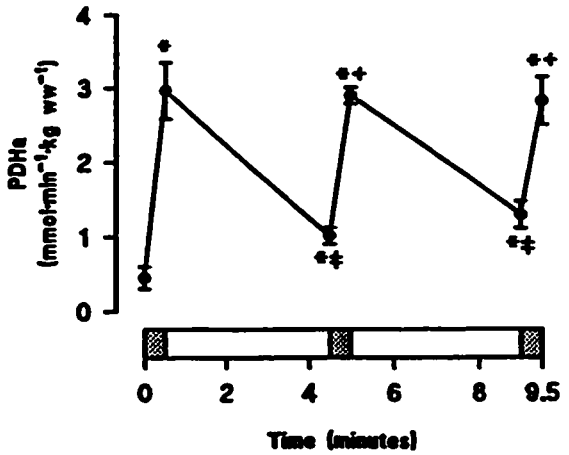

Fig. 2. Muscle pyruvate dehydrogenase activity (PDH_a) during maximal isokinetic cycling (shaded squares) and rest-recovery (open area). Data are means ± SE. Symbols as per Fig. 1.

tion (98.6 ± 8.3%). The following two bouts of maximal cycling also resulted in complete transformation, reaching 2.91 ± 0.11 (100.0 ± 6.9%) after *bout 2* and 2.82 ± 0.32 (92.7 ± 5.7%) after *bout 3*. In the two intervening rest-recovery periods PDH_a fell to 1.10 ± 0.11 (34.1 ± 3.3%) after *bout 1* and 1.28 ± 0.18 (44.5 ± 8.9%) in *bout 2*. During each of the rest-recovery periods PDH_a remained elevated 2.2- and 2.8-fold over the initial resting value. PDH_a for this group of subjects was 2.96 ± 0.17 mmol·min^{-1}·kg wet wt^{-1}.

Acetyl-CoA/CoASH

Acetyl-CoA/CoASH (Fig. 3) was 0.32 ± 0.04 at rest and rose as a result of maximal cycling to 0.57 ± 0.04, 0.96 ± 0.14 and 1.73 ± 0.75 after each of the three exercise bouts. In the two intervening rest periods this ratio recovered to resting levels. It was 0.47 ± 0.03 and 0.52 ± 0.03 at the end of the first and second recovery periods, respectively.

ATP/ADP

Muscle ATP (Table 1) remained stable during *bouts 1* and *2* but was reduced during *bout 3*. In contrast, muscle ADP (Table 1) alternately increased with each exercise bout and recovered to basal levels during the ensuing 4-min recovery period. Consequently the resultant ATP/ADP fell during maximal cycling and recovered during the rest periods (Fig. 3).

At rest ATP/ADP (Fig. 3) was 8.9 ± 0.5 and fell after *bout 1* of maximal cycling to 6.4 ± 0.9. After recovery in the first rest period (7.1 ± 0.9) it fell again after *bout 2* to 5.2 ± 0.6. During the second recovery period it was partially restored, reaching 6.4 ± 1.1, but did not completely recover to resting levels. After *bout 3* it decreased even further to 4.5 ± 0.7. Muscle AMP (Table 1) did not vary throughout the experimental protocol.

Mitochondrial and Cytosolic Redox State

NH_3 levels alternately rose and fell with successive bouts of maximal cycling, whereas changes in muscle glutamate were the opposite, falling with each successive bout (Table 1). Neither metabolite completely recovered to their preexercise resting values: NH_3 remained slightly elevated, whereas glutamate levels remained lower than the preexercise rest values. In contrast to these two metabolites, oxoglutarate decreased after *bout 1* and did not recover thereafter (Table 1).

Mitochondrial NADH/NAD (Fig. 3) was 25.7 ± 6.0 at rest and decreased to 9.6 ± 1.7 after *bout 1*. It partially recovered toward resting levels during the first recovery period, reaching 15.3 ± 2.6. However, *bout 2* of cycling resulted in a further decline to 4.9 ± 0.6; it remained low throughout the rest period (5.3 ± 0.7) and did not change after *bout 3* (4.8 ± 0.9). In contrast, the cytosolic compartment became more reduced (Table 1).

Lactate, Pyruvate, and [H⁺]

Intracellular accumulation of lactate, pyruvate, and H^+ (Fig. 4) occurred as a result of maximal cycling exercise. Muscle lactate (Fig. 4) increased as a result of cycling from 6.6 ± 0.8 mmol/kg dry wt at rest to 89.5 ± 11.6, 130.8 ± 13.8, and 106.6 ± 10.1 after each of the three bouts of maximal cycling. Lactate accumulation partially recovered during the first and second rest periods to 51.3 ± 8.6 and 81.7 ± 9.2 mmol/kg dry wt, respectively.

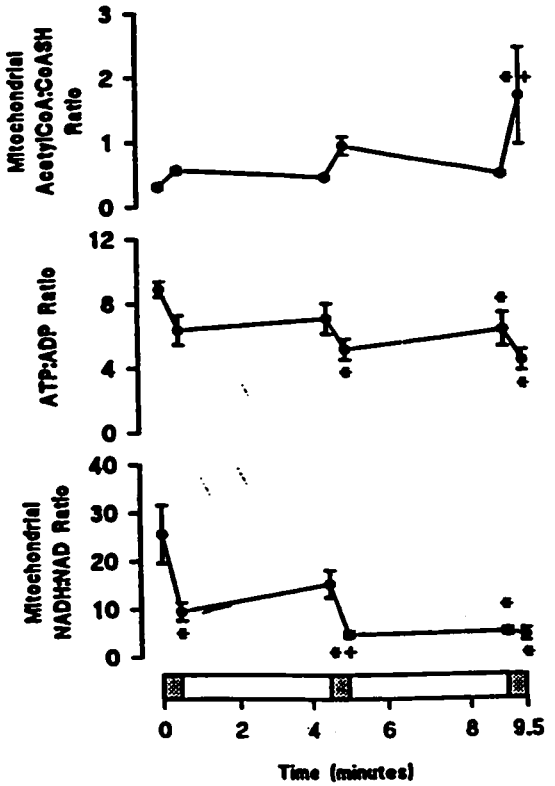

Fig. 3. Mitochondrial acetyl-CoA/CoASH, ATP/ADP, and mitochondrial NADH/NAD during maximal isokinetic cycling (shaded squares) and rest-recovery (open area). Symbols as per Fig. 1.

Table 1. *Muscle metabolite content in vastus lateralis at rest and during intermittent isokinetic cycling*

Measure	Bout 1		Bout 2		Bout 3	
	Pre	Post	Pre	Post	Pre	Post
NH_3	512 ± 88	1376 ± 164*	773 ± 48‡	1311 ± 130*†	988 ± 57* (6)	1696 ± 180*† (6)
Oxoglutarate	12.5 ± 1.0 (4)	7.5 ± 1.0*	6.9 ± 1.0*	7.3 ± 1.1* (6)	8.4 ± 1.2* (6)	5.3 ± 1.2* (5)
Glutamate	10.4 ± 1.4	7.0 ± 0.4*	5.9 ± 0.7*	4.2 ± 0.6*†‡	3.6 ± 0.5*‡ (6)	2.6 ± 0.5*‡ (5)
ATP	22.4 ± 1.0	23.1 ± 1.3	21.7 ± 2.2	18.5 ± 1.9	16.1 ± 1.7*‡ (6)	15.9 ± 1.4*‡ (6)
ADP	2.54 ± 0.13	3.53 ± 0.26*	3.21 ± 0.28	3.67 ± 0.23*	2.62 ± 0.22‡ (6)	3.69 ± 0.30*‡ (5)
AMP	0.16 ± 0.02	0.17 ± 0.02	0.20 ± 0.03	0.19 ± 0.01	0.18 ± 0.01 (6)	0.20 ± 0.03 (5)
PCr	75.9 ± 4.1	39.7 ± 3.0*	66.7 ± 2.3‡	27.8 ± 1.7*†‡	61.1 ± 5.6*‡ (6)	33.2 ± 2.2*† (6)
Cytosolic NAD/NADH	158 ± 37	142 ± 23	65 ± 15*	124 ± 22	60 ± 8* (6)	66 ± 15*‡ (6)

Data are means ± SE; $n = 7$ except where indicated in parentheses. Ammonia and oxoglutarate measures are expressed in μmol/kg dry wt; all other measures are expressed as mmol/kg dry wt. * Different from rest. † Different from pre of same bout. ‡ Different from post-*bout 1*.

Muscle pyruvate increased from 0.47 ± 0.09 mmol/kg dry wt at rest to 1.95 ± 0.34 and 1.77 ± 0.32 after *bouts 1* and *2*, respectively. In the intervening recovery period pyruvate fell to 0.77 ± 0.10, which did not differ from rest. During the second recovery period pyruvate decreased to 0.96 ± 0.12 but did not change after 30 s of maximal cycling in *bout 3* (0.93 ± 0.18).

Calculated $[H^+]$ (Fig. 4) was 65.6 ± 0.6 nmol/l at rest and increased to 216.5 ± 27.6, 277.8 ± 21.2 and 247.1 ± 21.1 after *bouts 1*, *2*, and *3* of maximal cycling. During the first and second recovery periods, $[H^+]$ partially recovered to 116.0 ± 11.2 and 167.1 ± 17.3 nmol/l, respectively.

Acetyl-Group Accumulation

Total CoA (Fig. 5) was relatively constant throughout, varying only slightly from 46.7 ± 5.38 μmol/kg dry wt at rest to 54.9 ± 3.93 after the first 30-s bout and 57.6 ± 7.33 after the third. Acetyl-CoA (Fig. 5) alternately rose and fell with successive bouts: it was 11.1 ± 1.6 μmol/kg dry wt at rest and rose to 19.7 ± 1.3, 24.7 ± 3.0 and 31.6 ± 5.2 after each of the three 30-s bouts of maximal cycling. During the two intervening 4-min rest periods acetyl-CoA partially recovered to 16.2 ± 1.6 and 18.1 ± 1.6 μmol/kg dry wt after the first and second rest period, respectively.

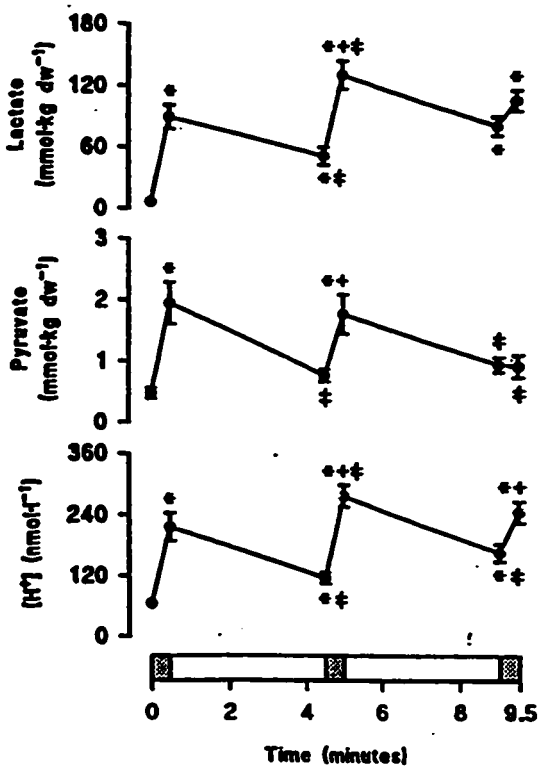

Fig. 4. Muscle lactate, pyruvate, and intracellular $[H^+]$ during maximal isokinetic cycling (shaded squares) and rest-recovery (open area). Symbols as per Fig. 1.

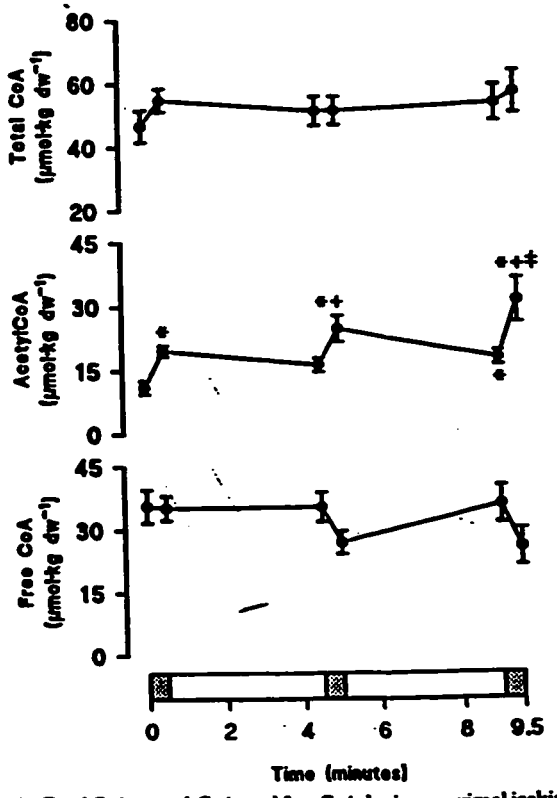

Fig. 5. Total CoA, acetyl-CoA, and free CoA during maximal isokinetic cycling (shaded squares) and rest-recovery (open area). Symbols as per Fig. 1.

Free CoA (CoASH) (Fig. 5) did not change during the first 30-s bout of cycling: it was 35.6 ± 3.9 μmol/kg dry wt at rest and 35.2 ± 2.9 after 30 s of isokinetic cycling. During the second and third exercise bouts, CoASH mirrored changes in acetyl-CoA, decreasing as the acetyl-CoA pool grew larger during exercise and rising as excess acetyl-CoA was metabolized during the rest periods. CoASH fell to 26.9 ± 2.7 μmol/kg dry wt after the second and 26.0 ± 4.4 after the third 30-s bout of cycling. It completely recovered to 36.1 ± 4.3 at the end of the second rest period.

The pattern of acetylcarnitine accumulation (Fig. 6) was similar to acetyl-CoA in that it rose as a result of 30 s of maximal cycling. However, it differed from the pattern of acetyl-CoA accumulation observed during the rest-recovery periods by continuing to rise throughout, indicating a transfer of acetyl groups to the larger carnitine pool. Acetylcarnitine was 4.0 ± 1.0 mmol/kg dry wt at rest and rose to 6.3 ± 0.7, 8.8 ± 0.7, and 8.9 ± 0.6 after *bouts 1*, *2*, and *3*, respectively. During the first and second recovery periods, acetylcarnitine rose continuously to 7.7 ± 1.4 and 9.1 ± 1.3 mmol/kg dry wt, respectively. Changes in free carnitine (Fig. 6) mirrored those of acetylcarnitine, decreasing continuously from 14.1 ± 1.8 mmol/kg dry wt at rest to 8.4 ± 1.0 after *bout 3*. Total carnitine (Fig. 6) did not change throughout; it was 18.4 ± 1.7 mmol/kg dry wt at rest and remained constant during three bouts of maximal exercise and the two rest periods. Total carnitine ranged from 19.3 ± 1.1 after *bout 1* to 17.1 ± 1.6 after *bout 3*.

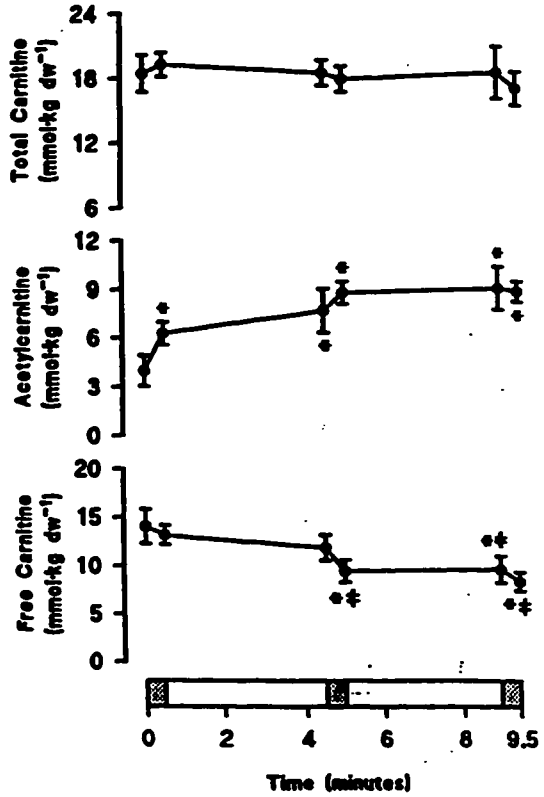

Fig. 6. Total carnitine, acetylcarnitine, and free carnitine during maximal isokinetic cycling (shaded squares) and rest-recovery (open area). Symbols as per Fig. 1.

Correlation and Multilinear Regression Analyses

Correlation analysis of PDH_a and the regulatory factors, ATP/ADP, NADH/NAD, PCr, pyruvate, and $[H^+]$ during rest and the recovery periods revealed the following. ATP/ADP ($r = -0.115$), NADH/NAD ($r = -0.514$), and PCr ($r = -0.733$) were negatively correlated with PDH_a, whereas pyruvate ($r = 0.536$) and H^+ ($r = 0.486$) were positively correlated. Furthermore, multilinear regression analysis using these independent variables accounted for 76.5% of the variation in PDH_a at rest and during recovery. The resulting regression equation was as follows

$$PDH_a = 1.88 + (0.0295 \times [ATP]/[ADP]) - (0.00473 \times [NADH]/[NAD]) - (0.0253 \times [PCr]) + (0.419 \times [pyruvate + (0.00305 \times [H^+])$$

$$(R^2 = 76.5\%, P < 0.006)$$

where pyruvate and PCr concentrations are expressed as millimoles per kilogram of dry weight and H^+ concentration as nanomoles per liter.

DISCUSSION

In two previous studies that employed the same protocol we found marked lactate production in the first 30-s bout, but in *bout 3* no change in lactate occurred (27, 34). We postulated that the reduction in lactate accumulation in the third bout was related to progressively greater PDH_a flux. The purpose of the present study was to examine skeletal muscle PDH_a regulation in humans during maximal intermittent exercise and to examine the role of PDH_a in regulating muscle lactate production. We also wanted to ascertain which regulatory factors were responsible for determining PDH_a in humans during exercise and to characterize the contribution of PDH_a flux to overall energy production during maximal contractions. The protocol employed was successful at altering muscle lactate content and PDH_a transformation during successive periods of exercise, and it provided a good model from which to examine factors regulating PDH_a transformation.

Lactate Metabolism

O_2 availability. The classic theory of muscle lactate accumulation during contraction has centered on the development of tissue hypoxia, which results in a substrate limitation of the electron transport chain at cytochrome oxidase and ultimately limits oxidative phosphorylation (39). Consequently, parallel increases in NADH/NAD and P_i and a decrease in the ATP/ADP ensue. Accumulation of ADP, AMP, and P_i in turn serve as stimuli for continued glycogenolytic and glycolytic flux. AMP activates glycogen phosphorylase, and the accumulation of P_i ensures that sufficient substrate is available for phosphorylase activity, whereas ADP, AMP,

and P_i provide a stimulus for increased phosphofructokinase activity and glycolytic flux. Catalyzed by lactate dehydrogenase, accumulated cytosolic NADH is then free to react reversibly with glycolytically generated pyruvate to form lactate.

Support for this theory was largely based on observations that muscle lactate production increases or decreases during exercise in response to breathing hypoxic or hyperoxic gas mixtures, respectively (see Ref. 22 for review). This explanation of lactate production is also supported by studies that have examined muscle cell redox state, which is purported to be a sensitive indicator of mitochondrial O_2 availability (22). Sahlin et al. (33) reported that muscle NADH accumulation coincided with muscle lactate production in humans during incremental cycling exercise under normoxic conditions. However, measurements of NADH and NAD by these investigators were made on whole cell extracts, which reflect total NAD and NADH but not the mitochondrial redox state, except in the case of circulatory occlusion. Furthermore, measurements of muscle Po_2 in exercising humans (3) demonstrated that in muscle which was accumulating lactate, tissue Po_2 was well above 0.1–0.5 mmHg, the critical level for the development of tissue hypoxia (6).

In contrast to whole cell measures of NAD and NADH, indicator measurements of the mitochondrial redox state using the glutamate dehydrogenase equilibrium reaction more accurately reflect tissue O_2 availability (15). In the present study, mitochondrial NADH/NAD decreased during the first bout and recovered during the ensuing rest period (Fig. 3). Although it also decreased after the second bout, it did not recover during the rest period but remained lower throughout the third bout. Concomitant with these changes during exercise was considerable lactate accumulation (Fig. 4). In contrast, muscle lactate decreased during the rest periods when the mitochondria became more reduced. These observations are consistent with muscle lactate accumulation occurring independent of O_2 availability in our subjects and are similar to previous reports of human muscle during cycling exercise (15) and in situ stimulated dog muscle (8, 9, 21, 35). In the absence of any apparent O_2 limitation to mitochondrial respiration, the decrease in ATP/ADP observed during each bout (Fig. 3) suggests that the controlling factor of mitochondrial respiration was either kinetic regulation by ADP alone or thermodynamic regulation by the cell phosphorylation potential (39).

Glycolytic rate, PDH_a, and lactate production. For the intensity of exercise employed in the present study most or all of the substrate for glycolytic ATP production is derived from muscle glycogen (18, 20, 27, 34). Thus glycolytic flux was initiated by the flux-generating enzyme glycogen phosphorylase and further regulated by the rate limiting enzyme phosphofructokinase. If it is assumed that most of the substrate utilized during exercise in the present study was from muscle glycogen (18, 20) and that 3 kg of intracellular water are equal to 1 kg dry weight of muscle, it is possible to calculate glycolytic flux for each bout as the difference between glycogen breakdown and the accumulation of hexose monophosphates, fructose 1,6-diphosphate, and glycerol 3-phosphate, which we have previously reported for this protocol (27, 34). Lactate release from the active muscle mass was not included in the present calculation, since it does not represent >10% of the total lactate produced (25). Glycolytic flux was 39 (27), 40 (34), and 11 (34) $mmol \cdot min^{-1} \cdot kg\ wet\ wt^{-1}$ in *bouts 1*, *2*, and *3*, respectively, which was similar to the sum of the rates of lactate and pyruvate accumulation and PDH_a flux in our subjects. The corresponding measures of PDH_a for *bouts 1*, *2*, and *3* were 3.0, 2.9, and 2.8 $mmol \cdot min^{-1} \cdot kg\ wet\ wt^{-1}$ (Fig 2). Thus glycolytic rate was 13-fold greater than PDH_a in *bouts 1* and *2* and 4-fold greater in *bout 3*. Because the PDH_a values reported here were 100% of PDH_t activity, they also represent the upper capacity for glycolytic flux into the TCA cycle. Given that mitochondrial O_2 was not limiting in our subjects, these data argue that muscle lactate production during maximal exercise in normoxia may simply be a function of widely different capacities between the ability to generate and to oxidize pyruvate.

During maximal cycling exercise 265, 254, and 80 mmol of lactate accumulated in the active muscle mass in *bouts 1*, *2*, and *3*, respectively. From a previous study (25) it is also possible to estimate that, during this protocol, 34, 28, and 7 mmol of lactate are released into the extracellular space during *bouts 1*, *2*, and *3*. Thus total lactate production was ~299, 282, and 87 mmol, during 30 s of cycling in each of *bouts 1*, *2*, and *3*, respectively. Thus, during *bouts 1* and *2*, 10% of the total lactate produced was released in the extracellular fluid space, whereas 8% was released during *bout 3*.

During the first and second recovery periods, the decrease in muscle lactate accumulation (Fig. 4) is consistent with greater PDH_a flux than glycolytic flux due to sustained PDH_a activity and inhibition of glycolytic rate. Measurements of muscle lactate in our subjects (Fig. 4) combined with arterial-venous lactate measures from a previous study (25) and with a leg blood flow of 10 l/min allow us to estimate the proportion of accumulated muscle lactate that was oxidized during recovery. Of the 265 mmol that accumulated in *bout 1*, 60 mmol were released into the extracellular space, 62 mmol were oxidized, and 143 mmol remained in the active muscle mass. Similarly, of the 254 mmol that accumulated in the active muscle mass during *bout 2*, 92 mmol were released into the extracellular space, 65 mmol were oxidized in the active muscle mass, and 97 mmol remained in the active muscle mass. Thus during 4 min of recovery from *bouts 1* and *2*, 23 and 26% of the accumulated lactate was removed by oxidation, respectively. A similar amount was also released into the extracellular fluid space: 23 and 36% of the accumulated lactate were released during recovery from *bouts 1* and *2*, respectively.

PDH_a Regulation

Rate of PDH_a transformation. Muscle contraction has previously been shown to result in transformation of PDH_t to PDH_a in humans (10, 12, 13, 29, 30), but the

maximum rate of PDH_a transformation during muscle contraction has not been characterized. Thus it was of interest in the present study to also examine the rate of change of PDH_a transformation ($\Delta PDH_a/\Delta t$). $\Delta PDH_a/\Delta t$ can be calculated from a previous report that employed electrical stimulation at 20 Hz (13). PDH_a was transformed from 26 to 64% of PDH_t after 16 s and 78% after 32 s. No further transformation occurred after 73 s of stimulation, when PDH_a was 79% of PDH_t. Thus at 20 Hz most of the transformation occurred in the first 16 s of stimulation and was equal to a $\Delta PDH_a/\Delta t$ of 3.1 $mmol \cdot min^{-2} \cdot kg\ wet\ wt^{-1}$ during this period. In contrast to that study, complete transformation occurred during each 30-s exercise bout in our subjects (Fig. 2). In *bout 1* this corresponded to a $\Delta PDH_a/\Delta t$ that was ≥ 5 $mmol \cdot min^{-2} \cdot kg\ wet\ wt^{-1}$ and is to our knowledge the largest observed rate of PDH_a transformation reported for human muscle. Given that each contraction was maximal in our subjects, it is possible that most of the transformation could have occurred early in the exercise bout (13), and the $\Delta PDH_a/\Delta t$ may be greater. The difference between the $\Delta PDH_a/\Delta t$ in the present study and that calculated from the data of others (13) is probably due to the more intense protocol that we employed.

Acetyl-CoA/CoASH, NADH/NAD, and ATP/ADP. Mitochondrial acetyl-CoA/CoASH, ATP/ADP, and NADH/NAD are important signals regulating the rate of aerobic metabolism and PDH_a transformation. The regulatory influence of these factors has recently been reviewed (37). NADH and acetyl-CoA inhibit PDH-phosphatase and increase the activity of PDH-kinase, whereas NAD and CoASH accumulation have the opposite effect, increasing the activity of PDH-phosphatase and inhibiting PDH-kinase activity. Thus an increase in either of acetyl-CoA/CoASH or NADH/NAD will activate PDH-kinase and inhibit PDH-phosphatase. Conversely, a decrease in NADH/NAD and acetyl-CoA/CoASH will inhibit PDH-kinase and activate PDH-phosphatase. The effects of acetyl-CoA/CoASH and NADH/NAD are thought to be primarily mediated through allosteric effects on the regulatory subunits (37).

Unlike the other regulatory ratios, ATP/ADP acts on PDH-kinase alone and does not exert its effect by allosteric interaction. Because ATP is the substrate for PDH-kinase, it is competitive with its product, ADP, which inhibits catalytic activity. Thus the net balance of acetyl-CoA/CoASH, NADH/NAD, and ATP/ADP are important determinants of the relative activities of PDH-kinase and PDH-phosphatase, which in turn determine the extent of PDH_t phosphorylation and PDH_a activity. When the activity of PDH-kinase is greater than that of PDH-phosphatase, PDH_a will decrease. Conversely, when the activity of PDH-phosphatase is greater, PDH_a will increase.

Acetyl-CoA/CoASH. In the present study, each exercise bout resulted in an increase in mitochondrial acetyl-CoA/CoASH (Fig. 3), which should have resulted in lower PDH_a. During the ensuing 4-min recovery periods, the changes that occurred in acetyl-CoA/CoASH during exercise were resolved. Oscillation of acetyl-CoA/CoASH (Fig. 3) and acetyl-CoA (Fig. 5) coincided with a continuous increase in acetylcarnitine and decrease in free carnitine throughout the exercise and rest periods (Fig. 6), which is consistent with the reversible transfer of acetyl groups from mitochondrial CoA to carnitine. The resulting acetylcarnitine is then transported to the cytosol for storage. This system of acetyl-group transfer has previously been characterized in human muscle (10, 12, 13, 17). In our subjects, it acted to buffer against excess acetyl-CoA when its production through PDH_a exceeded its rate of entry into the TCA cycle. The absence of any apparent inhibitory effect of acetyl-CoA/CoASH on PDH_a during cycling exercise indicates that other regulatory factors were sufficient to override this inhibitory stimulus.

During exercise, the continuous transfer of acetyl groups from CoA to carnitine attenuated the rise in mitochondrial acetyl-CoA (Fig. 5) and acetyl-CoA/CoASH (Fig. 3) and regenerated CoASH (Fig. 5) at a rate that was sufficient to sustain maximal PDH_a activity. During the recovery periods this mechanism of acetyl-group transfer allowed recovery of acetyl-CoA/CoASH to preexercise levels (Fig. 3), thus preventing complete inactivation of PDH_a (Fig. 2) and allowing continued PDH_a flux. During exercise these events would have been vital to ensuring maximal pyruvate oxidation and minimizing muscle lactate accumulation. In contrast, during the recovery periods continued PDH_a flux guaranteed net removal of accumulated lactate.

NADH/NAD. Before exercise, mitochondrial NADH/NAD was very large (Fig. 3), possibly due to a substrate limitation of ADP at oxidative phosphorylation, resulting in inhibition of the electron transport chain and NADH accumulation (39). Extensive NADH accumulation under rest conditions would have increased PDH-kinase activity and inhibited PDH-phosphatase, thus maintaining PDH_a in a phosphorylated state and sustaining low PDH_a.

NADH/NAD decreased twofold during *bout 1* and fourfold during *bout 2*, where it remained throughout the remainder of the protocol (Fig. 3). Consequently, the stimulatory and inhibitory effects of NADH on PDH-kinase and PDH-phosphatase, respectively, should have been removed, resulting in a shift toward greater PDH-phosphatase activity and greater PDH_a transformation (Fig. 2). A rise in NADH/NAD during the first rest period would have reversed this transformation and resulted in greater PDH-kinase activity and lower PDH_a. In contrast, maintenance of low NADH/NAD during the second rest period would have allowed PDH_a transformation to be maintained at a higher level.

Despite the fact that NADH/NAD was altered so as to support greater PDH_a during exercise, it is not likely to have exerted a major effect on PDH_a transformation, since its effects are apparently secondary to the potent stimulatory affects of Ca^{2+} (13). During exercise the role of NADH/NAD was probably relegated to one of support, acting in concert with the other regulatory ratios in PDH_a regulation. In contrast, during rest and recovery, when the influence of Ca^{2+} on PDH_a transformation is less, NADH/NAD appeared to be an important factor.

A continuous decrease in this ratio (Fig. 3) occurred from preexercise rest to the second recovery period, which coincided with an increase in PDH_a (Fig. 2). This is consistent with the gradual removal of the inhibitory effects of NADH/NAD with each successive recovery period, resulting in progressively greater PDH_a.

ATP/ADP. Fluctuations in the ATP/ADP (Fig. 3) were similar to changes in NADH/NAD (Fig. 3). At rest the ATP/ADP was ~9 and dropped by 33% to ~6 after *bout 1*. Similar to the effects of NADH/NAD, high ATP/ADP at rest would have stimulated PDH-kinase and served to maintain low PDH_a. A fall in this ratio during each exercise bout would have had the opposite effect, reducing PDH-kinase activity and allowing greater PDH_a transformation. In our subjects, the fall in ATP/ADP followed a step wise pattern with each successive bout, never fully recovering during the rest periods. Consequently, there was less of a stimulus for increased PDH-kinase activity with each successive exercise and recovery period. This would have resulted in greater PDH_a during the recovery periods and a faster rate of PDH_a transformation during *bouts 2* and *3*.

Although ATP/ADP reported here are of the whole cell, it is assumed that similar quantitative changes occur in the mitochondria, since mitochondrial and cytosolic compartments are in equilibrium (36). This is also supported by changes in whole cell PCr, which parallel changes in mitochondrial ATP/ADP (36): a reduction in muscle [PCr] reflects a lack of available mitochondrial ATP and a rise in ADP, whereas an increase in [PCr] reflects a relative increase in ATP over ADP. In our subjects, alterations in muscle [PCr] also paralleled changes in ATP/ADP.

Pyruvate. The primary substrate of PDH_a, pyruvate, is also a potent stimulus for PDH_a transformation, exerting its effects by inhibiting PDH-kinase, with an estimated inhibitory constant (K_i) of 0.5–2.0 mM (37). During *bouts 1* and *2*, muscle pyruvate increased fourfold over preexercise rest levels (Fig. 4), contributing to PDH_a transformation. In the first and second rest periods, pyruvate partially recovered but remained elevated, sustaining a substantial stimulus for greater PDH_a transformation. If it is assumed that the fluid volume of muscle is 75% of the total volume, it is possible to calculate that muscle pyruvate concentration was 0.2 mM at rest and reached a maximum concentration of 0.7 mM after *bout 1*, which is in the range of the K_i of pyruvate for PDH-kinase. Thus, in our subjects changes in muscle pyruvate concentration would have been in an optimal range to exert a significant inhibitory effect on PDH-kinase, stimulating rapid PDH_a transformation during exercise and slowing the conversion back to the inactive form during recovery.

[H+]. We used the relationship found by Sahlin and co-workers (32) between the sum of pyruvate plus lactate and muscle homogenate pH to estimate intramuscular [H^+]. Although many factors contribute to intracellular [H^+] in heavy exercise (24), they appear to change in parallel. Spriet et al. (34) found a closely similar relationship in maximal cycling exercise of the type used in the present study, supporting its validity, and allowing us to examine the influence of changes in [H^+] on PDH_a transformation.

Although few reports are available on the effects of [H^+] on PDH_a transformation, one study reported that acidosis increased PDH_a transformation in perfused rat heart (28). The increase in muscle [H^+] in our subjects (Fig. 4) should have inhibited glycogen phosphorylase (7) and phosphofructokinase (14), slowing the rate of lactate production. At the same time, increases in [H^+] would have increased PDH_a activity and oxidation of lactate. Because PDH_a transformation was rapid and complete during each exercise bout and there already exists a multitude of regulators of PDH_a transformation, it may seem redundant that this aspect of PDH_a regulation should be functionally significant in our subjects during very intense muscular contractions. However, the utility of this mechanism is highlighted during recovery from intense exercise when intramuscular acidosis inhibits lactate production in concert with sustained PDH_a activity and lactate oxidation to reduce muscle lactate concentration and alleviate the acidosis.

PDH_a flux. Under normal dietary conditions, measures of PDH_a are equal to in vivo flux (10, 12, 29, 30). For the present data, calculation of PDH_a flux was based on the following: *1*) 100 mmol of O_2 occupies 2.24 liters; *2*) 1 mmol of pyruvate is oxidized by 3 mmol of O_2; *3*) the active muscle mass is the primary consumer of the increased O_2 uptake during exercise; and *4*) the active muscle mass (13 kg) is equal to the average of three previously reported estimates (4, 27, 31). Subtracting resting from total O_2 uptake (mmol/min), converting the balance to the equivalent pyruvate flux, and correcting for the active muscle mass yield a calculated PDH_a flux of 3 mmol·min^{-1}·kg wet wt^{-1} for all three bouts. This is similar to the measured values of PDH_a during this time which were 3.0, 2.9 and 2.8 mmol·min^{-1}·kg wet wt^{-1} (Fig. 2) in *bouts 1*, *2*, and *3*, respectively. Thus the factors that determined PDH_a transformation during exercise also determined PDH_a flux.

Similar calculations for each of the recovery periods resulted in a calculated PDH_a flux of 1 mmol·min^{-1}·kg wet wt^{-1}, which was twofold lower than the average measured PDH_a for the first and second recovery periods (Fig. 2). During recovery from maximal exercise, the average measured PDH_a may have been greater than PDH_a flux if the enzyme activity decreased in a linear fashion over the 4-min period (Fig. 2). If so, this suggests that during recovery PDH_a transformation did not present a limitation to oxidation of the lactate load as factors that determined PDH_a ensured that the potential for flux was twofold greater than actual flux. However, if PDH_a actually decreased to ~1 mmol·min^{-1}·kg wet wt^{-1} upon cessation of exercise, then measured PDH_a would have been equal to PDH_a flux. It is likely that PDH_a actually decreased to 1.10 and 1.28 mmol·min^{-1}·kg wet wt^{-1} (Fig. 2) almost immediately after cessation of exercise in *bouts 1* and *2*, respectively, since the quantity of lactate oxidized during each of the recovery periods would have required an average PDH_a flux of ~1 mmol·min^{-1}·kg wet wt^{-1}. This suggests that under these conditions, the factors

that determined PDH_a transformation also determined PDH_a flux. Lactate was probably used as a metabolic fuel during recovery from exercise to replenish the PCr pool (16) and support intracellular ATP requirements during this period.

Contribution of PDH_a flux to ATP production. During intense muscular contractions of the duration employed in the present study, the major sources of ATP production have traditionally been thought to come from PCr breakdown and anaerobic glycolysis (20). However, it is not clear what proportion of ATP production is supported by aerobic metabolism during 30 s of maximal sprint activity. Thus it was of interest in the present study to determine the proportions of the various fuels utilized to generate ATP and determine what proportion was supplied by flux through PDH_a. ATP production from both anaerobic metabolism (PCr and glycolysis) and aerobic glucose oxidation are summarized in Table 2.

In *bouts 1* and *2*, ~67–71% of the ATP was generated from anaerobic sources: 16% was from PCr and 51–55% was from anaerobic glycolysis. Meanwhile, aerobic glycolysis contributed 29–33% of the ATP. Because there were no apparent differences between the amount or proportions of fuels utilized to supply ATP, the drop in power output from 19.3 kJ in *bout 1* to 16.3 in *bout 2* was probably related to the development of muscle fatigue (24). In contrast, during *bout 3* total ATP production decreased, contributing to a further decrease in power output to 14.2 kJ. In *bout 3*, only 37% of the ATP was generated from anaerobic sources while generation from aerobic glycolysis increased to 63%. The difference in total ATP generation and the proportion of fuels utilized between *bout 3* and *bouts 1* and *2* were primarily attributed to a 3-fold decrease in ATP production from anaerobic glycolysis and a 1.4-fold increase in aerobic glycolysis. Flux through PDH_a provided an increasing amount of energy with each bout (220 mmol ATP in *bout 1*, 255 mmol ATP in *bout 2*, and 357 mmol ATP in *bout 3*) and was the result of greater PDH_a transformation during each recovery period.

Summary of PDH_a regulation. During intense cycling exercise PDH_c was rapidly transformed to PDH_a. The primary stimulus for this transformation was probably Ca^{2+}, which activates PDH-phosphatase and inhibits PDH-kinase (37). During exercise, greater PDH_a may have been further supported by falls in NADH/NAD and ATP/ADP, increases in pyruvate and H^+ concentrations, and attenuation of the rise in acetyl-CoA/CoASH. However, the physiological relevance of these changes on PDH_a regulation may not be realized until recovery when intracellular Ca^{2+} decreases and the alterations in these regulatory factors persist. During exercise PDH_a flux matched measured values of PDH_a. It is suggested that the role of PDH_a is to supply oxidizable substrate to the TCA cycle for generation of ATP and that this source of energy becomes increasingly important with each successive bout of maximal isokinetic cycling because anaerobic sources become increasingly inhibited.

During recovery, the influence of Ca^{2+} was removed but PDH_a remained elevated due to lower NADH/NAD and ATP/ADP, greater pyruvate and H^+ concentrations, and recovery of acetyl-CoA/CoASH. Measured PDH_a was also similar to PDH_a flux during recovery, allowing a significant portion of the accumulated lactate to be oxidized. Correlation and multilinear regression analysis were able to explain 76.5% of the variation in PDH_a, suggesting that all of the regulatory factors examined may have been important determinants of PDH_a transformation and PDH_a flux during rest and recovery from exercise.

Table 2. *Sources of ATP generation during three consecutive 30-s bouts of maximal isokinetic exercise separated by 4 min of rest-recovery*

Bout		Anaerobic			Aerobic	
		PCr	Glycolysis	Total	Glycolysis	Total
1	ATP, mmol	118	410	528	220	748
	% of total	16	55	71	29	100
2	ATP, mmol	126	390	516	255	771
	% of total	16	51	67	33	100
3	ATP, mmol	91	122	212	357	569
	% of total	16	21	37	63	100

Data are expressed as mmol of ATP and are also summarized as % of total ATP generated. ATP production from PCr and anaerobic glycolysis were calculated from the breakdown of PCr and the accumulation of lactate, respectively. Production of ATP from aerobic glycolysis was calculated from total acetyl-CoA production as the area under the PDH_a curves for each bout; 1 mmol of acetyl-CoA from glycogenolysis was equal to 19.5 mmol of ATP. Contribution of fat fuels was assumed to be negligible (20).

Summary

The present study examined PDH_a regulation and lactate metabolism in human muscle during repeated bouts of maximal isokinetic cycling. Transformation of PDH_a was rapid and complete after each of three 30-s maximal sprints. Lactate accumulation during exercise was attributed to differences in the maximal fluxes of glycolysis and PDH_a, but no evidence was found to indicate that lactate production was dependent on O_2 availability. Thus the results of the present study support our hypothesis that lactate accumulation in maximally contracting human skeletal muscle results from a greater rate of glycolytic pyruvate production than pyruvate oxidation by PDH_a. During exercise, glycolytic flux was much greater than PDH_a flux, resulting in muscle lactate accumulation. Conversely, during recovery from exercise, maintenance of muscle PDH_a flux while glycolytic flux was slowed appears to be responsible for net lactate oxidation.

The primary determinant of PDH_a during exercise appears to have been Ca^{2+}, whereas the other regulatory factors examined were secondary. Conversely, during rest and recovery from exercise, the important determinants of PDH_a were found to be ATP/ADP and NADH/NAD as well as the concentrations of pyruvate, PCr and H^+. The contribution of PDH_a flux to total ATP production during repeated 30-s bouts of maximal sprint activity became increasingly important with successive bouts, accounting for 29, 33, and 63% of total energy production, respectively. The progressive increase in intramuscular $[H^+]$ during the recovery periods served to simultaneously inhibit glycolysis and maintain greater PDH_a

transformation, accounting for the progressive reduction in muscle lactate accumulation.

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APPENDIX B:

EFFECTS OF SHORT-TERM SUBMAXIMAL TRAINING IN HUMANS ON MUSCLE METABOLISM IN EXERCISE

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Effects of short-term submaximal training in humans on muscle metabolism in exercise

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[1]*Department of Medicine, McMaster University Medical Centre, Hamilton, Ontario L8N 3Z5;* [3]*Department of Kinesiology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada; and* [2]*Department of Clinical Chemistry, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden*

Putman, C. T., N. L. Jones, E. Hultman, M. G. Holliday-Horvat, A. Bonen, D. R. McConachie, and G. J. F. Heigenhauser. Effects of short-term submaximal training in humans on muscle metabolism in exercise. *Am. J. Physiol.* 275 (*Endocrinol. Metab.* 38): E132–E139, 1998.—Muscle metabolism, including the role of pyruvate dehydrogenase (PDH) in muscle lactate (Lac^-) production, was examined during incremental exercise before and after 7 days of submaximal training on a cycle ergometer [2 h daily at 60% peak O_2 uptake ($\dot{V}O_{2peak}$)]. Subjects were studied at rest and during continuous steady-state cycling at three stages (15 min each): 30, 65, and 75% of the pretraining $\dot{V}O_{2peak}$. Blood was sampled from brachial artery and femoral vein, and leg blood flow was measured by thermodilution. Biopsies of the vastus lateralis were obtained at rest and during steady-state exercise at the end of each stage. $\dot{V}O_{2peak}$, leg O_2 uptake, and the maximum activities of citrate synthase and PDH were not altered by training; muscle glycogen concentration was higher. During rest and cycling at 30% $\dot{V}O_{2peak}$, muscle Lac^- concentration ($[Lac^-]$) and leg efflux were similar. At 65% $\dot{V}O_{2peak}$, muscle $[Lac^-]$ was lower (11.9 ± 3.2 vs. 20.0 ± 5.8 mmol/kg dry wt) and Lac^- efflux was less [−0.22 ± 0.24 (one leg) vs. 1.42 ± 0.33 mmol/min] after training. Similarly, at 75% $\dot{V}O_{2peak}$, lower muscle $[Lac^-]$ (17.2 ± 4.4 vs. 45.2 ± 6.6 mmol/kg dry wt) accompanied less release (0.41 ± 0.53 vs. 1.32 ± 0.65 mmol/min) after training. PDH in its active form (PDH_a) was not different between conditions. Calculated pyruvate production at 75% $\dot{V}O_{2peak}$ fell by 33%, pyruvate reduction to lactate fell by 59%, and pyruvate oxidation fell by 24% compared with before training. Muscle contents of coenzyme A and phosphocreatine were higher during exercise after training. Lower muscle lactate production after training resulted from improved matching of glycolytic and PDH_a fluxes, independently of changes in muscle O_2 consumption, and was associated with greater phosphorylation potential.

lactate; oxygen uptake; pyruvate dehydrogenase; glucose transporters; glycogen; leg blood flow; free fatty acids; phosphorylation potential

LONG-TERM ENDURANCE (submaximal) training carried out over periods of at least several weeks leads to a reduction in muscle glycogen utilization and lactate production, with greater use of fats as fuel during submaximal exercise (17). These changes have been ascribed to improvements in oxygen delivery through increases in muscle capillarization, greater metabolic oxidative capacity related to increases in mitochondrial content, and increased activity of enzymes taking part in the citric acid cycle and fat oxidation. Recently, short-term (6–10 days) endurance training has also been shown to shift metabolism from carbohydrates to fats, with less lactate production, before any increases in mitochondrial oxidative capacity have occurred (15, 16, 24), calling into question the importance of increasing oxygen delivery and oxidative capacity in explaining the effects of training on the choice of fuels for exercise. Muscle biopsy and whole body substrate turnover studies have shown that muscle glycogen and glucose utilization are decreased and less lactate is produced (8, 15, 16, 21, 23, 24). Intramuscular triglycerides are oxidized to a greater extent, without increases in the oxidation of plasma-derived free fatty acids (20, 21, 23, 24).

A reduction in blood lactate accumulation is uniformly found after endurance training (17), and because traditionally lactate formation has been considered to be secondary to exercise-induced tissue hypoxia, improvements in oxygen delivery and utilization have been considered to be the causal mechanisms. An alternative view of lactate formation (29) is that it results from an imbalance between the rates of pyruvate production by glycolysis and of pyruvate oxidation by the pyruvate dehydrogenase enzyme complex (PDH_c) (26) and enzymes of oxidative phosphorylation (29). Because the equilibrium constant of the lactate dehydrogenase system markedly favors lactate over pyruvate, small changes in pyruvate concentration are associated with large increases in lactate formation.

Recently, it has been demonstrated (8) that the reduction in muscle lactate production after short-term training is associated with a reduction in glycogenolytic rate, related to posttransformational downregulation of glycogen phosphorylase activity, modulated by decreases in both PO_4^{2-} and AMP. In all these previous studies, reductions in lactate accumulation were larger than the accompanying reductions in glycogenolysis. Recent lactate tracer studies have shown that after short-term training (22), there is in addition to a lower muscle lactate production a greater rate of lactate clearance from the blood.

Because PDH_c controls the rate of acetyl-CoA formation from pyruvate, the effects of training on lactate metabolism may be explained by an increase in the active form of PDH_c (PDH_a). Greater conversion of pyruvate into acetyl-CoA may allow greater oxidative phosphorylation and lower pyruvate concentration, accompanied by lower lactate formation. Because previous studies of short-term training have shown no increase in mitochondrial enzymes, any increases in lactate clearance and pyruvate oxidation are likely to

result from altered PDH_a regulation at submaximal workloads, rather than increases in the total PDH_c activity (PDH_t). However, the role of increased transformation of PDH_c to PDH_a in attenuating net muscle lactate production after short-term training has not been established. The purpose of the present study was to examine the regulation of PDH_a during exercise before and after this type of training.

METHODS

Subjects

Seven male subjects [age 23.4 ± 1.5 (SE) yr, height 181 ± 2 cm, weight 81.4 ± 4.1 kg] participated in this study. Approval was obtained from the ethics committees of McMaster University and the McMaster University Medical Centre. Written informed consent was obtained from all subjects after an explanation of the attendant risks associated with the study protocol.

Training Protocol

Before training, subjects completed a progressive exercise test on a cycle ergometer to determine peak oxygen uptake ($\dot{V}O_{2peak}$) and maximum work capacity. Measurements of O_2 uptake and CO_2 output were made using either a metabolic cart (Quinton Q-plex 2; Quinton Instrument, Seattle, WA) or by mass spectrometry. On a separate day, the subjects were studied at rest and during three levels of continuous steady-state exercise (30, 65, and 75% of $\dot{V}O_{2peak}$), each maintained for 15 min. Each subject then completed either 7 or 8 consecutive days of training on a cycle ergometer at 60% of their pretraining $\dot{V}O_{2peak}$ for 2 h daily. On the day after completion of the training period, $\dot{V}O_{2peak}$ was again determined, and on the next day experiments were repeated at rest and at the same absolute workloads as before training.

Experimental Protocol

On the morning of each experiment, subjects ate a standard light meal, consisting primarily of carbohydrate, and reported to the laboratory 1–2 h later. The femoral vein was catheterized percutaneously by means of the Seldinger technique, after administration of 3–4 ml of xylocaine, as described by Bernéus et al. (4). The brachial artery was catheterized percutaneously (4) with a radiopaque Teflon catheter after local anesthesia with 0.5 ml of xylocaine. Catheters were maintained patent with sterile nonheparinized saline solution. Leg blood flow was determined using the thermodilution method, as described by Andersen and Saltin (1): 10 ml of nonheparinized saline were injected, and leg blood flow was determined from the change in temperature as a function of time by use of a portable CO monitor (Spacelab, Redmond, WA). At least three measures were recorded at each time point and averaged.

Before and after training, four biopsy sites were prepared superficial to the vastus lateralis after local anesthesia. Subjects remained sitting and inactive for 20 min (preexercise), followed by cycling for 15 min each at 30, 65, and 75% of their pretraining $\dot{V}O_{2peak}$. Respiratory measurements were made during the last 5 min of the preexercise period and between 8 and 12 min of each exercise stage. Blood samples from the femoral vein and the brachial artery were simultaneously drawn at rest and at 9 and 13 min of each exercise stage. Leg blood flow was measured immediately after blood sampling at both 9 and 13 min. Biopsies of the vastus lateralis were obtained at rest just before the start of exercise and at the end of each exercise stage, and these were immediately frozen in liquid nitrogen and stored in liquid nitrogen until analyzed.

Blood Sampling and Analysis

Arterial and venous blood samples were drawn in heparinized plastic syringes and placed on ice. One portion of each sample was deproteinized in 6% perchloric acid (PCA) and stored at −20°C until analysis for glucose, lactate, and glycerol according to the methods of Bergmeyer (3) adapted for fluorometry. The second portion of blood was immediately centrifuged at 15,900 *g* for 2 min; the plasma supernatant was frozen and later analyzed for free fatty acids (Wako NEFA C test kit, Wako Chemical, Montréal, QC, Canada). A third portion of blood was analyzed for O_2 content (AVL 995 Automatic Blood Gas Analyzer, Intermedico, Markham, ON, Canada). Hematocrit (Hct) was determined using heparinized microcapillary tubes centrifuged for 5 min at 15,000 *g*.

Muscle Analysis

PDH_a and PDH_t were analyzed as previously described (10, 26). To compensate for contamination of muscle homogenates with blood or connective tissue, PDH_a and PDH_t measures were corrected to the highest total creatine in a series of biopsies obtained from each subject on a given day. Muscle glycogen was determined fluorometrically using the enzymatic end-point method described by Bergmeyer (3). Neutralized PCA extracts of freeze-dried, dissected, and powdered muscle samples were analyzed for acetyl-CoA, free CoASH, total CoA, acetylcarnitine, free carnitine, and total carnitine according to the method of Cederblad et al. (7). ATP, ADP, glucose, glucose 6-phosphate (G-6-*P*), fructose 6-phosphate (F-6-*P*), glycerol 3-phosphate (G-3-*P*), pyruvate, lactate, and phosphocreatine (PCr) were determined by the methods of Bergmeyer adapted for fluorometry. PCA extracts were corrected to the highest total creatine concentration within a series of biopsies obtained from each subject on a given day. Muscle wet-to-dry ratios were determined by weighing the frozen muscle samples before and again after freeze-drying. Maximal citrate synthase (CS) activity was determined according to Bergmeyer. Maximal CS activity was measured on each biopsy in duplicate on two different occasions for each subject. Because no differences were found between the two sets of independent measures, between conditions, or across time within a condition, the data were pooled and presented as pre- and posttraining. Muscle GLUT-1 and GLUT-4 transporter contents were determined by Western blot analysis (25).

Leg Uptake and Release of Metabolites and O_2

Uptake and release of metabolites (glucose, free fatty acids, glycerol, and lactate) and O_2 were calculated from their whole blood contents in arterial and venous blood and leg blood flow. Because there were no differences in Hct over time within a condition or between matched arterial and venous samples, blood samples were not corrected for fluid shifts. Also, because subjects were in steady state, with no significant differences in blood flows or metabolite concentrations between 9 and 13 min of each exercise stage, the two values at these time points were averaged to obtain one value for each.

Calculation of Pyruvate Production and Oxidation and Lactate Production

Pyruvate production was calculated from the sum of the rates of glycogen breakdown and glucose uptake minus the

sum of the rates of accumulation of muscle glucose, G-6-*P*, and F-6-*P* by use of wet weight concentration differences and with the assumption that working muscle amounted to 4.3 kg/leg. Because net lactate release across the leg was measured during the steady state of each workload, with most lactate being produced earlier in exercise, net lactate release over the complete exercise period was instead calculated from changes in arterial blood lactate concentration by assuming a distribution volume of 0.6 × body weight. Pyruvate oxidation was calculated as pyruvate production minus lactate production.

Statistical Analysis and Summary of Data

Data were analyzed using two-way ANOVA with repeated measures over time, except where otherwise stated. When a significant *F* ratio was found, the Newman-Keuls post hoc analysis was used to compare means over time and between conditions. The following data were analyzed using a 2-tailed paired dependent-samples Student's *t*-test: relative and absolute $\dot{V}O_{2max}$, body weight, maximal CS activity, PDH_a, GLUT-1, GLUT-4, mean total CoA, mean total carnitine, pyruvate production, pyruvate oxidation, and lactate production. Because we specifically hypothesized that respiratory exchange ratio (RER) and glycogen utilization would decrease as a result of training, these data were analyzed using a 1-tailed paired dependent-samples Student's *t*-test. Data are summarized as means ± SE. Differences were considered significant at $P < 0.05$.

RESULTS

$\dot{V}O_{2max}$ and Respiratory Gas Exchange Responses to Training and Cycling Exercise

No changes were observed in maximum aerobic capacity as a result of submaximal training, as indicated by no changes in muscle CS activity and in absolute or relative $\dot{V}O_{2max}$ (Table 1). Whole body $\dot{V}O_2$ (Table 1) and O_2 uptake across the leg (see Table 5) were not changed by training. In contrast, ventilation ($\dot{V}E$) was lower at 75% $\dot{V}O_{2max}$ and CO_2 uptake ($\dot{V}CO_2$) was lower at 65% $\dot{V}O_{2max}$ in the posttraining condition. After training, RER (Table 2) was lower during cycling at 30 and 65% $\dot{V}O_{2max}$.

Muscle Metabolism

ATP, ADP, and PCr. Muscle ATP and ADP concentrations (Table 3) were unaltered by exercise or as a result of training. Before training, PCr concentration ([PCr]) decreased with each increase in exercise intensity from 30 to 75% $\dot{V}O_{2max}$. However, after training, [PCr] did not decrease further from 65 to 75% $\dot{V}O_{2max}$, being higher at this level of exercise than before training (Table 3).

Glycogen. After training, intramuscular glycogen was greater at rest and throughout each stage of the exercise protocol (Table 3). Glycogen utilization during cycling at 30 and 65% of $\dot{V}O_{2max}$ was not different between the pretraining and posttraining conditions. However, at 75% $\dot{V}O_{2max}$, pretraining glycogen utilization (113.2 ± 14.6 mmol/kg dry wt) was 42.5 mmol/kg dry wt greater ($P < 0.02$) than in the posttraining condition (70.7 ± 13.9 mmol/kg dry wt).

Glucose, G-6-P, F-6-P, and G-3-P. Intramuscular accumulation of glucose was similar between conditions at each stage, except at 75% $\dot{V}O_{2max}$, where it was lower posttraining (Table 3). Muscle G-6-*P* and G-3-*P* contents were also lower after training (Table 3). F-6-*P* (Table 3) was not different between the pre- and posttraining conditions.

Lactate and pyruvate. Muscle lactate concentration ([lactate]) (Fig. 1) was significantly lower after training (main effect $P < 0.03$). Before training, muscle [lactate] increased progressively from 4.3 ± 0.5 mmol/kg dry wt at rest to 9.0 ± 3.8 at 30%, 20.0 ± 5.8 at 65%, and 45.2 ± 6.6 mmol/kg dry wt at 75% $\dot{V}O_{2max}$. In the posttraining condition, muscle [lactate] did not increase above the initial preexercise value of 4.2 ± 0.4 at 30% (4.3 ± 0.3) but then increased to 11.9 ± 3.2 and 17.2 ± 4.4 mmol/kg dry wt at 65 and 75% of $\dot{V}O_{2max}$, respectively. Muscle pyruvate concentration ([pyruvate]) progressively increased to the same extent, with increasing workload in both the pre- and posttraining conditions up to 65% $\dot{V}O_{2max}$ (Fig. 1). At 75% $\dot{V}O_{2max}$, however, posttraining muscle [pyruvate] (0.57 ± 0.07 mmol/kg dry wt) was 32% lower than the pretraining value (0.75 ± 0.06 mmol/kg dry wt) (Fig. 1).

PDH_a. PDH_a was similar before and after training (Table 1). Before training, the preexercise PDH_a level (Fig. 2) was 0.87 ± 0.11 mmol·min^{-1}·kg wet wt^{-1} and increased to 1.81 ± 0.29, 3.58 ± 0.76, and 3.89 ± 0.87 mmol·min^{-1}·kg wet wt^{-1} after cycling at 30, 65, and 75% $\dot{V}O_{2max}$, respectively. After training, PDH_a was not

Table 1. *Maximal oxygen uptake, body weight, maximal enzyme activities, and skeletal muscle GLUT-1 and GLUT-4 glucose transporters before and after short-term training*

Measure	Pretraining	Posttraining
$\dot{V}O_{2max}$, l/min	3.66 ± 0.20	3.77 ± 0.25
Relative $\dot{V}O_{2max}$, ml·kg^{-1}·min^{-1}	45.2 ± 2.0	46.4 ± 3.2
Body weight, kg	81.1 ± 4.1	81.6 ± 3.8
CS, mmol·min^{-1}·kg wet wt^{-1}	15.1 ± 1.44	15.9 ± 1.09
PDH_a, mmol·min^{-1}·kg wet wt^{-1}	3.22 ± 0.40	3.52 ± 0.40
GLUT-1, %	100	99 ± 7
GLUT-4, %	100	143 ± 9*

Values are means ± SE. $\dot{V}O_{2max}$, maximal O_2 uptake; PDH_a, total or maximal activity of pyruvate dehydrogenase complex; CS, maximal citrate synthase activity. GLUT-1 and GLUT-4 are expressed as % of pretraining values, where pretraining values were set to 100%. *Significantly different from pretraining.

Table 2. *Respiratory measures before and after short-term training*

Measure	Training State	Preexercise	$\dot{V}O_{2max}$, % 30	65	75
$\dot{V}O_2$, l/min	Pre	0.36 ± 0.02	1.49 ± 0.07	2.61 ± 0.14	3.09 ± 0.16
	Post	0.29 ± 0.02	1.46 ± 0.07	2.52 ± 0.10	3.03 ± 0.06
$\dot{V}CO_2$, l/min	Pre	0.33 ± 0.02	1.37 ± 0.07	2.52 ± 0.13	2.99 ± 0.13
	Post	0.26 ± 0.03	1.29 ± 0.06	2.36 ± 0.06*	2.89 ± 0.09
$\dot{V}E$ STPD, l/min	Pre	10.8 ± 0.5	31.3 ± 1.6	54.7 ± 3.0	74.5 ± 5.3
	Post	12.0 ± 1.0	28.9 ± 1.2	52.1 ± 1.6	67.4 ± 3.7*
RER	Pre	0.92 ± 0.05	0.92 ± 0.02	0.97 ± 0.02	0.97 ± 0.02
	Post	0.97 ± 0.06	0.88 ± 0.01*	0.94 ± 0.01*	0.95 ± 0.01

Values are means ± SE. $\dot{V}E$ STPD, ventilation expressed at standard temperature and pressure, dry; RER, respiratory exchange ratio; $\dot{V}CO_2$, CO_2 uptake. *Posttraining significantly different from pretraining.

Table 3. *Muscle metabolite measures and muscle wet-to-dry ratios before and after training*

Measure	Training State	Precxercise	$\dot{V}O_{2max}$, % 30	65	75
Glycogen, mmol glucose U/kg dry wt	Pre	454.1 ± 13.9	427.4 ± 21.8	348.4 ± 42.8	235.1 ± 31.5
	Post	600.3 ± 52.8*	591.2 ± 57.2*	520.2 ± 38.9*	449.5 ± 43.4*
Glucose, mmol/kg dry wt	Pre	2.02 ± 0.25	2.73 ± 0.64	3.23 ± 0.62 (5)	4.98 ± 1.00 (5)
	Post	1.82 ± 0.22	2.55 ± 0.47 (5)	2.96 ± 0.60	2.97 ± 0.64*
Glucose 6-phosphate, mmol/kg dry wt	Pre	2.04 ± 0.19	2.48 ± 0.19	3.82 ± 0.39	4.53 ± 0.52 (5)
	Post	1.13 ± 0.22*	1.57 ± 0.22 (5)*	2.86 ± 0.37*	3.34 ± 0.51*
Fructose 6-phosphate, mmol/kg dry wt	Pre	0.17 ± 0.03	0.24 ± 0.05	0.46 ± 0.08	0.38 ± 0.11 (5)
	Post	0.14 ± 0.06	0.15 ± 0.03 (5)	0.38 ± 0.07	0.40 ± 0.11
Glycerol 3-phosphate, mmol/kg dry wt	Pre	0.62 ± 0.08	0.90 ± 0.22	1.45 ± 0.44	2.15 ± 0.36 (5)
	Post	0.62 ± 0.09	0.53 ± 0.05 (5)	0.92 ± 0.19	1.01 ± 0.22*
ATP, mmol/kg dry wt	Pre	24.0 ± 1.0	22.2 ± 0.8	23.1 ± 1.1	23.5 ± 0.6
	Post	22.7 ± 0.6	22.4 ± 0.4	21.7 ± 0.9	23.4 ± 0.4
ADP, mmol/kg dry wt	Pre	2.99 ± 0.39	2.20 ± 0.51	3.54 ± 0.56	4.03 ± 0.46
	Post	3.19 ± 0.56	3.23 ± 0.44	3.61 ± 1.07	3.34 ± 0.37
PCr, mmol/kg dry wt	Pre	86.6 ± 3.4	75.0 ± 5.1	66.9 ± 3.7	40.0 ± 5.6
	Post	83.8 ± 3.8	80.8 ± 2.8 (5)	62.4 ± 4.8	53.7 ± 4.8*
Muscle wet/dry ratio	Pre	4.22 ± 0.10	4.36 ± 0.14	4.50 ± 0.11	4.66 ± 0.06
	Post	4.16 ± 0.08	4.28 ± 0.07	4.51 ± 0.05	4.44 ± 0.07

Values are means ± SE; $n = 6$ for all data except where indicated in parentheses. PCr, phosphocreatine. *Significantly different from pretraining.

different from the pretraining condition at matched time points, being 0.84 ± 0.10 mmol·min^{-1}·kg wet wt^{-1} before cycling exercise and 1.65 ± 0.24, 2.60 ± 0.13, and 3.78 ± 0.36 mmol·min^{-1}·kg wet wt^{-1} at 30, 65, and 75% $\dot{V}O_{2max}$, respectively.

CoA, carnitine, and acetylated forms. Total muscle CoA content increased as a result of short-term training (Table 4). When averaged across all four biopsies within each condition, this amounted to a 25% increase ($P < 0.03$) in the mean total CoA content posttraining (pre- vs. posttraining: 75.4 ± 5.4 vs. 94.6 ± 11.2 mmol/kg dry wt). Acetyl-CoA increased to the same extent with increasing exercise intensity in both conditions (Table 4). Free CoASH behaved in a reciprocal fashion, decreasing as a function of increasing exercise intensity (Table 4). Total muscle carnitine content was not altered by cycling exercise or by training (Table 4), the averages of four biopsies taken before and after training being 20.3 ± 1.6 and 20.3 ± 2.0 mmol/kg dry wt, respectively. Acetylcarnitine followed a similar pattern as acetyl-CoA, increasing as a function of increasing exercise intensity, with the exception that acetylcarnitine accumulation was attenuated at 65 and 75% $\dot{V}O_{2max}$ posttraining (Table 4). Free carnitine decreased in a reciprocal manner and did not differ between

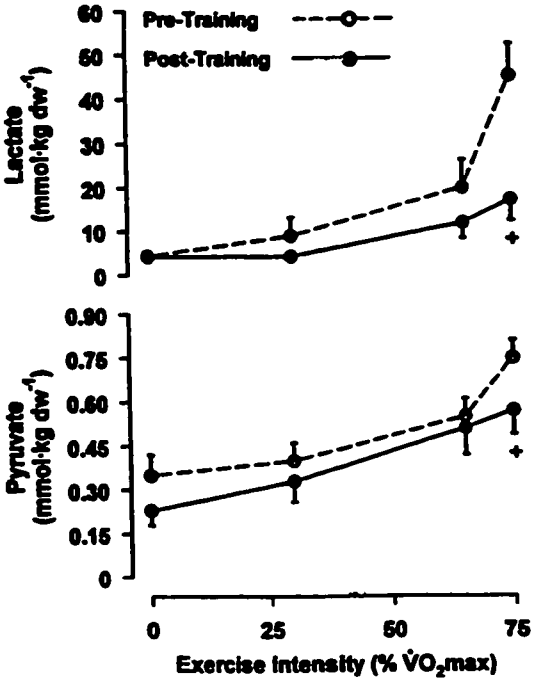

Fig. 1. Muscle lactate and pyruvate concentrations before and after training. $\dot{V}O_{2max}$, maximal O_2 uptake. +Significantly different from pretraining at matched time points. There was a significant main effect over time for posttraining lactate data and for both pre- and posttraining pyruvate data. dw, Dry wt.

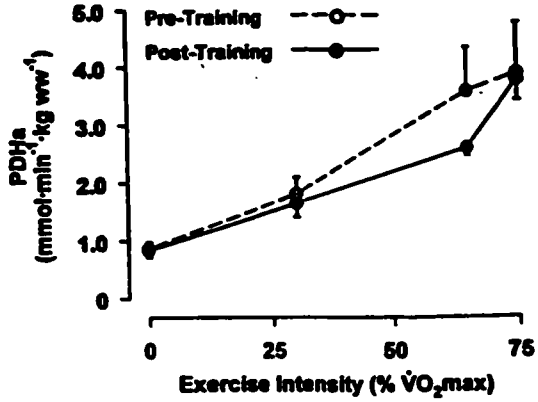

Fig. 2. Muscle pyruvate dehydrogenase activity (PDH_a) before and after training. There was a significant main effect over time for both pre- and posttraining conditions. ww, Wet wt.

Table 4. *Muscle CoA, carnitine, and their acetylated forms before and after training*

Measure	Training State	Preexercise	$\dot{V}O_{2max}$, % 30	65	75
Total CoA, μmol/kg dry wt	Pre	82.2 ± 7.6	89.6 ± 6.4	72.3 ± 5.8	77.4 ± 4.6
	Post	101.7 ± 13.7*	96.1 ± 10.8*	94.2 ± 12.4*	86.5 ± 10.2
Acetyl-CoA, μmol/kg dry wt	Pre	8.9 ± 1.9	10.4 ± 1.7	19.8 ± 1.9	23.9 ± 2.5
	Post	7.0 ± 0.9	11.2 ± 1.3	17.6 ± 2.6	25.9 ± 2.6
Free CoASH, μmol/kg dry wt	Pre	73.3 ± 6.7	59.2 ± 6.0	52.5 ± 5.4	53.5 ± 3.3
	Post	94.7 ± 13.8*	84.9 ± 10.4*	76.5 ± 11.6*	60.5 ± 8.4
Total carnitine, mmol/kg dry wt	Pre	20.5 ± 1.3	18.7 ± 1.7	20.2 ± 1.7	21.7 ± 2.0
	Post	20.5 ± 2.1	20.9 ± 2.3	19.9 ± 2.0	20.0 ± 1.9
Acetylcarnitine, mmol/kg dry wt	Pre	3.1 ± 0.8	4.2 ± 0.6	11.3 ± 1.9	15.8 ± 2.4
	Post	2.1 ± 0.6	3.5 ± 0.3	7.8 ± 1.0*	12.6 ± 1.1*
Free carnitine, mmol/kg dry wt	Pre	16.3 ± 1.9	14.3 ± 1.3	9.2 ± 1.3	6.0 ± 1.1
	Post	18.3 ± 1.8	16.4 ± 1.9	12.2 ± 1.9*	7.0 ± 0.9

Values are means ± SE; $n = 6$ for all measures. *Significantly different from pretraining.

conditions, except at 65% $\dot{V}O_{2max}$ posttraining, when it was greater.

Metabolite Exchange Across the Leg

Leg blood flow and O_2 uptake. Leg blood flow progressively increased from preexercise (i.e., rest) to 65% $\dot{V}O_{2max}$ to the same extent in the pre- and posttraining conditions (Table 5). However, at 75% $\dot{V}O_{2max}$, leg blood flow was 0.84 l/min greater after training. O_2 uptake across the leg was not altered by training (Table 5).

Blood lactate. At rest and during cycling at 30% $\dot{V}O_{2max}$, arterial [lactate] (Table 5) did not differ within or between conditions, but at 65 and 75% $\dot{V}O_{2max}$, there was a progressive increase in both conditions. However, after training, arterial [lactate] values were lower at 65 and 75% $\dot{V}O_{2max}$ (Table 5).

During rest and cycling at 30% $\dot{V}O_{2max}$, there were no differences in the net release of lactate between the pre- and posttraining conditions (Fig. 3). At 65 and 75% $\dot{V}O_{2max}$, net lactate release was less at posttraining (Fig. 3).

Blood glucose and muscle glucose transporters. Arterial glucose concentration ([glucose]) was similar at all power outputs in both pre- and posttraining conditions (Table 5). Leg glucose uptake (Fig. 3) did not differ between conditions. Training resulted in a 43% increase in GLUT-4 glucose transporter content but no change in GLUT-1 content (Table 1).

Blood free fatty acids and glycerol. Arterial free fatty acid (FFA) concentrations were not altered by training or as a result of the exercise protocol (Table 5). No changes were observed between conditions in the net uptake or release of FFA before and during cycling exercise (Fig. 4).

Arterial glycerol concentration increased with increasing exercise intensity, but there were no differences between the pre- and posttraining conditions (Table 5). Similarly, there were no significant differences in glycerol release between conditions (Fig. 4).

Pyruvate production, oxidation, and conversion to lactate. During cycling exercise at 75% $\dot{V}O_{2max}$, pyruvate production was reduced by 33% posttraining, whereas pyruvate oxidation decreased by 24%; lactate production was reduced by 59% (Table 6).

DISCUSSION

The present study examined the effects of short-term training on some biochemical responses to 45 min of

Table 5. *Arterial concentrations of blood-borne substrates, leg blood flow, and leg O_2 uptake before and after training*

Measure	Training State	Preexercise	$\dot{V}O_{2max}$, % 30	65	75
Lactate, mmol/l	Pre	0.70 ± 0.11	1.06 ± 0.15	3.57 ± 0.45	5.66 ± 0.67
	Post	0.67 ± 0.04	0.84 ± 0.10	2.49 ± 0.34*	3.76 ± 0.41*
Glucose, mmol/l	Pre	5.16 ± 0.20	4.94 ± 0.18	4.59 ± 0.18	4.50 ± 0.15
	Post	4.93 ± 0.15	4.87 ± 0.19	4.50 ± 0.16	4.33 ± 0.09
FFA, mmol/l	Pre	0.74 ± 0.14	0.58 ± 0.09	0.53 ± 0.06	0.63 ± 0.09
	Post	0.57 ± 0.10	0.46 ± 0.05	0.42 ± 0.05	0.46 ± 0.07
Glycerol, μmol/l	Pre	64 ± 14	69 ± 14	96 ± 15	138 ± 26
	Post	47 ± 10	71 ± 9	109 ± 14	147 ± 21
Leg blood flow, l/min	Pre	0.90 ± 0.21	3.64 ± 0.52	4.16 ± 0.52	4.73 ± 0.46
	Post	0.68 ± 0.19	3.29 ± 0.31	4.36 ± 0.34	5.57 ± 0.36*
Leg O_2 uptake, mmol/min	Pre	2.1 ± 0.42	17.8 ± 3.85	22.4 ± 4.44	21.7 ± 3.30
	Post	1.6 ± 0.22	15.0 ± 1.61	19.8 ± 2.54	24.5 ± 2.36

Values are means ± SE; $n = 7$ for all measures. FFA, free fatty acids. Leg blood flow and O_2 uptake are for one leg only. *Significantly different from pretraining.

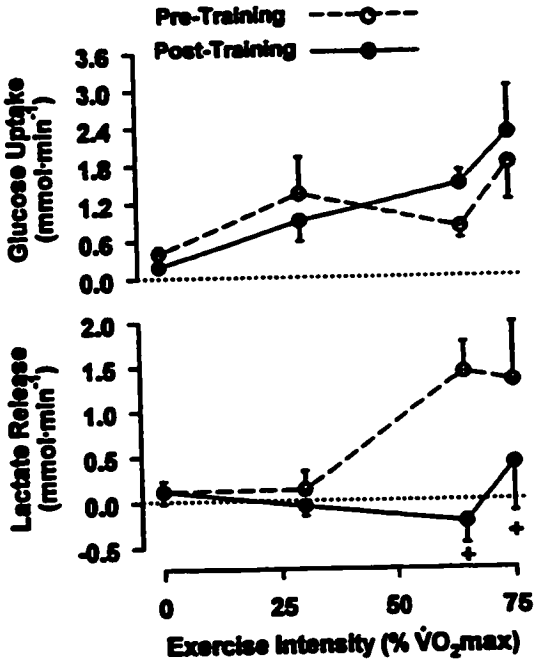

Fig. 3. Glucose uptake and lactate release across the leg before and after training. *Significantly different from pretraining at matched time points. There was a significant main effect over time for both pre- and posttraining glucose data and posttraining lactate data.

continuous exercise, consisting of 15 min each at 30, 65, and 75% $\dot{V}O_{2max}$. Although there were no significant differences in glycogen utilization and lactate production at 30% $\dot{V}O_{2max}$, lactate accumulation in muscle and blood was lower at 65 and 75% $\dot{V}O_{2max}$ after training. The most marked changes associated with training were observed at the highest load; there was a 33%

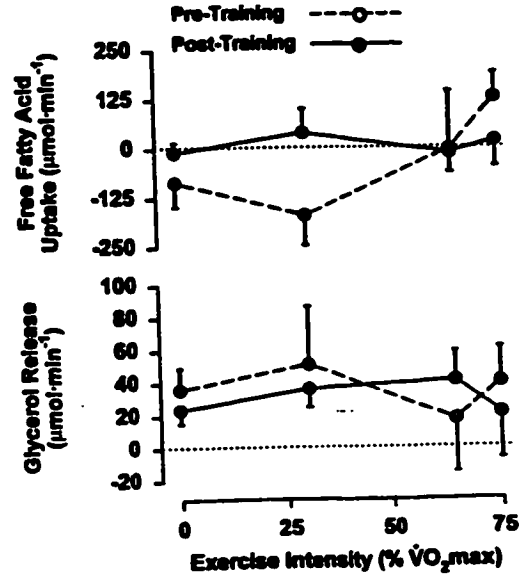

Fig. 4. Free fatty acid uptake and glycerol release across the leg before and after training.

Table 6. *Pyruvate metabolism at 75% $\dot{V}O_{2max}$ before and after training*

Measure	Pretraining	Posttraining
Pyruvate production	248 ± 28.3	167 ± 8.2*
Pyruvate oxidation	184 ± 20.4	141 ± 8.6*
Lactate production	64 ± 13.1	26 ± 1.8*

Values are means ± SE; n = 5. Units are mmol/leg. *Posttraining value is less than pretraining.

decrease in pyruvate production, a 59% decrease in pyruvate conversion to lactate, and a 24% decrease in pyruvate oxidation (Table 6). The decrease in pyruvate production at this load was explained by a 30% decrease in glycogen degradation and a small, nonsignificant reduction in glucose utilization. Pulmonary and leg O_2 uptake and the activity of mitochondrial CS were unchanged. The total activity of the PDH_t and the proportion in its active form (PDH_a) were unchanged. An increase in the total muscle content of CoA was observed, and [PCr] was also higher after training.

Previous studies of short- and long-term training have suggested that the decrease in muscle and blood lactate concentrations is the result of both a decrease in lactate production from glycolysis and an increase in its metabolic clearance from the circulation. Isotope tracer studies in rats (11, 12) and humans (19, 22) have demonstrated that reductions in blood lactate with training are associated with increases in clearance of lactate from the plasma pool rather than reductions in its appearance into plasma from muscle. In the present study, after training, lower blood and muscle [lactate] values at 75% $\dot{V}O_{2max}$ during steady state were associated with a lower rate of glycolysis, and efflux of lactate from muscle (appearance) was associated with a smaller increase in pyruvate oxidation (clearance). The lower activation of glycogenolysis and glycolysis posttraining may be related to the higher [PCr], itself an indication of higher phosphorylation potential of the high-energy phosphate pool (ATP, ADP, AMP, and PCr), concomitant with a lower PO_4^{2-} concentration (8, 15, 22, 24). PO_4^{2-} is known to act as a substrate for phosphorylase a, having a relatively high Michaelis-Menten constant that is decreased by increases in AMP concentration (27). The phosphorylation potential will thus have a direct effect on the rate of glycogen degradation, as well as a direct influence on the activity of phosphofructokinase via the ATP/ADP ratio.

An increase in phosphorylation potential has been observed, both after short-term training (8, 15, 22, 24) and when the availability of FFA for muscle metabolism is increased by infusion of fat emulsion (13) and caffeine ingestion (28). In all these situations, increased [PCr] and decreased calculated concentrations of free ADP and AMP have been accompanied by glycogen sparing during exercise. Similar findings were reported by From et al. (14) in studies of heart muscle. Thus, when FFA availability is increased, oxidative phosphorylation is enhanced, glycogen utilization and lactate formation decrease, and there is a lower PCr degradation.

A reduction in glycogenolytic rate during exercise after training was associated with reductions in pyruvate oxidation. As this reduction was unaccompanied by changes in PDH_a (Table 1) or PDH_a (Fig 2), we infer that it is due to a reduction in pyruvate availability secondary to a lower rate of glycogenolysis. The decrease in pyruvate oxidation may be compensated for by an increase in FFA oxidation. This argument is supported by calculations of the amount of FFA (palmitate) that would be needed to account for this difference in oxidative ATP production, showing that only 4.5 mmol of palmitate would be required for each leg during 15 min of exercise at 75% $\dot{V}O_{2max}$ posttraining. Small increases in fat oxidation may not have a significant effect on measured RER (seen at 30 and 65% $\dot{V}O_{2max}$ but not at 75% $\dot{V}O_{2max}$), because this measurement is too insensitive to reveal such small increases in fat oxidation. Increases in fat oxidation during exercise posttraining have been observed in many studies (17, 20), including those employing short periods of training (8, 9, 21, 23, 24). The 25% increase in total CoA observed in the present study may have an impact on fatty acid utilization, because both the acylation and the transport of fatty acids are dependent on CoA availability.

The results of isotope studies carried out pre- and posttraining (19, 22) were interpreted to indicate an increase in pyruvate oxidation posttraining. Because PDH_a catalyzes the flux-generating step of pyruvate oxidation by the TCA cycle (2), and in view of the isotope studies, we hypothesized that lower net lactate production after training resulted from an increase in PDH_a. Although we did not find changes in PDH_a to account for reductions in lactate accumulation posttraining, our measurements were made after 15 min of cycling at a given power; thus it is still possible that PDH_a or the allosteric regulators of PDH_a transformation were altered during the transition to a steady state, from rest to exercise or from one load to a higher one. Recently, Timmons and co-workers (30, 31) employed the isolated dog gracilis model to study the effects of increases in the rate of PDH activation by dichloroacetate at the onset of muscle contraction. They found that PDH activation, with concomitant increases in acetylcarnitine concentration before muscle stimulation, resulted in less lactate production during ischemic contractions. However, in the present study after training, acetylcarnitine accumulation was less just before the onset of the highest exercise intensity (75% $\dot{V}O_{2max}$), in which the reduction in lactate production was greatest. Thus the association between increases in fat utilization and reductions in lactate production after short-term training observed in the present study may be mediated through different mechanisms from those in the animal model used by Timmons et al. (31).

Isotopic studies of the effects of training suggested that training was associated with an increase in lactate clearance from the plasma pool, with no change in the appearance rate of lactate into the pool. This finding is in contrast to the results of the present study, which utilized measurements of intramuscular concentration of metabolites with arteriovenous lactate concentrations and flow. These measurements suggested that there was both a reduction in muscle lactate production and a lower efflux of lactate from muscle. A narrowing of the concentration gradient for lactate between arterial blood and muscle is presumably accounted for by an increase in the content of the sarcolemmal monocarboxylate transporter (MCT1) found in the present subjects and reported elsewhere (5). This increase in MCT1 would increase bidirectional exchange of lactate between the plasma and intracellular compartments and also account for the increases in lactate clearance from the plasma pool that were observed in isotope studies.

In the present study, leg blood flow during exercise was increased by training (Table 5). This finding is consistent with increased capillary density associated with type IIa fibers recently reported (16), which provides a morphological basis for the physiological change observed in our subjects (Table 5). Thus it is possible that the uptake of exogenous blood-borne substrates and/or endogenous fat utilization may be enhanced during the non-steady-state period at the onset of cycling exercise after training.

Previous studies using short-term training to examine the early adaptations of muscle glucose metabolism during submaximal aerobic cycling have reported greater GLUT-4 content (25) and maximal hexokinase activity (9, 24). Although such changes should serve to increase plasma glucose uptake and utilization, tracer studies (9, 21, 23) have paradoxically reported a reduction in whole body plasma glucose turnover during cycling exercise after training. Lower rates of glucose appearance from hepatic gluconeogenesis and disappearance from the plasma pool were thought to result from lower hormonally induced hepatic glucose production and lower glucose utilization by trained muscle (9, 21, 23), respectively. However, the use of labeled glucose tracers to determine glucose turnover may underestimate glucose utilization, because labeled glucose can be converted to metabolites that are not oxidized (18). Furthermore, short-term training may increase this conversion, creating the impression of lower glucose utilization posttraining. In the present study, there were no differences in the preexercise rates of glucose uptake (Fig. 3), arterial glucose concentrations (Table 5), or muscle glucose content (Table 3), but muscle glycogen levels (Table 3) were considerably greater posttraining. Whereas there were no changes in the content of the GLUT-1 glucose transporter (Table 1), GLUT-4 content (Table 1) increased by 43% after training. Thus greater preexercise glycogen levels posttraining can be attributed to enhanced glycogen synthesis mediated by greater insulin-stimulated glucose uptake by GLUT-4 and greater maximal activities of hexokinase and glycogen synthase during the recovery periods between each training session. This suggestion is supported by a previous report demonstrating similar changes after 7 consecutive days of chronic low-frequency electrical stimulation (6).

Summary and Conclusions

This study examined mechanisms of altered muscle lactate production during cycling exercise in humans

after 7–8 days of training on a cycle ergometer at 60% $\dot{V}O_{2max}$. Training resulted in a significant reduction in net muscle lactate production during steady-state cycling exercise at 65 and 75% $\dot{V}O_{2max}$ without change in muscle oxygen consumption during exercise or maximum muscular oxidative potential. Lower muscle lactate production after training resulted from the attenuation of glycogenolysis without a change in PDH_a, leading to improved matching of glycolytic and PDH_a fluxes. Changes leading to lower muscle lactate production coincided with improved maintenance of cell phosphorylation potential and greater use of fat as a fuel.

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APPENDIX C:

EFFECTS OF INCREASED FAT AVAILABILITY ON FAT-CARBOHYDRATE INTERACTION DURING PROLONGED EXERCISE IN MEN

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Effects of increased fat availability on fat-carbohydrate interaction during prolonged exercise in men

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Odland, L. Maureen, George J. F. Heigenhauser, Denis Wong, Melanie G. Hollidge-Horvat, and Lawrence L. Spriet. Effects of increased fat availability on fat-carbohydrate interaction during prolonged exercise in men. *Am. J. Physiol.* 274 (*Regulatory Integrative Comp. Physiol.* 43): R894–R902, 1998.—The study examined the existence and regulation of fat-carbohydrate interaction during low- and moderate-intensity exercise. Eight males cycled for 10 min at 40% and 60 min at 65% maximal O_2 uptake ($\dot{V}O_{2max}$) while infused with either Intralipid and heparin (Int) or saline (Con). Before exercise, plasma arterial free fatty acid (FFA) was 0.69 ± 0.04 mM (Int) vs. 0.25 ± 0.04 mM (Con). Muscle biopsies were taken at rest and at 10, 20, and 70 min of exercise. Arterial and femoral venous blood samples and expired gases were collected simultaneously throughout exercise, and blood flow was estimated from pulmonary O_2 uptake and the leg arterial-venous O_2 difference. Respiratory exchange ratio was higher in Con (0.94 ± 0.01) compared with Int (0.91 ± 0.01). Mean net leg FFA uptake was higher in Int (0.16 ± 0.03 vs. 0.04 ± 0.01 mmol/min), and net lactate efflux was reduced (Int, 1.55 ± 0.36 vs. Con, 3.07 ± 0.47 mmol/min). Leg net glucose uptake was unaffected by Int. Muscle glycogen degradation was 23% lower in Int [230 ± 29 vs. 297 ± 36 mmol glucosyl units/kg dry muscle (dm)]. Pyruvate dehydrogenase activity in the *a* form (PDH*a*) was lower during Int (1.61 ± 0.17 vs. 2.22 ± 0.24 mmol·min^{-1}·kg wet muscle^{-1}), and muscle citrate was higher (0.59 ± 0.04 vs. 0.48 ± 0.04 mmol/kg dm). Muscle lactate, phosphocreatine, ATP, acetyl-CoA, acetyl-carnitine, and P_i were unaffected by Int. Calculated free AMP was significantly lower in Int compared with Con at 70 min of exercise (3.3 ± 0.8 vs. 1.5 ± 0.3 μmol/kg dm). The high FFA-induced reduction in glycogenolysis and carbohydrate oxidation at 65% $\dot{V}O_{2max}$ appears to be due to regulation at several sites. The reduced flux through phosphorylase and phosphofructokinase during Int may have been due to reduced free AMP accumulation and increased cytoplasmic citrate. The mechanism for reduced PDH transformation to the *a* form is unknown but suggests reduced flux through PDH.

glucose-fatty acid cycle; glycogen phosphorylase; pyruvate dehydrogenase; citrate; acetyl-coenzyme A; free adenosine monophosphate; phosphofructokinase

CURRENT CONTROVERSY EXISTS regarding the mechanisms that regulate substrate choice for oxidation in skeletal muscle during aerobic exercise (for review, see Ref. 36). The concept of the glucose-fatty acid (G-FA) cycle was originally introduced by Randle et al. (28, 29) to explain the interaction between carbohydrate (CHO) and fat metabolism. It was proposed that the increased delivery of free fatty acids (FFA) to muscle tissue enhanced the rate of fat oxidation, which led to increased acetyl-CoA and citrate production and resulted in downregulation of the rate-limiting carbohydrate metabolizing enzymes, pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK), respectively. Decreased glycolytic activity also resulted in glucose 6-phosphate (G-6-*P*) accumulation, which in turn decreased hexokinase activity and reduced glucose uptake (29).

Much of the original support for the G-FA cycle was obtained from perfused contracting heart muscle or resting diaphragm muscle bathed in a high-FFA concentration ([FFA]) medium (14, 15, 28, 29). Contracting heart and resting diaphragm muscles perform constant duties, which requires the majority of oxidizable substrate to come from exogenous sources. Conversely, in skeletal muscles other than diaphragm, the high energy demand during exercise dictates that fuel must also be provided from the endogenous glycogen store. Recent studies in humans have shown that CHO use is reduced in the presence of high [FFA], but the classic regulation of the G-FA cycle in skeletal muscle during 15 min of intense aerobic cycling [80–85% maximal O_2 uptake ($\dot{V}O_{2max}$)] does not occur (12, 13). High FFA provision did not alter muscle acetyl-CoA and citrate levels and had no effect on the transformation of PDH to the more active *a* form. Rather, downregulation appeared to exist at the level of glycogen phosphorylase (Phos), not by altering the transformation to the more active *a* form but via posttransformational regulation. Enhanced FFA availability and oxidation appeared to provide a better match between energy demand and provision at the onset of exercise, which led to reduced accumulations of free AMP (allosteric activator of Phos *a*) and P_i (substrate for Phos) and a resultant lower flux through Phos (13).

However, because smaller alterations in the energy status of the cell, and therefore in the requirement for glycogenolysis, would be expected to occur at the onset of lower-intensity exercise, it is possible that the regulation of fat/CHO interaction at lower power outputs (i.e., <85% $\dot{V}O_{2max}$) may occur as classically proposed. Very few studies have examined both the presence and regulation of the G-FA cycle during low-to-moderate aerobic exercise in humans (20, 21).

The purpose of this study was to enhance FFA provision to human skeletal muscle during low (40% $\dot{V}O_{2max}$)- and moderate (65% $\dot{V}O_{2max}$)-intensity aerobic exercise and determine *1*) whether CHO sparing occurred (reduced muscle glycogen use and/or leg glucose uptake) and *2*) whether regulation of CHO sparing was as classically proposed. On the basis of our previous findings during intense aerobic exercise (12, 13), it was

hypothesized that elevated FFA before exercise would result in significant sparing of muscle glycogen with no concomitant change in muscle citrate, acetyl-CoA, or PDH activation and no reduction in leg glucose uptake compared with control.

METHODS

Subjects. Eight males volunteered to participate in the study. Three subjects were well trained, three were highly active, and two were untrained (means ± SD: age, 24.5 ± 1.6 yr; height, 175 ± 9 cm; mass, 76.1 ± 9.6 kg). Written consent was obtained after the experimental procedures, and possible risks and benefits were explained. The study was approved by the Human Ethics Committees of both universities.

Preexperimental protocol. All participants initially performed an incremental $\dot{V}O_{2max}$ test on a cycle ergometer. The mean $\dot{V}O_{2max}$ for the group was 3.96 ± 0.18 l/min. Each subject also participated in a practice trial to determine the power outputs required to elicit 40 and 65% $\dot{V}O_{2max}$. Daily food records were kept for 48 h preceding each test session, and subjects were instructed to refrain from caffeine consumption and intense physical activity for 24 h before testing. No difference was observed in the subjects' diets 48 h before each trial [CHO ingestion before the control and Intralipid (Int) trials was 55–60% of total caloric intake].

Experimental protocol. Each subject participated in two experimental trials, separated by 1–2 wk. On the morning of each trial day, subjects reported to the laboratory having eaten a meal high in CHO content 1–2 h before arrival. In addition, all participants consumed 1–2 bagels and 250–500 ml Gatorade −2–3 h before exercise to ensure low resting levels of plasma FFA. Subjects cycled for 10 min at 40% $\dot{V}O_{2max}$ and 60 min at 65–70% $\dot{V}O_{2max}$ (Fig. 1) while receiving, in random order, an infusion of 20% Int or saline of similar volume (Con). Int (Clintec Nutrition, Mississauga, ON) is composed of 20% soybean oil (50% linoleic acid), 1.2% egg phospholipids, and 2.25% glycerol. The Int infusion rate was 100 ml/h at rest (30 min) and 75 ml/h during exercise. A total of 2,000 U of sterile heparin was administered (Fig.1) during Int to facilitate hydrolysis of the infused triacylglycerol to FFA. The Int infusion ended after 40 min of exercise.

Before exercise, the radial artery was catheterized percutaneously with a Teflon catheter (20 gauge, 3.2 cm; Baxter, Irvine, CA) after local anesthesia with 0.5 ml of 2% lidocaine (without epinephrine) as previously described (3). The femoral vein was catheterized percutaneously (Thermodilution catheter, model no. 93-135-6F, Baxter) with the use of the Seldinger technique after administration of 3–4 ml lidocaine (3). A third catheter, in the antecubital or basilic arm vein, was used for Int or Con infusion. Catheters were maintained patent with nonheparinized isotonic saline.

Arterial and femoral venous resting blood samples (~9 ml each) were taken simultaneously at −30 min (Fig. 1), after which infusion was begun. In the following 30 min, one leg was prepared for percutaneous needle biopsy of the vastus lateralis. Three incisions of the skin were made through to the deep fascia under local anesthesia (2% lidocaine without epinephrine) as described by Bergstrom (2). Immediately before exercise (0 min), a resting muscle sample was obtained with the subject lying on a bed. The subject was then seated on the cycle ergometer (Quinton Excalibur; Quinton, Seattle, WA), and catheter lines were adjusted such that the subject could move freely without discomfort. A second set of preexercise blood samples were then drawn (0 min, Fig. 1). The time lapse between the preexercise biopsy and the onset of exercise was ~2 min.

$\dot{V}O_2$, CO_2 output ($\dot{V}CO_2$), and respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$) were determined throughout exercise using a metabolic cart (Quinton Q-plex 1, Quinton). Arterial and femoral venous blood samples and muscle biopsies from the vastus lateralis were taken at the time points indicated in Fig. 1.

Muscle sampling. Muscle samples were frozen immediately in liquid N_2. A small piece (10–35 mg) was chipped from each biopsy (under liquid N_2) for measurement of the fraction of PDH in the more active α form (PDHα) (8, 27). The remainder of the sample was freeze-dried, dissected free of blood and connective tissue, and powdered. A portion of powdered muscle (4–6 mg) from the resting (0 min) and 70-min exercise biopsies was alkaline extracted and used for enzymatic determination of muscle glycogen (18). A further portion of dry muscle (~5 mg) was extracted in 0.5 M perchloric acid and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$, and analyzed for acetyl-CoA, acetyl-carnitine (6), phosphocreatine, creatine (Cr), citrate, ATP, and lactate (1, 18). Muscle metabolites were normalized to the highest total Cr content for a given individual [$\bar{x}$ total Cr = 130.6 ± 4.4 mmol/kg dry muscle (dm)] to correct for nonmuscle contamination. Free contents of ADP and AMP were calculated from the near-equilibrium Cr kinase and adenylate kinase reactions, respectively, as previously described (13), and H^+ concentration ($[H^+]$) was determined from the regression equation between lactate accumulation and $[H^+]$ for dynamic exercise (34). Free phosphate content (P_i) at rest was assumed to be 10.8 mmol/kg dm (7). This value was added to the difference between rest and exercise phosphocreatine contents to estimate exercise $[P_i]$.

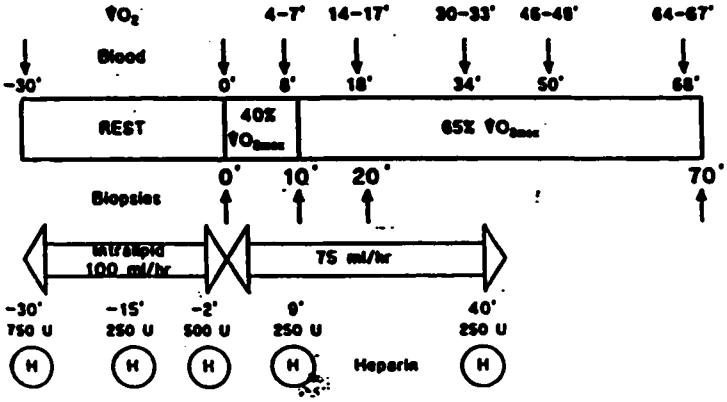

Fig. 1. Schematic diagram of experimental protocol. $\dot{V}O_{2max}$, maximal O_2 uptake. Prime indicates minutes.

Blood sampling and analysis and calculation of blood flow. Blood samples (~9 ml) were drawn from the radial artery and femoral vein in heparinized plastic syringes and placed on ice. Each blood sample was analyzed for O_2 and CO_2 contents (Cameron Instrument, Port Arkansas, TX), hemoglobin (OSM3 Hemoximeter; Radiometer, Copenhagen, Denmark), and hematocrit (centrifugation method).

Plasma for FFA determination (Wako NEFA C test kit, Wako Chemical) was obtained by immediate centrifugation of heparinized blood and incubation at 56°C in the presence of NaCl (13). This procedure denatures the lipoprotein lipase released into the blood by heparin injection, and thus avoids falsely elevated FFA measures. A 200-μl aliquot of whole blood was deproteinized in 1.0 ml of 0.6 M $HClO_4$, and the supernatant was used for fluorometric determination of whole blood glucose, lactate, and glycerol (1). Leg blood flow was not measured in this study as it has been demonstrated to be unaffected by Int and heparin infusion (17). However, leg blood flow was estimated from the pulmonary O_2 uptake and the leg arterial-venous (a-v) O_2 difference as described by Jorfeldt and Wahren (22). The leg respiratory quotient (RQ) was calculated from

$$\frac{\text{leg } CO_2 \text{ production (blood flow} \times \text{v-a } CO_2 \text{ content difference)}}{\text{leg } O_2 \text{ uptake (blood flow} \times \text{a-v } O_2 \text{ content difference)}}$$

Blood substrate uptake and release data were calculated according to the Fick equation: blood flow × a-v substrate concentration difference.

Statistical analyses. Experimental data are presented as means ± SE and were analyzed by two-way analysis of variance with repeated measures over time. When a significant F ratio was obtained, the Tukey post hoc test was used to compare means. Pre- and postexercise glycogen contents and glycogen utilization during exercise were compared between conditions using a paired-samples t-test. Significance was accepted at $P < 0.05$.

RESULTS

Respiratory gas exchange, blood gases, and blood flow. Whole body $\dot{V}O_2$ was similar in the two conditions (Table 1). However, RER was lower throughout the elevated FFA condition: 0.92 ± 0.02 vs. 0.89 ± 0.01 at 40% $\dot{V}O_{2max}$ and 0.94 ± 0.01 vs. 0.91 ± 0.01 at 65% $\dot{V}O_{2max}$ in Con and Int, respectively. The blood a-v O_2 content difference and leg blood flow data were not different between trials. Calculated RQ across the working leg muscles was consistently lower during Int, but this difference was not statistically significant (Table 1).

Blood metabolites. Int infusion led to an elevation in plasma arterial FFA from 0.25 ± 0.03 to 0.69 ± 0.04 mM at rest (Fig. 2*A*). During exercise, FFA was significantly higher during Int at all time points and peaked at 0.89 ± 0.05 mM at 50 min. In the Con trial, FFA was significantly elevated above rest only at 68 min (Fig. 2*A*). Changes in plasma FFA during Int were reflected by significant increases in whole blood glycerol (Fig. 2*B*). Arterial glycerol levels during Con were significantly less than Int at all time points and increased over time such that values were greater than rest by 50 min exercise (Fig. 2*B*). No significant differences occurred in whole blood arterial glucose or lactate between conditions (Table 2). Blood glucose decreased at all exercise time points, and blood lactate increased above rest levels at both power outputs during both conditions (Table 2). Blood data were not corrected for fluid shifts during exercise, as no differences in hemoglobin concentration or hematocrit occurred between conditions (data not shown).

Blood metabolite exchange. Elevated arterial plasma FFA resulted in an approximately fourfold increase in FFA uptake during Int over Con (Table 3). Interestingly, the increased arterial glycerol levels during Int appeared to reverse glycerol exchange from net release during Con to net uptake during Int. Int infusion resulted in a significant reduction in the rate of lactate efflux at 18 and 34 min of exercise compared with Con (Fig. 3*A*) but had no effect on glucose uptake (Fig. 3*B*).

Muscle metabolites. Resting glycogen levels were not different between conditions (Table 4). During exercise, muscle glycogen degradation was 297 ± 36 mmol

Table 1. *Respiratory variables, blood a-v O_2 content difference, leg blood flow, and leg RQ during 70-min cycle exercise at 40 and 65% $\dot{V}O_{2max}$ following Intralipid infusion or control*

	40% $\dot{V}O_{2max}$ 8 min	65% $\dot{V}O_{2max}$ 18 min	34 min	50 min	68 min	Σ
$\dot{V}O_2$, l/min						
Con	1.56 ± 0.09	2.36 ± 0.11	2.55 ± 0.12	2.60 ± 0.13	2.67 ± 0.13	2.54 ± 0.06
Int	1.58 ± 0.09	2.67 ± 0.09	2.57 ± 0.09	2.66 ± 0.10	2.70 ± 0.12	2.57 ± 0.06
RER						
Con	0.92 ± 0.02	0.99 ± 0.01	0.94 ± 0.01	0.93 ± 0.01	0.92 ± 0.01	0.94 ± 0.01
Int	0.89 ± 0.01	0.95 ± 0.01	0.90 ± 0.01	0.89 ± 0.01	0.90 ± 0.01	0.91 ± 0.01*
a-vO_2 diff, ml/100 ml						
Con	14.51 ± 0.6	16.62 ± 0.41	17.2 ± 0.37	17.93 ± 0.36	17.36 ± 0.40	17.2 ± 0.21
Int	15.43 ± 0.5	17.00 ± 0.78	17.35 ± 0.68	17.37 ± 0.67	16.64 ± 0.71	17.09 ± 0.4
LBF, l/min						
Con	3.48 ± 0.23	4.76 ± 0.23	5.01 ± 0.26	4.92 ± 0.31	5.20 ± 0.29	4.97 ± 0.14
Int	3.28 ± 0.15	4.69 ± 0.11	4.99 ± 0.13	5.19 ± 0.18	5.52 ± 0.31	5.10 ± 0.12
Leg RQ						
Con	0.91 ± 0.05	1.03 ± 0.03	0.99 ± 0.03	0.97 ± 0.03	0.99 ± 0.03	1.00 ± 0.02
Int	0.87 ± 0.05	0.93 ± 0.04	0.93 ± 0.03	0.95 ± 0.02	0.97 ± 0.02	0.94 ± 0.01

Data are means ± SE; n = 7. RER, respiratory exchange ratio; a-vO_2 diff, arterial-venous O_2 content difference; LBF, leg blood flow; RQ, respiratory quotient. $\dot{V}O_{2max}$, maximal O_2 uptake; Int, Intralipid infusion; Con, control. *Significantly different from Con.

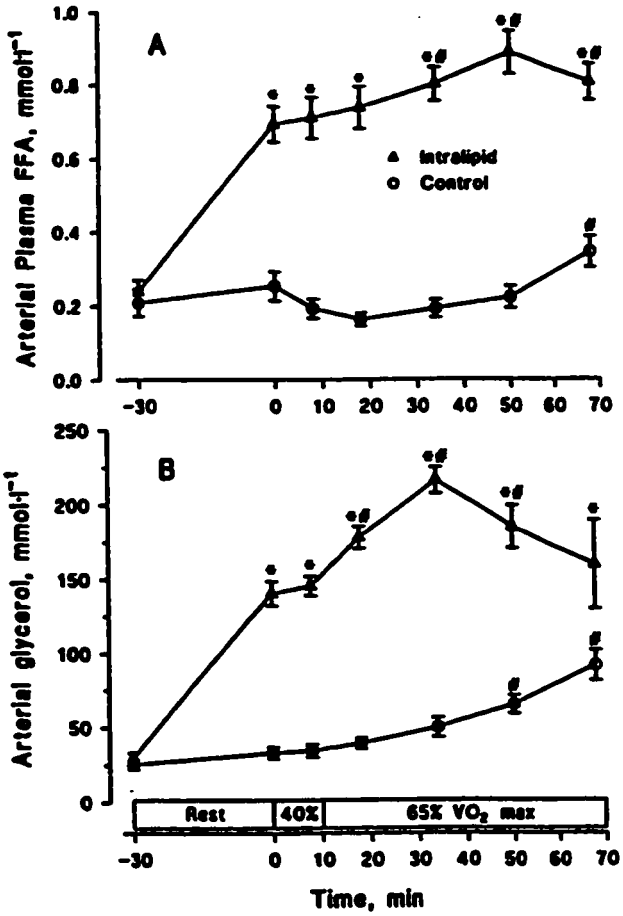

Fig. 2. Plasma arterial free fatty acid (FFA; *A*) and glycerol (*B*) concentrations at rest and during 70 min of cycling at 40 and 65% $\dot{V}O_{2max}$ with Intralipid infusion or control. Values are means ± SE. *Significantly different from control; #significantly different from 0 min within same condition.

glucosyl units·min^{-1}·kg dm^{-1} in the Con trial vs. 230 ± 29 mmol·min^{-1}·kg dm^{-1} following Int infusion. This corresponded to a 22.6% sparing during Int.

Muscle ATP remained constant throughout exercise in both conditions (Table 5). Phosphocreatine decreased significantly from rest during exercise at 65% $\dot{V}O_{2max}$, but was not affected by Int. Similarly, muscle lactate increased above rest levels at 65% $\dot{V}O_{2max}$ and revealed no differences between conditions. Muscle acetyl-CoA and acetyl-carnitine both increased from rest to 40% $\dot{V}O_{2max}$, and from 40 to 65% $\dot{V}O_{2max}$, but were unaffected by Int infusion (Table 5). Elevation of FFA levels with Int infusion resulted in a concomitant increase in muscle citrate at rest, which remained elevated throughout exercise (Fig. 4).

Calculated values for free ADP, AMP, and P_i were similar between conditions at rest and at 10 and 20 min exercise, but were significantly reduced at 68 min during exercise in the presence of elevated FFA (Fig. 5, *A* and *B*). P_i accumulation was also reduced at 68 min during Int, but this difference failed to reach significance (Fig. 5*C*).

The transformation of PDH into the more active PDH*a* was significantly reduced at all exercise time points during the Int trial (Fig. 6).

DISCUSSION

The present study demonstrated that elevated plasma FFA levels produced a significant reduction in glycogenolysis (23%) during 68 min of moderate cycle exercise. This result is consistent with previous investigations (9, 38) in which subjects exercised at 70% $\dot{V}O_{2max}$ for 30 and 60 min and used 40 and 28% less muscle glycogen compared with low-fat control trials. In the present study, the RER (pulmonary) and RQ data (measured across the working leg muscles) also indicated reduced CHO use and increased fat oxidation in the Int trial.

Classic regulation of the G-FA cycle is based on the premise that increased availability of FFA will enhance the rate of fat oxidation and lead to increased acetyl-CoA and citrate concentrations (28). These fat-induced increases in citrate and acetyl-CoA are proposed to downregulate PFK and PDH, respectively, and consequently lead to a decline in the amount of CHO oxidized. Decreased activity of PFK should result in accumulation of G-6-*P*, which in turn is proposed to decrease hexokinase activity and lead to reduced glucose uptake (29).

In addition to glycogen sparing, Int infusion in the present study resulted in an elevated muscle citrate content and reduced transformation of PDH to the more active *a* form. These results are consistent with the classic description of the G-FA cycle (29). However, there was no concomitant increase in muscle acetyl-CoA content and no reduction in glucose uptake associated with increased FFA availability. Calculated free

Table 2. *Arterial glucose and lactate concentrations during 70-min cycle exercise at 40 and 65% $\dot{V}O_{2max}$ following Intralipid infusion or control*

	Rest		40% $\dot{V}O_{2max}$	65% $\dot{V}O_{2max}$			
	−30 min	0 min	8 min	18 min	34 min	50 min	68 min
Glucose							
Con	4.62 ± 0.30	5.05 ± 0.14	3.96 ± 0.20*	4.06 ± 0.22*	4.01 ± 0.14*	3.91 ± 0.18*	3.80 ± 0.19*
Int	5.28 ± 0.17	4.87 ± 0.26	4.28 ± 0.16*	4.01 ± 0.14*	4.18 ± 0.15*	4.25 ± 0.21*	4.36 ± 0.14*
Lactate							
Con	0.45 ± 0.12	0.20 ± 0.03	1.08 ± 0.21*	2.98 ± 0.53*	3.17 ± 0.62*	2.95 ± 0.52*	2.31 ± 0.37*
Int	0.42 ± 0.14	0.20 ± 0.10	0.66 ± 0.18*	2.65 ± 0.29*	3.00 ± 0.44*	3.01 ± 0.46*	3.12 ± 0.62*

Data are means ± SE in mM; n = 7. *Significantly different from 0 min.

Table 3. *Net leg FFA and glycerol uptake / release during 70-min cycle exercise at 40 and 65% $\dot{V}O_{2max}$ following Intralipid infusion or control*

	40% $\dot{V}O_{2max}$	65% $\dot{V}O_{2max}$			
	8 min	18 min	34 min	50 min	68 min	$\bar{x}$
FFA, mmol·min^{-1}·leg^{-1}						
Con	0.04 ± 0.01	0.03 ± 0.02	0.04 ± 0.03	0.03 ± 0.02	0.10 ± 0.02	0.05 ± 0.01
Int	0.18 ± 0.05*	0.16 ± 0.04*	0.31 ± 0.09*	0.20 ± 0.09*	0.24 ± 0.06*	0.23 ± 0.04*
Glycerol, μmol·min^{-1}·leg^{-1}						
Con	−1.66 ± 5.29	−6.23 ± 4.0	−10.13 ± 7.6	−7.42 ± 6.9	−6.69 ± 10.3	−7.62 ± 3.85
Int	28.9 ± 14.5	30.8 ± 23.3	13.7 ± 35.7	−11.3 ± 19.7	47.7 ± 20.0	19.2 ± 14.0

Data are means ± SE; n = 7. FFA, plasma free fatty acids. Negative values indicate net release. * Significantly different from Con.

ADP and AMP contents were also reduced in Int compared with Con at 68 min of exercise. The reduced flux through Phos during Int may have been due to reduced free AMP and P_i contents during exercise at 65% $\dot{V}O_{2max}$, and PFK may have been inhibited by increased cytoplasmic citrate. The mechanism for reduced transformation of PDH to the more active a form (which was not related to acetyl-CoA) is unknown, but suggests reduced flux through PDH. Therefore, although downregulation of CHO metabolism in the presence of high [FFA] is partially regulated as classically proposed, other regulatory factors appear to contribute.

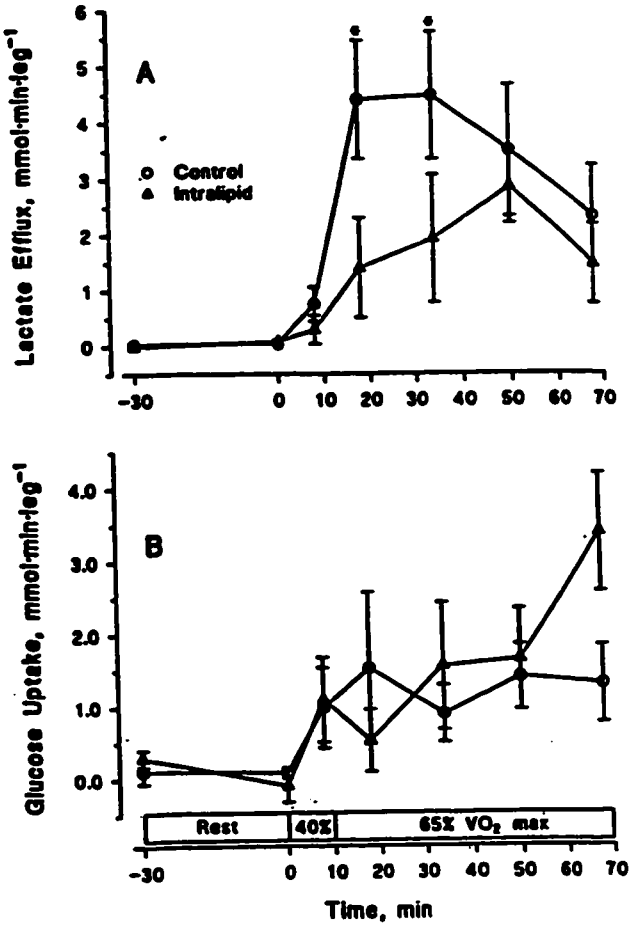

Fig. 3. Net leg lactate efflux (*A*) and glucose uptake (*B*) during 70 min of cycling at 40 and 65% $\dot{V}O_{2max}$ with Intralipid infusion or control. Values are means ± SE. *Significantly different from Intralipid.

Glucose uptake. Elevation of plasma FFA did not reduce glucose uptake at rest or during exercise (Fig. 3*B*). Previous studies have demonstrated reduced whole body glucose disposal (4), leg muscle glucose uptake (17, 23), and forearm glucose oxidation (39) at rest following Int infusion. Insulin was not measured in this study, although a similar protocol reported no change in resting insulin levels with Int (17). Also, some of the studies demonstrating a reduction in glucose disposal were done under conditions of hyperinsulinemia/high FFA for a much longer time than the 30 min in the present study (4, 23). Last, although the glucose uptake values were not statistically different between trials (Con, 0.09 ± 0.07 mmol·min^{-1}·leg^{-1}; Int, 0.03 ± 0.11 mmol·min^{-1}·leg^{-1}), they were similar to the resting data reported by Hargreaves et al. (17) (Con, 0.11 ± 0.02 mmol·min^{-1}·leg^{-1}; Int, 0.04 ± 0.01 mmol·min^{-1}·leg^{-1}).

Few researchers have investigated glucose uptake during exercise in humans. Romijn et al. (33) reported that blood glucose disappearance (stable-isotope tracer) was unaffected by elevated plasma FFA during cycling at 85% $\dot{V}O_{2max}$. A significant reduction in glucose uptake was, however, reported during 60 min of leg extension exercise at 80% work capacity in response to elevated FFA (17). The discrepancy in results is difficult to explain but may be due to differences in experimental protocols.

In the Hargreaves et al. (17) study, all Con trials were performed immediately before Int trials (opposite legs) and subjects were fasted overnight, producing high

Table 4. *Muscle glycogen contents pre- and postcycling and glycogen utilization during 10 min of cycling at 40% and 60 min of cycling at 65% $\dot{V}O_{2max}$ following Intralipid infusion or control*

	Pre	Post	Δ	%Sparing
Con	472 ± 38	175 ± 27*	297 ± 36	
Int	436 ± 33	206 ± 32*	230 ± 29†	22.6

Data are means ± SE in mmol glucosyl units/kg dry muscle. Pre, before cycling; Post, after cycling; Δ, glycogen degradation. * Significantly different from pre; † significantly different from Con.

Table 5. *Muscle metabolites during cycle exercise (10 min at 40% and 60 min at 65% $\dot{V}O_{2max}$) in control and Intralipid trials*

	Preexercise	40% $\dot{V}O_{2max}$ 10 min	65% $\dot{V}O_{2max}$ 20 min	65% $\dot{V}O_{2max}$ 70 min
ATP				
Con	26.6±1.2	26.1±3.0	24.3±2.9	24.6±1.3
Int	24.1±2.9	25.4±3.1	25.9±0.6	24.6±0.9
PCr				
Con	83.4±1.4	76.6±8.8	54.4±6.5*†	43.5±4.6*†
Int	81.7±9.4	74.3±9.2	50.1±3.4*†	55.5±4.6*†
Lactate				
Con	6.2±1.7	7.2±2.4	29.0±5.7*†	25.6±6.1*†
Int	8.2±1.6	10.1±2.4	30.2±6.2*†	25.2±4.3*†
Acetyl-CoA				
Con	8.8±0.9	14.8±1.8*	23.2±3.3*†	26.7±1.9*†
Int	8.6±1.2	16.8±2.8*	27.0±2.1*†	26.6±2.3*†
Acetyl-carn				
Con	1.2±0.2	5.4±0.9*	11.4±1.7*†	13.3±1.0*†
Int	1.1±0.2	5.4±0.9*	12.2±0.8*†	13.6±1.0*†

Data are means ± SE in mmol/kg dry muscle (acetyl-CoA in μmol/kg dry muscle). Acetyl-carn, acetyl-carnitine; PCr, phosphocreatine. *Significantly different from preexercise; †significantly different from 10 min.

resting control levels of plasma FFA (0.60 ± 0.07 vs. 0.25 ± 0.04 mM, present study). Interestingly, no increase in FFA uptake and no reduction in muscle glycogen use occurred during knee-extensor exercise in response to elevated FFA (17). The knee-extensor model has increased muscle blood flow relative to power output compared with conventional dynamic exercise (i.e., cycling or running). This, combined with the relatively high control FFA levels, may have accounted for the inability of Int to increase FFA uptake, as the delivery of FFA to the muscles was already high in the Con trial. The Int infusion had no independent effect on leg blood flow because measured values were the same in the Con and Int trials (17).

Hargreaves et al. (17) did not measure muscle citrate but found similar G-6-*P* and glucose accumulations and leg citrate release between trials. They suggested that reduced glucose uptake was due to direct inhibition of glucose transport rather than by the classical G-FA cycle. If elevated plasma FFA exert a direct effect on glucose transport, this effect was not observed in the present study, or a similar protocol at a higher power output (85% $\dot{V}O_{2max}$) (33).

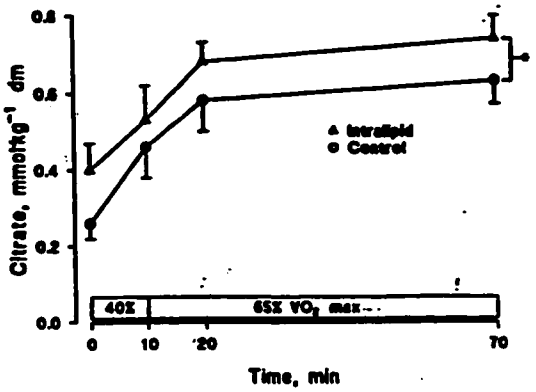

Fig. 4. Muscle citrate content at rest and accumulation during 70 min of cycling at 40 and 65% $\dot{V}O_{2max}$ with Intralipid infusion or control. Values are means ± SE. dm, Dry muscle. *Significantly different from control.

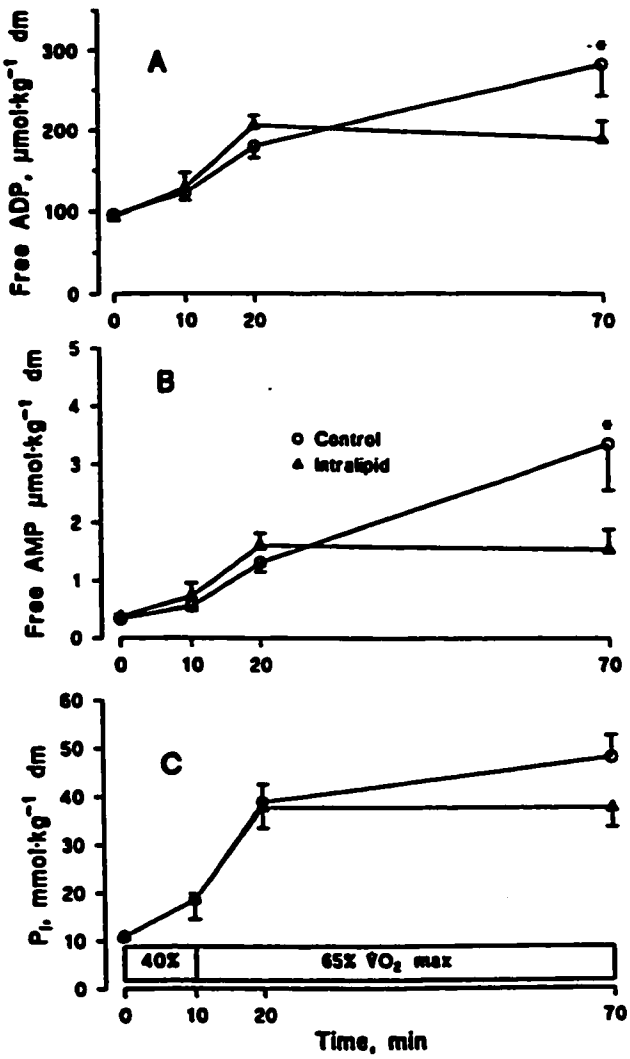

Fig. 5. Muscle free ADP (*A*), free AMP (*B*), and P_i (*C*) contents during 70 min of cycling at 40 and 65% $\dot{V}O_{2max}$ with Intralipid infusion or control. Values are means ± SE. *Significantly different from control.

Control of glycogen Phos. In the present study, glycogen Phos activity was reduced during exercise in the high-fat condition (less glycogen use), independent of inhibition of PFK or PDH and the regulatory scheme proposed in the classic G-FA cycle. Phos exists in two interconvertible forms: a more active *a* and a less active *b* form. Transformation from Phos *b* to *a* is thought to occur at the onset of exercise, primarily due to stimulation of Phos kinase by increased cytoplasmic [Ca^{2+}] and to a minor degree by epinephrine (7). Phos *a* is then regulated posttransformationally by free AMP, an allosteric modulator, and substrate availability (P_i and glycogen) (32).

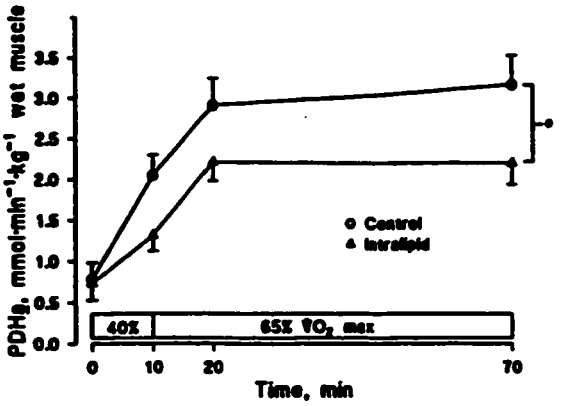

Fig. 6. Muscle pyruvate dehydrogenase activity of the α form (PDHα) during 70 min of cycling at 40 and 65% $\dot{V}O_{2max}$ with Intralipid infusion or control. Values are means ± SE. *Significantly different from control.

The transformational state of Phos was not measured in the present study but should have been similar between trials, given the constant power outputs (Ca^{2+}), and similar preexercise glycogen contents and epinephrine concentrations (13). In addition, previous investigations from our laboratory reported no change in Phos transformation despite a 45% sparing of muscle glycogen with elevated FFA during exercise at 85% $\dot{V}O_{2max}$ (12, 13). We suggested that the reduction of Phos activity was largely due to posttransformational regulation. Calculated P_i accumulation was similar between trials at all time points during exercise, but tended to be lower at 68 min during Int (Fig. 5*C*). Calculated free AMP was significantly lower at 68 min exercise during the Int trial compared with control (Fig. 5*B*). This suggests that the decreased accumulation of AMP during Int (combined with the tendency for reduced P_i) could have reduced Phos activity sometime between 20 and 68 min of exercise. The time course of the reduced glycogenolysis in the Int trial cannot be determined because glycogen was measured only at rest and 68 min. As previously mentioned, RER and PDH transformation were reduced by 10 min of exercise, and lactate efflux was reduced during Int at 18 and 34 min. These results suggest reduced glycogenolysis and CHO use occurred, both early in exercise and beyond 20 min during Int. The mechanisms that may have reduced Phos activity early in exercise are unknown.

Muscle citrate and PFK activity. The classic G-FA cycle suggests that increased fat oxidation results in citrate-mediated inhibition of PFK, accumulation of G-6-*P*, and subsequent inhibition of glucose uptake (28, 29). In this study, glucose uptake was unaffected by Int, but muscle citrate was slightly (but significantly) elevated at rest and during exercise. Because muscle G-6-*P* and glucose levels were not measured, it is difficult to assess whether PFK activity was downregulated by the accumulation of muscle citrate or lower due to the reduced Phos α activity higher up in the pathway. However, the unaffected glucose uptake suggests that either PFK/hexokinase activities were not inhibited, or, if enzyme activities were reduced by citrate, this reduction had no effect on glucose uptake.

Previous investigations of muscle citrate accumulation in response to increased FFA availability during exercise have produced variable results. Studies involving high-intensity exercise (75–85% $\dot{V}O_{2max}$) have consistently reported no effect of FFA elevation on muscle citrate levels, despite the occurrence of significant glycogen sparing (12, 13, 27, 37). Two earlier studies reported increased muscle citrate and decreased muscle glycogenolysis during cycle exercise at 65% $\dot{V}O_{2max}$ in response to 5 days of high-fat diet and aerobic training, respectively (20, 21). Studies at lower power outputs (<65% $\dot{V}O_{2max}$) have not measured muscle citrate (24, 30), while others have reported increased resting muscle citrate with elevated FFAs (13, 20, 27, 37).

Citrate is produced in the mitochondria and must move to the cytoplasm to affect PFK. Whole muscle measurements of citrate do not allow for partitioning of citrate to the different cellular compartments. Therefore, an increase in total muscle citrate may not reflect increased cytoplasmic citrate.

Early in vitro investigations overestimated the potency of citrate to inhibit PFK activity during exercise, because increases in positive regulators override its inhibitory effect (5, 25). Recent in vitro work reported that the citrate-mediated inhibition of PFK appears to be most powerful at rest and that additional increases that occur in response to increased FFA availability and exercise would not increase PFK inhibition at rest or during exercise (25). In the present study, we cannot conclusively state whether the small increases in muscle citrate with high [FFA] decreased CHO metabolism but predict that they would have little effect on PFK.

Control of PDH activity. In response to Int infusion, the transformation of PDH to the more active α form was reduced at all exercise time points, while acetyl-CoA increased to the same level in both conditions. PDH activity is regulated by a complex phosphorylation cycle. It is dephosphorylated and activated (to PDHα) by PDH phosphatase, and phosphorylated and inactivated by PDH kinase. Several metabolites are purported to be regulators of the PDH complex (see Refs. 19 and 31 for review). Ca^{2+} is a potent activator of PDH phosphatase, whereas PDH kinase can be inhibited by pyruvate and a high NAD^+-to-NADH ratio and activated by high ATP-to-ADP and acetyl-CoA-to-CoA ratios.

The concept of the G-FA cycle is partially based on the premise that increased fat oxidation will result in the accumulation of acetyl-CoA, which in turn will activate PDH kinase and downregulate PDH (28). Acetyl-CoA control of PDH activity appears to exist in humans at rest (4, 27), but several recent investigations have suggested that this potential regulatory effect is overridden by other factors during exercise (8, 12, 13, 27). Because acetyl-CoA increased at a time when PDH was transformed to the α form during exercise in both trials, our results support this concept.

There are other potential regulators of PDH that may account for the lower transformation of PDH to the

more active α form during Int. Ca^{2+} activates PDH phosphatase (10), but Ca^{2+} was assumed to be similar between trials because power output was constant. PDH kinase activity can be inhibited by ADP, which may accumulate in the mitochondria during exercise. The calculated accumulation of free ADP was reduced during Int only at 68 min of exercise and therefore was unlikely to contribute early in exercise. Also, the intramitochondrial [ADP] is unknown, making it difficult to assess the potential effect of ADP on PDH transformation.

PDH kinase can also be inhibited by pyruvate, and, although it was not measured, the reduced glycogenolysis with Int suggests that less pyruvate per unit time was available to PDH. A feed-forward mechanism could exist whereby reduced glycogenolytic flux is directly linked to downregulation of PDHα. Support for this suggestion was reported in a study that utilized dietary manipulations to alter plasma FFA levels and found reduced glycogenolysis, pyruvate content, and PDHα during cycle exercise at 75% $\dot{V}O_{2max}$ following a low-CHO diet (27). Measurements of muscle pyruvate in control and high-FFA conditions are needed to examine this possibility.

Evidence from in vitro studies suggests that a decrease in the NAD^+-to-NADH ratio decreases the transformation of PDH to the more active α form (26). Direct measurements of the mitochondrial reduction-oxidation (redox) state of the NAD^+/NADH couple in skeletal muscle during contraction have produced inconsistent results. Using the glutamate dehydrogenase reaction, a substantial increase in estimated mitochondrial redox state (increased NAD^+-to-NADH ratio) was reported during cycle exercise in humans (75 and 100% $\dot{V}O_{2max}$) (16). In contrast, Duhaylongsod et al. (11) monitored redox state in stimulated canine gracilis muscle using near-infrared spectroscopy and reported a decreased redox state at all power outputs. Furthermore, NADH measured in human muscle has been shown to decrease at 45% $\dot{V}O_{2max}$ and to increase at higher power outputs (35). No attempt was made to estimate the mitochondrial redox state in the present study, but it is possible that NADH accumulation was increased by elevated FFA availability during Int. This would reduce PDHα transformation if NADH exerts a significant regulatory effect on PDH kinase.

In summary, the present study confirms previous findings that elevated FFA levels result in significant sparing of muscle glycogen during moderate-intensity exercise. Although certain aspects of the classical regulation of fat/CHO interaction were present, other regulatory factors were involved. No difference in glucose uptake occurred between trials; thus regulation of CHO use occurred at the level of glycogen Phos, PFK, and/or PDH. Reduced flux through Phos with high [FFA] may have been due to reduced free AMP and P_i accumulation, which occurred late in exercise, and PFK may have been inhibited by increased cytoplasmic citrate. PDH transformation to the more active α form during exercise was reduced following Int infusion, but the decrease was not due to increased acetyl-CoA accumulation. The mechanisms for the reduced transformation of PDH are unknown but may be due to reduced pyruvate and/or NADH accumulation during exercise with Int infusion.

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APPENDIX D:

EFFECTS OF DICHLOROACETATE INFUSION ON HUMAN SKELETAL MUSCLE METABOLISM AT THE ONSET OF EXERCISE

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Effects of dichloroacetate infusion on human skeletal muscle metabolism at the onset of exercise

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Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1; Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3Z5; and Department of Clinical Chemistry, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden

Howlett, Richard A., George J. F. Heigenhauser, Eric Hultman, Melanie G. Hollidge-Horvat, and Lawrence L. Spriet. Effects of dichloroacetate infusion on human skeletal muscle metabolism at the onset of exercise. *Am. J. Physiol.* 277 (*Endocrinol. Metab.* 40): E18–E25, 1999.—This study investigated whether dichloroacetate (DCA) decreases the reliance on substrate level phosphorylation during the transition from rest to moderate-intensity exercise in humans. Nine subjects cycled at ~65% of maximal oxygen uptake ($\dot{V}O_{2max}$) after a saline or DCA (100 mg/kg body wt) infusion, with muscle biopsies taken at rest and at 30 s and 2 and 10 min of exercise. DCA infusion increased pyruvate dehydrogenase (PDH) activation at rest (4.0 ± 0.3 vs. 0.9 ± 0.1 mmol·kg wet wt^{-1}·min^{-1}) and at 30 s (3.6 ± 0.2 vs. 2.5 ± 0.4 mmol·kg^{-1}·min^{-1}) of exercise. As a result, acetyl-CoA (45.9 ± 5.9 vs. 11.3 ± 1.5 μmol/kg dry wt) and acetylcarnitine (13.1 ± 1.0 vs. 1.6 ± 0.3 mmol/kg dry wt) were markedly increased by DCA infusion at rest. These differences were maintained at 30 s and 2 min for both acetyl-CoA and acetylcarnitine. Resting muscle lactate and phosphocreatine (PCr) were not different between trials, but DCA infusion resulted in lower lactate accumulation throughout exercise, especially at 2 min (21.6 ± 3.1 vs. 44.6 ± 8.0 mmol/kg dry wt). PCr utilization in the initial 30 s (16.9 ± 0.4 vs. 31.7 ± 2.6 mmol/kg dry wt) and 2 min (27.8 ± 4.7 vs. 45.1 ± 2.6 mmol/kg dry wt) of exercise was decreased with DCA. This resulted in a lower accumulation of free inorganic phosphate at 30 s (25.4 ± 2.0 vs. 36.4 ± 2.8 mmol/kg dry wt) and 2 min (34.6 ± 4.7 vs. 50.5 ± 2.2 mmol/kg dry wt) with DCA and decreased glycogenolysis over 10 min. The data from this study support the hypothesis that increased provision of substrate by DCA infusion increases oxidative metabolism during the rest-to-work transition, resulting in decreased PCr utilization and an improved cellular energy state at the onset of exercise. The transitory improvement in energy state decreased glycogenolysis and lactate accumulation during moderate-intensity exercise.

glycogenolysis; lactate; phosphocreatine; inorganic phosphate; oxidative metabolism; pyruvate dehydrogenase activity; acetyl-coenzyme A; acetylcarnitine

THE CAUSES OF LACTATE PRODUCTION during steady-state, moderate power output exercise are controversial (7), with two main mechanisms being proposed. Increases in lactate have been suggested to be due to limitations in O_2 delivery to the mitochondrial electron transport chain (19, 34); others hypothesize that O_2 is never limiting and that increased lactate content is simply a consequence of the reduced energy state of the cell (6, 36). The latter hypothesis, often called the "mass action effect," suggests that the rate of lactate production is determined by the balance between pyruvate production and oxidation. When pyruvate production exceeds the rate at which it can be converted to acetyl-CoA via pyruvate dehydrogenase (PDH), it begins to accumulate, and because lactate dehydrogenase is a near-equilibrium enzyme, it is converted to lactate. In support of this hypothesis, a recent study at 65% maximal oxygen uptake ($\dot{V}O_{2max}$) showed that significant lactate accumulation occurred in the working muscle, even when PDH was not totally transformed to its active form. Therefore, glycogenolytic flux was greater than PDH flux, even though PDH could potentially have oxidized more pyruvate (17).

During the transition from rest to exercise, there is a transient mismatch between the rates of ATP degradation and aerobic synthesis. To maintain ATP levels, substrate level phosphorylation of ADP occurs from both glycogenolysis and phosphocreatine (PCr) breakdown, resulting in accumulation of free inorganic phosphate (P_i), ADP, AMP, and lactate. The magnitude of this mismatch determines how great the PCr utilization and free metabolite accumulations are, and these in turn determine the rate of glycogenolysis and lactate accumulation. The lag between the onset of ATP breakdown and the activation of oxidative ATP production will be one factor in determining the severity of this mismatch.

Dichloroacetic acid (DCA) increases the activation of PDH to its active "a" form (PDH_a) by inhibiting PDH kinase (35). It has been used experimentally in animals (20) and clinically in humans for the treatment of lactic acidosis (29). Previous human studies utilizing DCA have shown reduced blood lactate accumulations in patients with lactic acidosis (29) and healthy volunteers during exercise (3), suggesting that enhanced PDH activation decreased lactate production and/or enhanced lactate clearance. Until recently, little or no work had been done with skeletal muscle to determine the biochemical mechanisms by which these changes occur. In a recent series of studies by Timmons et al. (30–33), DCA was used to test the hypothesis that substrate availability determines the rate at which aerobic ATP production begins, and that increased oxidative substrate at the start of exercise, specifically acetyl-CoA derived from acetylcarnitine, decreased the

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reliance on substrate level phosphorylation from PCr and glycogenolysis. Using contracting dog muscle (32, 33), contracting ischemic human muscle (31), and healthy human subjects performing leg-extensor exercise (30), these studies showed that DCA infusion resulted in increased resting PDH activation, less PCr utilization, and decreased glycogen breakdown. The decreased glycogenolysis decreased the amount of lactate accumulation, presumably as a better match between pyruvate production and oxidation occurred.

The present study was also designed to test the above hypothesis during the onset (first 2 min) of whole body, moderate-intensity aerobic exercise with normal blood flow in humans. Rapid biopsy sampling and a higher DCA dose (100 mg/kg) were selected to provide additional information on the time course (i.e., the rest-to-work transition) of the effects and to increase resting PDH_a to maximal exercise levels.

METHODS

Subjects. Nine (2 female, 7 male) healthy subjects volunteered to participate in this study. Their mean (±SE) age, height, weight, and $\dot{V}O_{2max}$ were 21.8 ± 0.6 yr, 178.6 ± 2.3 cm, 72.6 ± 4.2 kg, and 3.7 ± 0.3 l/min (50.5 ± 2.4 ml·kg^{-1}·min^{-1}), respectively. None of the subjects were well trained, nor were they regularly active (i.e., <2 aerobic workouts per week). Subjects were informed of possible risks involved in the study, and informed consent was received from all subjects. The study was approved by the ethics committees of both universities.

DCA. DCA (monosodium salt) was obtained from TCI America (Portland, OR) under the supervision of Dr. N. L. Jones. It was prepared at a concentration of 100 mg/ml (pH 7.0) by the pharmacy at McMaster University Medical Center, filtered, assayed to ensure sterility and lack of pyrogens, and checked for purity by HPLC in an independent laboratory. It was delivered intravenously to subjects in the dose of 100 mg/kg by use of ~500 ml of normal saline solution over the course of 1 h immediately before exercise. Although the infusion of DCA was designed to fully activate PDH, it is possible that it exerted effects on other enzymes. However, it is unlikely that the effect of DCA on other enzymes would be important during 10 min of exercise at ~65% $\dot{V}O_{2max}$ in skeletal muscle.

Preexperimental protocol. Subjects underwent a continuous incremental exercise test on a bicycle ergometer (Excalibur, Quinton Instruments, Seattle, WA) to determine $\dot{V}O_{2max}$ with a metabolic cart (model 2900, SensorMedics, Yorba Linda, CA). From this test, the power output required to elicit ~65% of $\dot{V}O_{2max}$ was calculated. On a separate day, subjects cycled for the required 10 min to confirm that the correct percentage of $\dot{V}O_{2max}$ was reached. Mean power output for the trials was 169 ± 14 W. It was assumed that basal oxygen uptake ($\dot{V}O_2$) and $\dot{V}O_{2max}$ were not altered by DCA.

Experimental protocol. On two separate experimental days (1–2 wk apart), subjects arrived at the laboratory at the same time of day. Subjects ate a high-carbohydrate meal 2 h previous to arriving at the laboratory, as they would do in preparation for exercise. They were also asked to consume their normal diet before the test days and to refrain from strenuous exercise for 24 h before each trial. On test days, the subjects received either DCA or saline infusion in a randomized order. One hour before each exercise trial, a catheter was inserted into the antecubital vein of each subject, and a preinfusion blood sample was taken. The control or DCA infusion was then started, and the subject rested quietly on a bed. A second blood sample was taken at 30 min of infusion, at which time subjects had one leg prepared for needle biopsies, with four incisions made through the skin superficial to the vastus lateralis muscle under local anesthesia (2% lidocaine without epinephrine) (2). A resting biopsy and blood sample were taken at 60 min, and then subjects moved to an electronically braked cycle ergometer and began pedaling at the prescribed power output. Exercise biopsies were taken at 30 s, 2 min, and 10 min while the subject remained on the cycle ergometer. Samples were immediately frozen in liquid N_2 (3–5 s from the insertion of the needle), removed from the needle, and stored in liquid N_2 until analysis. Exercise blood samples were taken at 3 and 9 min. Expired gases were collected to measure $\dot{V}O_2$ and carbon dioxide production between 4 and 6 min.

One (~1-ml) aliquot of blood, for free fatty acid (FFA) analysis, was centrifuged for 1 min at 10,000 rpm, and 400 μl of the plasma (supernatant) were added to 100 μl of 5 M NaCl and immediately put into a 56°C waterbath for 30 min to inhibit lipoprotein lipase activity. A second aliquot of 200 μl was added to 800 μl 0.6 N perchloric acid (PCA), vortexed, and spun at 10,000 rpm for 1 min. The supernatant was removed for subsequent analysis.

Analyses. A small piece of frozen wet muscle (20–30 mg) was removed under liquid N_2 for the determination of PDH activation (PDH_a), as described by Constantin-Teodosiu et al. (9) and modified by Putman et al. (25). The remainder of the biopsy sample was freeze-dried, dissected of all visible blood, connective tissue, and fat, and powdered for subsequent analysis.

One aliquot of freeze-dried muscle was extracted with 0.5 M $HClO_4$ (containing 1 mM EDTA) and neutralized with 2.2 M $KHCO_3$. This extract was used for determination of creatine, PCr, ATP, glucose 6-phosphate (G-6-*P*), lactate, glycerol 3-phosphate (G-3-*P*), and glucose by enzymatic spectrophotometric assays (1, 16). Pyruvate was determined on this extract fluorometrically (21). Acetyl-CoA and acetylcarnitine were determined by radiometric measures (4). Muscle glycogen content was determined on a second aliquot of freeze-dried muscle from resting and 10-min samples (16).

Plasma FFA concentrations were determined using a WAKO nonesterified fatty acid (NEFA C) assay kit (WAKO Chemicals, Osaka, Japan). Whole blood glucose and lactate were determined on the PCA extract samples by the methods of Bergmeyer (1).

Calculations. Free ADP and AMP concentrations were calculated by assuming equilibrium of the creatine kinase and adenylate kinase reactions as previously described (12). Free ADP was calculated using the measured ATP, PCr, and creatine content, and H^+ concentration was estimated from the muscle lactate content according to the regression equation of Sahlin et al. (28). Free AMP was calculated from the estimated free ADP and ATP using the adenylate kinase reaction. Free P_i was calculated by adding the estimated resting free phosphate of 10.8 mmol/kg dry measure to the difference in PCr content minus the accumulation of glycolytic intermediates G-6-*P* and G-3-*P* between rest and each exercise time point. All metabolite contents and the activity of PDH_a were normalized to the highest total creatine measurement in the eight biopsies from each subject.

PDH flux was calculated for the first 30 s and subsequent 90 s of exercise. It was assumed that *1*) ATP turnover was equal in both trials as power output was the same and *2*) any reduction in ATP from substrate level phosphorylation was due to oxidative ATP production. First, the "baseline" PDH flux to oxidative metabolism was estimated, with the assump-

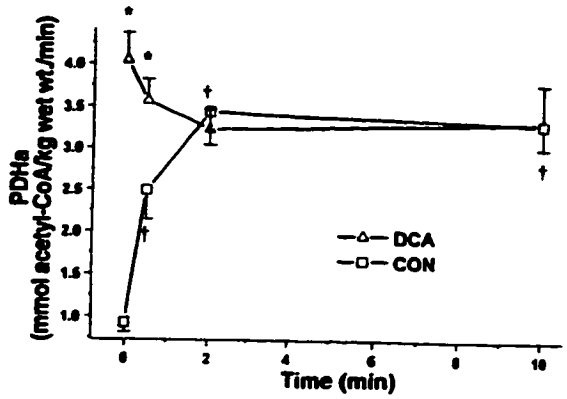

Fig. 1. Pyruvate dehydrogenase activation to the active "a" form (PDH_a) during control (CON) and dichloroacetate (DCA) trials. Significantly different from: *CON; †rest for the same trial.

tion that it was equal to the average PDH_a in the control (CON) trial, as previously shown by others (10, 17, 23, 25). Next, the amount of pyruvate directed to oxidative phosphorylation to account for the reduction in substrate-level phosphorylation during the DCA trial was calculated by adding the ATP equivalents of the decrease in PCr utilization and lactate accumulation, with the assumption of an ATP yield of 15 mmol of ATP per millimole of pyruvate. Finally, this "extra" PDH flux in the DCA trial was added to the flux that was directed to the increased accumulation of acetylcarnitine. The sum of these three fluxes is equal to the total PDH flux in the two trials.

Statistics. All data are presented as means ± SE. For net glycogen usage between trials, a paired *t*-test was used to determine significant difference. For all other dependent variables, a two-way ANOVA (time × trial) with repeated measures was employed. Significance was set at $\alpha = 0.05$, and when obtained, Tukey's post hoc test was used to identify where significant differences occurred.

RESULTS

$\dot{V}O_2$ and respiratory exchange ratio. During the DCA trial, $\dot{V}O_2$ was 2.21 ± 0.16 l/min or 60.4 ± 1.1% $\dot{V}O_{2max}$, whereas CON $\dot{V}O_2$ was 2.27 ± 0.15 l/min or 62.3 ± 1.3% $\dot{V}O_{2max}$. Respiratory exchange ratio values between 4 and 6 min in the DCA and CON trials were 1.00 ± 0.01 vs. 0.97 ± 0.01, respectively.

PDH_a. DCA infusion at rest resulted in a very marked (4.04 ± 0.32 vs. 0.9 ± 0.11 mmol·kg wet wt^{-1}·min^{-1}) and significant increase in resting PDH_a compared with CON (Fig. 1). Resting PDH_a in the DCA trial was similar to that reported for maximum total PDH activity during intense activity (14, 17, 22, 23). During exercise, despite a small decrease in PDH_a from rest in the DCA trial, the significant difference between trials remained at 30 s. However, there was no difference in PDH_a at subsequent time points, as PDH_a in the CON trial increased to DCA trial levels after 2 min of exercise.

Muscle metabolites. ATP levels were not significantly different at any time, regardless of trial (Table 1). Resting PCr was similar between trials but was significantly lower at both 30 s and 2 min during exercise in CON (Fig. 2). Resting lactate levels were similar between trials, but there was a significant main effect for lower lactate accumulation during exercise over 10 min in the DCA trial, with a significant interaction at 2 min (Fig. 3).

Resting acetyl-CoA and acetylcarnitine contents were both significantly elevated well above CON by DCA infusion. The DCA-induced levels were comparable to maximal exercise values (17, 23) and did not change with exercise in the DCA trial. Despite significant increases in acetyl-CoA and acetylcarnitine from rest in CON, the difference between DCA and CON trials remained significant after 2 min of exercise, but by 10 min, no differences existed (Fig. 4, *A* and *B*).

Resting G-3-*P*, G-6-*P*, and free glucose contents were not different between trials. However, the contents of these metabolites rose significantly during exercise, reflecting the increased glycolytic flux (Table 1).

Free P_i accumulation was lower at 30 s and 2 min in DCA vs. CON (Fig. 5). The contents of free ADP and

Table 1. *Muscle metabolite contents during CON and DCA trials*

Metabolite	Condition	0 min	30 s	2 min	10 min
ATP	DCA	26.5 ± 0.8	27.6 ± 0.6	26.7 ± 1.6	25.4 ± 0.5
	CON	26.3 ± 0.9	27.5 ± 0.6	26.4 ± 1.2	24.2 ± 1.3
Pyruvate	DCA	0.22 ± 0.1	0.33 ± 0.1	0.60 ± 0.1*†	0.44 ± 0.1*
	CON	0.31 ± 0.1	0.47 ± 0.1	0.42 ± 0.1	0.51 ± 0.1*
Glucose	DCA	2.6 ± 0.4	3.8 ± 0.4	4.5 ± 0.8	5.6 ± 0.9*
	CON	2.6 ± 0.3	3.8 ± 0.6	5.4 ± 0.6*	7.7 ± 1.8*†‡
G-3-*P*	DCA	2.1 ± 0.2	4.7 ± 0.7*	4.6 ± 1.2*	4.8 ± 0.9*
	CON	1.3 ± 0.2	3.6 ± 0.3*	5.0 ± 1.2*	5.0 ± 1.1*
G-6-*P*	DCA	1.3 ± 0.4	2.2 ± 0.5	3.4 ± 0.4*	3.6 ± 0.7*†
	CON	1.3 ± 0.2	3.9 ± 0.8*	2.7 ± 0.3*	3.3 ± 0.6*
Free ADP	DCA	91.6 ± 4.8	149.0 ± 9.4*	184.9 ± 28.6*	280.5 ± 36.3*†
	CON	84.2 ± 6.1	186.0 ± 17.7*	218.0 ± 29.5*	251.6 ± 30.5*†
Free AMP	DCA	0.31 ± 0.1	0.79 ± 0.1	1.46 ± 0.5*	3.41 ± 0.9*†‡
	CON	0.27 ± 0.1	1.30 ± 0.3	2.00 ± 0.4*	2.71 ± 0.5*†

All values are means ± SE. CON, control; DCA, dichloroacetate. Pyruvate, glucose 6-phosphate (G-6-*P*), glycerol 3-phosphate (G-3-*P*) and glucose are expressed in mmol/kg dry wt, and free ADP and AMP are in μmol/kg dry wt. Significantly different from: *rest for the same trial; †30 s for the same trial; ‡2 min for the same trial.

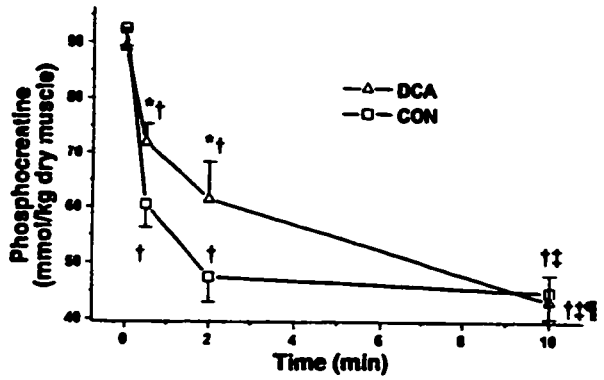

Fig. 2. Phosphocreatine degradation during CON and DCA trials. Significantly different from: *CON; †rest for the same trial; ‡30 s for the same trial; ¶2 min for the same trial.

AMP were not different between trials at any time, but there was a significant main trial effect for their levels to be lower during the DCA trial (Table 1).

Resting muscle glycogen content was similar between CON and DCA before (479.2 ± 39.6 vs. 512.2 ± 396.9 mmol/kg dry wt) and after (322.5 ± 36.5 vs. 396.9 ± 41.8 mmol/kg dry wt) exercise. However, the use of glycogen during the exercise period was significantly reduced in DCA vs. CON (115.3 ± 19.2 vs. 156.6 ± 24.9 mmol/kg dry wt).

Blood metabolites. Resting blood lactate was higher in the CON trial than the DCA trial after 30 and 60 min of infusion. During exercise, blood lactate was significantly lower in the DCA than in the CON trial at 9 min. Blood glucose and plasma FFA were not different between trials at any time (Table 2).

PDH flux. Total PDH flux during the first 30 s of exercise was 46% higher in the DCA trial compared with CON (Table 3). Of this increase in total PDH flux, nearly all was directed toward oxidative metabolism to reduce the dependence on substrate level phosphorylation, as the contribution to acetylcarnitine accumulation was virtually identical between trials (Tables 3 and 4). However, during the subsequent 90 s of exercise, total PDH flux was actually 16% higher in the CON trial (Table 3), as the flux directed to "sparing" oxidative phosphorylation fell in the DCA trial, and large amounts of flux were directed to increasing acetylcarnitine accumulation in the CON trial (Tables 3 and 4).

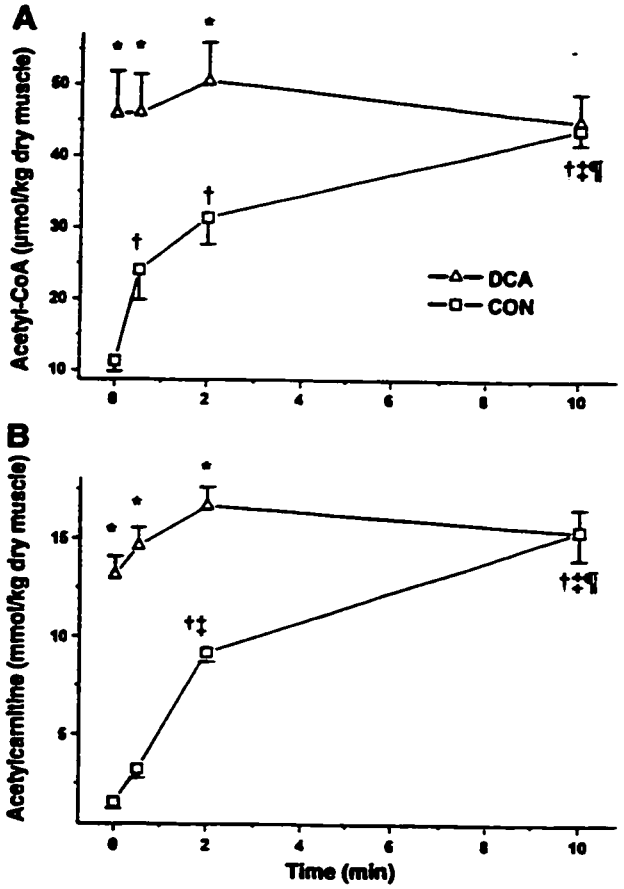

Fig. 4. Acetyl-CoA accumulation (*A*) and acetylcarnitine accumulation (*B*) during CON and DCA trials. Significantly different from: *CON; †rest for the same trial; ‡30 s for the same trial; ¶2 min for the same trial.

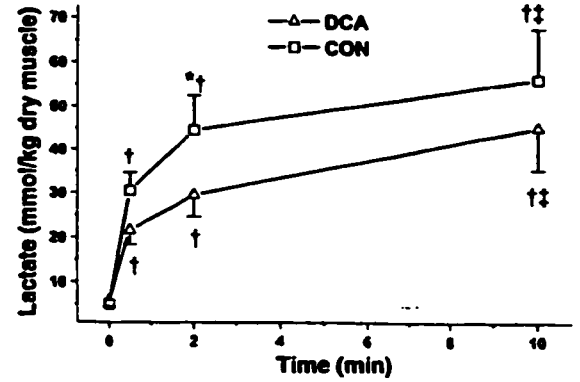

Fig. 3. Lactate accumulation during CON and DCA trials. Significantly different from: *CON; †rest for the same trial; ‡30 s for the same trial.

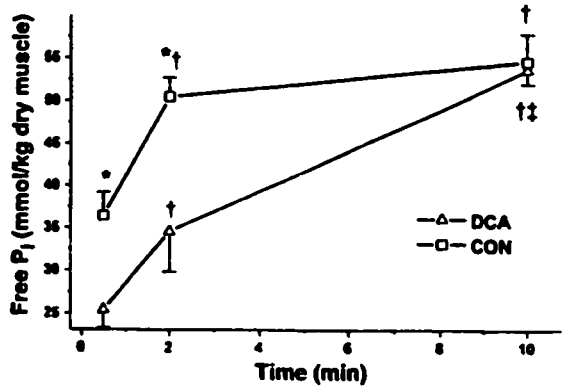

Fig. 5. Free inorganic phosphate (P_i) accumulation during CON and DCA trials. Significantly different from: *CON; †rest for the same trial; ‡30 s for the same trial.

Table 2. *Whole blood lactate and glucose concentrations and plasma FFA concentrations during CON and DCA trials*

Metabolite	Condition	−60 min	−30 min	0 min	3 min	9 min
Lactate	DCA	1.9 ± 0.3	0.6 ± 0.2*†	0.2 ± 0.1*†	2.1 ± 0.2‡	4.6 ± 0.3*†‡¶
	CON	2.1 ± 0.1	1.2 ± 0.1†	1.0 ± 0.1†	2.5 ± 0.2‡	5.6 ± 0.3†‡¶
Glucose	DCA	4.7 ± 0.2	4.6 ± 0.3	4.6 ± 0.2	4.7 ± 0.1	4.5 ± 0.1
	CON	4.5 ± 0.3	4.9 ± 0.2	4.5 ± 0.2	4.4 ± 0.2	4.3 ± 0.1
FFA	DCA	0.21 ± 0.04	0.23 ± 0.04	0.33 ± 0.04	0.37 ± 0.05	0.31 ± 0.03
	CON	0.23 ± 0.05	0.27 ± 0.05	0.36 ± 0.07	0.34 ± 0.06	0.33 ± 0.06

Values are means ± SE expressed in mmol/l. FFA, free fatty acid. Significantly different from: *CON; † −60 min for the same trial; ‡0 min for the same trial; ¶3 min for the same trial.

DISCUSSION

DCA infusion before moderate-intensity whole body exercise resulted in maximal activation of PDH to its active form (PDH_a) and subsequent acetylation of the free CoA and carnitine pools to levels seen at maximal exercise (8, 23). During the initial 2 min of exercise, DCA infusion resulted in decreased PCr breakdown and P_i accumulation and decreased glycogenolysis and lactate accumulation. These results are consistent with previous results from studies utilizing different models (30–33), which demonstrated that increasing the amount of carbohydrate-derived substrate that can be oxidized during the transition from rest to exercise improves the energetic state of the working muscle. This improved energy state results in lower glycogenolysis and decreased lactate accumulation in the muscle. The greater substrate availability increased the amount of oxidative metabolism used in the work-to-rest transition, decreasing the reliance on substrate level phosphorylation and resulting in the above changes.

In the present study, the 100 mg/kg dose of DCA produced resting PDH_a activation values (4.04 ± 0.32 mmol·kg wet $wt^{-1}\cdot min^{-1}$) that were higher than the significant increases previously reported in humans (1.3 ± 0.1 to 2.2 ± 0.3 mmol·kg wet $wt^{-1}\cdot min^{-1}$) with a lower DCA dose (50 mg/kg) (11, 13, 30). The larger dose in this study successfully increased resting PDH_a to levels seen during maximal exercise (14, 17, 22, 23). The resting acetylcarnitine contents were similar to previous DCA studies on humans (30, 31), and resting muscle acetyl-CoA content was also similar to maximal exercise values (23).

Table 3. *Total PDH flux and direction of PDH flux to various fates in DCA and CON during first 30 s and subsequent 90 s of exercise at 65% $\dot{V}O_{2max}$*

Time	Condition	Total PDH Flux	"Baseline" PDH Flux	PDH Flux to Acetylcarnitine	"Extra" PDH Flux to Oxid. Phos.
0–30 s	DCA	1.25	0.48	0.34	0.43
	CON	0.86	0.48	0.37	
30 s–2 min	DCA	3.73	3.13	0.42	0.17
	CON	4.46	3.13	1.32	

Fluxes are expressed as mmol pyruvate/kg wet muscle. PDH, pyruvate dehydrogenase. $\dot{V}O_{2max}$, maximal O_2 uptake. Oxid. Phos., oxidative phosphorylation. See text for details on calculations.

During exercise, the decrease in PCr degradation in the present study was significantly lower with DCA, similar to previous studies on human volunteers (30). The accompanying decrease in lactate accumulation seen in the present study was not observed with DCA infusion at 8 min in humans undergoing voluntary exercise, although they were using lower-intensity leg extensor exercise (30). However, decreased muscle lactate has been observed previously with DCA infusion (31).

Increased muscle lactate content has often been assumed to be a result of tissue oxygen insufficiency (19, 34). However, another line of evidence suggests that lactate accumulation results from an imbalance between pyruvate oxidation and pyruvate production (6). Pyruvate oxidation is controlled by the entry of pyruvate into the mitochondria through PDH, whereas pyruvate production during exercise is a function of glycogenolysis, or flux through glycogen phosphorylase. Therefore, the regulation of these enzymes is crucial in the control of lactate production.

PDH exists in active and inactive forms, acutely regulated by an allosterically modulated phosphatase/kinase system in human skeletal muscle (18, 35). During exercise, PDH is transformed to its active "a" form at the start of exercise, primarily by increases in Ca^{2+} and pyruvate. The extent of PDH activation is highly dependent on power output; thus, during exercise, independently calculated PDH flux has been shown to correlate with the catalytic activity of PDH_a (10, 17, 23, 25). Because skeletal muscle pyruvate (27) and Ca^{2+} contents increase with increased power output, they provide possible signals for increased PDH activation during exercise. In the present study, DCA infusion transformed PDH maximally at rest (22), resulting in

Table 4. *Percentage of total PDH flux directed to oxidative phosphorylation or acetylcarnitine production in DCA and CON during first 30 s and subsequent 90 s of exercise at 65% $\dot{V}O_{2max}$*

Time	Condition	Oxidative Phosphorylation	Acetylcarnitine Production
0–30 s	DCA	73	27
	CON	57	43
30 s–2 min	DCA	89	11
	CON	70	30

complete acetylation of the carnitine and free CoA pools, and allowing maximal acetyl-CoA formation from pyruvate at the onset of exercise. When tricarboxylic acid (TCA) cycle flux during maximal exercise was compared with the catalytic activity of PDH_a, it was very similar (14), suggesting that PDH_a could be limiting for the entry of pyruvate-derived substrate into the TCA cycle (18).

Using the data from the present study and the correlation between PDH flux and PDH_a measurements (10, 17, 23, 25), we estimated total PDH flux and the portion of this flux that went to various cellular fates for the initial 30 s and the subsequent 90 s of exercise (Table 3). The decreased reliance on substrate level phosphorylation, calculated from lactate accumulation and PCr utilization and expressed in ATP equivalents, resulted in a difference between DCA and CON trials of 29.4 mmol/kg dry wt at 30 s and 41.4 mmol/kg dry wt at 2 min.

During the first 30 s of exercise, PDH flux was 46% higher in the DCA trial (Table 3). This increase in PDH flux was directed to oxidative phosphorylation, and not acetylcarnitine production (Table 4), as the rate of acetylcarnitine accumulation was similar between trials despite the very different resting acetylcarnitine contents. These data suggest that the provision of substrate is limiting the increase in oxidative phosphorylation in the CON trial, as increased flux through PDH during the DCA trial allowed for greater oxidative phosphorylation, accounting for the decreased substrate level phosphorylation during the first 30 s of exercise.

Conversely, for the following 90 s, from 30 s to 2 min of exercise, the results are quite different. As most (71%) of the sparing of nonoxidative ATP production occurred during the first 30 s, the dependence on substrate level phosphorylation was much lower during the subsequent 90 s. Therefore, much of the increase in PDH flux in the CON trial was directed at increasing the acetylcarnitine pool. Because this pool was already very high in the DCA trial, and less extra flux to oxidative phosphorylation is required to account for the further attenuation of substrate level ATP production, the total PDH flux in this trial was lower than in CON. However, the percentage of this flux to oxidative phosphorylation was still higher in the DCA trial than in CON, as much of the PDH flux in the latter was used for acetylcarnitine production (Table 4). These data suggest that, during this 90-s period, there is no limitation in substrate utilization in the CON trial, but the use of PDH flux to increase acetylcarnitine limits the amount of PDH flux that can be used for oxidative phosphorylation. The lower amount of PDH flux committed to acetylcarnitine during DCA allowed for further decreases in substrate level phosphorylation during this time period, despite a lower total PDH flux.

Increased flux through PDH at the start of exercise may not be the only factor in the improved oxidative metabolism. The large resting acetyl-CoA and acetylcarnitine contents have been hypothesized to increase the substrate available for the TCA cycle and oxidative metabolism. DCA infusion has resulted in a decrease in the high acetyl-CoA levels early in exercise in some (31–33) but not all studies (30). In the present study, we observed no decrease in the acetylcarnitine content at any time point in either trial, but because very little acetylcarnitine utilization would be required for large changes in oxidative metabolism, it is possible that our measurements were not sensitive enough to detect the small changes over a shorter time course than previous studies.

Unfortunately, it is not possible to determine with the present results whether maximal PDH activation at the start of exercise causes the adjustments in cell metabolism via the greater pyruvate availability during the work-to-rest transition, or whether the increased oxidative substrate (acetyl-CoA and acetylcarnitine) produced at rest is responsible. A previous study involving acetate infusion caused large increases in acetyl-CoA and acetylcarnitine at rest without subsequent differences in the muscle energetic state during exercise (24). However, in that case, the increased acetyl-CoA also decreased PDH_a at rest (but not during exercise), possibly counteracting the increases in resting substrate.

Flux through glycogen phosphorylase (glycogenolysis) is determined by two factors. The first is the extent of activation of phosphorylase to its more active "a" form by activation of phosphorylase kinase. The second level of regulation is via the concentrations of other modulators, especially free AMP and free P_i, a phosphorylase a allosteric modulator and a phosphorylase substrate, respectively (26). Because power outputs were the same between trials in the present study, DCA infusion would not affect the extent of phosphorylase activation, as the initial burst in activation is primarily due to Ca^{2+}. Likewise, it has recently been shown that the activation state of phosphorylase is not affected by power output and is not a good indicator of phosphorylase flux (17). However, previous studies have shown a strong correlation between free P_i content and the glycogenolytic rate in contracting skeletal muscle, as P_i is a substrate for the glycogen phosphorylase reaction (17, 26). By decreasing the degradation of PCr, DCA infusion in the present study caused a decreased accumulation of free P_i and a subsequent decrease in the breakdown of glycogen compared with the CON trial. Likewise, free AMP is an allosteric activator of phosphorylase a, and there was a strong trend toward an attenuated rise (~50%) in free AMP during DCA.

Our data and those of others (30–33) suggest that the ability of metabolic processes to be activated limits O_2 use in the transition from rest to steady-state aerobic exercise in the control state. DCA infusion improves the rate at which oxidative metabolism is activated. However, the degree to which DCA improves the cellular energy state was not as large as expected. Despite impressive resting changes in acetyl-CoA and acetylcarnitine, and total PDH activation, there was still significant PCr utilization and lactate production. There appears to be an inability to totally utilize the preexercise stores of acetylated compounds. It appears that

this inability could be related to oxygen limitation, slower TCA cycle flux increases, and/or mass-action effects.

It is often assumed that O_2 is not limiting at moderate power outputs (5), but during the rest-to-work transition, it is possible that there is a lag in O_2 provision, or that mitochondria are sensitive to O_2 pressure levels well above the level assumed to be limiting for cytochrome turnover (36). Any substrate level ATP production and the subsequent PCr breakdown and lactate formation would tend to blunt the effect of DCA. There is also the possibility of a lag in increasing TCA cycle flux, despite the high resting acetyl-CoA content. Although citrate synthase, the enzyme that utilizes acetyl-CoA, is not considered a near-equilibrium enzyme, its flux appears to be highly dependent on delivery of oxaloacetate, its other substrate (15). Resting oxaloacetate concentrations are extremely low and rise quickly with exercise via anaplerotic reactions (15), but the two carbon acetyl-CoA cannot increase the amount of TCA intermediates at rest. In fact, because pyruvate appears to be very important in increasing TCA intermediates, the increased oxidation of pyruvate with DCA could actually decrease anaplerosis, although this has presently been demonstrated only in resting skeletal muscle (11). Finally, it is possible that the lactate accumulation seen even with DCA administration at this power output, despite increased PDH_a, is merely a consequence of mass action. Accumulations of free ADP, AMP, and P_i determine the rate of oxidative phosphorylation (36) but also regulate the glycogenolytic rate. As the rate of oxidative phosphorylation increases, there is a necessary increase in the delivery of pyruvate (30). Because of the near-equilibrium nature of lactate dehydrogenase as the concentration of pyruvate rises, there may be an obligatory increase in lactate content.

In summary, the present study demonstrated that DCA infusion increased resting PDH activation and resting muscle contents of acetyl-CoA and acetylcarnitine to values indicative of maximal exercise. These resting changes, which increase the amount of substrate available for oxidative metabolism, caused a blunting in PCr degradation and the accumulation of free ADP, AMP, and P_i during the initial 2 min of the transition to steady state during whole body exercise at ~65% $\dot{V}O_{2max}$. The improved cellular energy state decreased glycogen breakdown and subsequent lactate accumulation. These data concur with the findings of Timmons et al. (30–33), suggesting that the provision of oxidative substrate is one factor limiting oxidative metabolism early in exercise, and that increasing the availability of substrate early in exercise allows for increased oxidative metabolism and decreased reliance on substrate level phosphorylation.

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