# $\dot{V}O_2$ ON-KINETICS IN SKELETAL MUSCLE IN NORMOXIA AND HYPOXIA WITH MATCHED CONVECTIVE $O_2$ DELIVERY

by

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Abstract

The aim of this investigation was to evaluate $O_2$ uptake ($\dot{V}O_2$) on-kinetics under hypoxic conditions while holding convective $O_2$ delivery constant in isolated canine muscles *in situ*. Canine gastrocnemius muscles (n=7) were surgically isolated and then electrically stimulated via the sciatic nerve (8 V, 0.2-ms duration, 50Hz, 200-ms trains) eliciting contractions at a rate of 1 contraction every 2 seconds (about 70% of peak $\dot{V}O_2$). The dogs inspired 3 different $O_2$ fractions: 21% (normoxia), 12% (hypoxia), and 8% (severe hypoxia). Convective $O_2$ delivery [blood flow ($Q_m$) x arterial $O_2$ content ($CaO_2$)] to the muscle was held constant by elevating muscle blood flow via a perfusion pump. Blood flow was recorded with an ultrasonic flowmeter while venous $O_2$ saturation was monitored continuously in order to allow measurement of contraction-by-contraction muscle $VO_2$. Relative muscle deoxygenation (ΔHHbMb) was assessed with continuous near-infrared spectroscopy (NIRS). Arterial blood $PO_2$ (torr) was measured during each stimulation period and the mean per condition was 111 ± 11 for 21% inspired $O_2$, 41 ± 3 for 12% inspired $O_2$, and 30 ± 3 for 8% inspired $O_2$. Venous blood $PO_2$ (torr) measured prior to the end of contractions was significantly different ($P< 0.05$) among the three conditions (21% = 20 ± 5, 12% = 16 ± 5, 8% = 13 ± 5). The arterial $O_2$ content ($CaO_2$) was significantly lower in the hypoxic conditions and pump perfusion was significantly higher in the hypoxic conditions resulting in matched convective $O_2$ delivery ($ml/Kg^{-1}/min^{-1}$) that were not different ($P > 0.05$) among the three different $O_2$ fractions, (21% = 202 ± 57, 12% = 206 ± 57 and 8% = 205 ± 59). There was also no difference in tau, the primary time constant for the $VO_2$

on-kinetics among treatments (21% = 10.9 ± 2.4s, 12% = 10.1 ± 1.8s, 8% = 9.2 ± 1.9s ). Additionally, the NIRS data showed no significant time difference in the Δ HHb time course, mean response time (MRT) (21% = 15.3 ± 2.7s, 12 % = 14.7 ± 4.4s, 8% = 13.9 ± 3.0s) for each treatment. The primary component of both $VO_2$ and ΔHHbMb on-kinetics were characterized by a monoexponential function. Thus in the presence of unchanged (or "matched") convective $O_2$ delivery with arterial hypoxemia, skeletal muscle $\dot{V}O_2$ on-kinetics were not significantly impaired indicating there is not a peripheral diffusion limitation due to lower mean capillary $PO_2$.

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Table of Contents

List of Tables

List of Figures

# I. INTRODUCTION

Movement, whether it involves foraging for food, running for your life or scratching an itch, requires muscles to contract. Muscles require some form of energy to power these contractions and this energy comes from the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate ($P_i$). At rest the levels of ATP are relatively stable, about 5-8 mmol per kilogram (Kg) of wet muscle tissue (15). However, once muscles begin to contract, ATP is used rapidly and must be rapidly replaced for contractions to continue. ATP replenishment comes from three main chemical sources: the phosphogen system, also known as the immediate system, anaerobic glycolysis/glycogenolysis, and oxidative phosphorylation. The latter is the most important source of ATP replacement for mammals (129). Oxidative phosphorylation has the capacity to create substantially more ATP than other metabolic systems; however, there are limitations to the system (34, 46).

As early as 1913 Krogh and Lindhard (100) described these "changes in ventilation, blood flow, pulse rate, respiratory exchange and alveolar $CO_2$ tension," at the onset of light and heavy exercise. From this starting point, investigations have looked at humans and animals working and exercising in various environments to access $\dot{V}O_2$. Studies have examined; maximal $O_2$ consumption ($\dot{V}O_{2max}$ for whole body work/exercise or $\dot{V}O_{2peak}$ for isolated muscle), the rate of recovery to resting $O_2$ consumption levels after cessation of that work/exercise and the relatively slow $O_2$ uptake kinetics at the beginning of work/exercise. Krogh and Lindhard (100) labeled the relatively slow rise in $O_2$ consumption at the beginning of exercise as an "oxygen deficit." They went on to say that the $O_2$ deficit was not compensated for during exercise but

was repaid after work ceased, as an "$O_2$ debt" (99, 101). Some 40 years later, in the 1950s the time course of the oxygen uptake was described by Henry et al. (61, 83, 138). Later, in 1970, di Prampero et al.(61) and Whipp et al. (142) described $O_2$ deficit as having a mono-exponential time curve during moderate work rates. $O_2$ deficit is now often described in terms the rate of change or time it takes to reach steady state $\dot{V}O_2$ and thus called oxygen uptake kinetics or $\dot{V}O_2$ on-kinetics (46, 79, 85).

From this early work, exercise physiologist have continued to attempt to identify the factor or factors that limit the increase in $O_2$ consumption during the transition from rest to work. The investigations have examined two main theories; 1) metabolic activation – which states that there must be a buildup of metabolic substrates in the muscle before oxidative phosphorylation is turned on - a feedback system (45) and 2) $O_2$ availability - which states that the finite time required to move $O_2$ from the lungs to the muscle and then for the diffusion from the capillary to the mitochondria limits $\dot{V}O_2$ - a feed forward system (115). Researchers have conducted experiments with humans and animals using a wide array of technologies to examine whole body, individual muscle and single muscle fiber responses during rest-work transitions. Previous research in our laboratory has used canine skeletal muscle *in situ,* a model originally used by Stainsby et al. (131) and Piiper et al. (112). This model offers several advantages including; control of blood flow (thus $O_2$ delivery) to the muscle, the ability to measure both arterial and venous $O_2$ saturation at the muscle to derive muscle oxygen consumption ($\dot{V}O_{2M}$) and the ability to stimulate the muscle tetanically, recruiting all muscle fibers during each contraction. The research from our lab has examined $\dot{V}O_2$ on-kinetics using convective $O_2$ delivery (44, 49, 52), chemical treatments (50-51, 53, 57) and different exercise intensities (52, 62). The current study will use this same model to examine $\dot{V}O_2$ on-kinetics in normoxia and

hypoxia with matched convective $O_2$ delivery in an attempt to ascertain if there is an $O_2$ delivery or a diffusion limitation of $O_2$ that is responsible for the a slowing of $\dot{V}O_2$ on-kinetics in normally seen under hypoxic conditions.

## II. REVIEW OF LITERATURE

### $\dot{V}O_2$ ON-KINETICS

Humans are rarely in a steady metabolic state. The normal activities of life require movement and movement requires muscle contractions. During the greater part of the day human metabolic energy demand is in transition, i.e. going from sitting to walking or from walking to a more vigourous movement. Traditionally ATP demand during these transitions has been characterized as an immediate square wave increase (Fig. 1). Recent studies (148) has questioned the square wave demand, describing a slower rise in ATP turnover during the first 25 seconds of contractions. This needs to be investigated further, however, whether the increase is square wave or slightly less rapid, there is an immediate increase in ATP demand at the onset of contractions that must be met. In skeletal muscle the supply of ATP is limited; ≈ 5-8 mmoles per Kg of wet weight and this ATP is only capable of supplying energy for a limited number of contractions. Estimates of ATP demand by muscles per contraction cover a wide range from .15 mM (13) to 3.0 mM (25). To allow contractions to continue ATP must be replenished and within mammalian bodies there are three main sources: phosphocreatine (PCr); anaerobic glycolysis and the comparatively slow, oxidative phosphorylation. ATP is hydrolyzed with each muscle contraction to ADP and organic phosphate (Pi). In the presence of PCr and creatine kinase ADP is recombined with inorganic phosphate from PCr to make ATP in a reversible reaction based on the law of mass action. The equation that describes the process is

$$ADP + PCr \longleftrightarrow ATP + Cr$$

reserves of PCr are higher than ATP in skeletal muscle but still only sufficient for few contractions (144). In all out exercise, such as a 200 meter running event, peak output occurs in ≈ 1 sec (40) and decreases rapidly over 30 sec(15). This reaction provides a "temporal buffer" allowing both the glycolytic pathway and the aerobic pathway to begin to supply sufficient ATP to muscle (106). This "metabolic capacitance" provides the muscle cell with readily available means to meet a wide range of energy demands (134). As PCr is providing ATP extremely quickly the glycolytic pathway is activated. Glycolysis/glycogenolysis, the conversion of glucose or glycogen to ATP produces pyruvate which in the presence of lactate dehydrogenases is quickly converted to lactate (41). Oxidative phosphorylation begins to rise with the first contractions and continues to rise depending on ATP demand. In order that oxidative phosphorylation works there needs to be synchronization between the cardiovascular system, the pulmonary system and the tissues. Oxygen consumption or oxygen uptake is discussed in terms of oxygen uptake per minute or $\dot{V}O_2$ and can described for the whole body by the Fick principle:

$$\dot{V}O_2 = Q \times (CaO_2 - CvO_2).$$

Cardiac output, Q, represents blood flow to the tissue. Cardiac output is the result of heart rate (HR) times the stroke volume (SV) of the heart minus any peripheral resistance to the blood flow within the arteries. $CaO_2$ represents the arterial blood oxygen content that is being delivered via arteries to arterioles and then to capillaries where $O_2$ can diffuse into tissue cells. Arterial oxygen content depends on alveolar oxygen pressure ($PO_2$), hematocrit, hemoglobin (Hb) and the ability of Hb to bind to available oxygen (47, 85). $CvO_2$ represents mixed venous blood that consists of blood that has off loaded $O_2$ to the cells and blood that has not. The difference between the $O_2$ concentration in arterial blood and mixed venous blood gives the oxygen extraction by the tissue. This equation can be applied to the whole body or to a particular set of

tissues such as the liver, heart, or muscles. In previous research in our laboratory we have been investigated $\dot{V}O_2$ in isolated muscle *in situ*, examining muscle blood flow to a muscle, $Q_M$, and the arterial and venous difference across that muscle.

With an increase to in muscle energy demand such as going from a resting state to some less than exercise intensity below $\dot{V}O_2$ max, oxidative phosphorylation normally meets the ATP demand in 2-3 minutes(88) . The rate change for oxidative metabolism can be seen in Figure 1. Note at the start of contractions, time 0, oxidative phosphorylation is providing ATP to meet previous steady state ATP demand and begins to rise with the new demand. In this stylized diagram, there is no time delay or Phase I (this will be discussed shortly) depicted and the rise in $O_2$ consumption follows a curvilinear path that can be described by a monoexponential function. The area above and to the left of the oxidative area on chart the from start of contractions to the steady state is the oxygen deficit.(88)

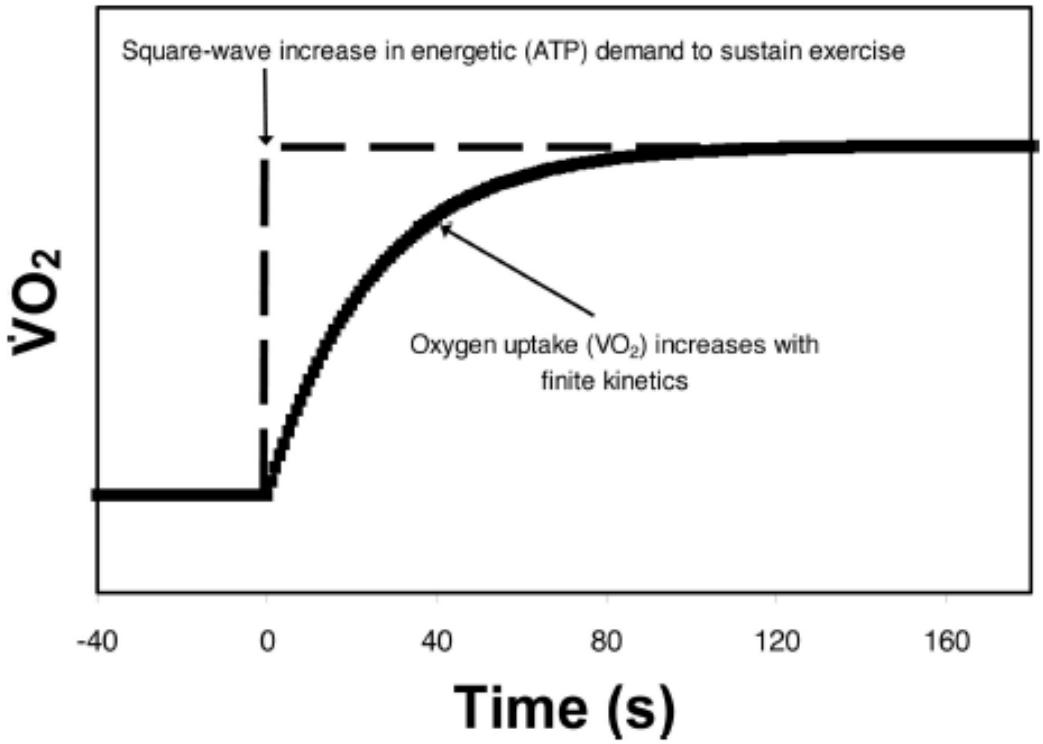


Figure 1. (88) Stylized diagram showing typical $VO_2$ response to a step increase in work rate to a new metabolic steady state. The initial ATP demand at the muscle is shown as a square wave and must be met by the muscle's immediate energy systems. Under normal conditions oxidative phosphorylation meets the steady state energy demand after ≈ 2 minutes in healthy individuals. A recent paper has questioned if the ATP demand at the start of exercise is actually a square wave response (148) however whether square wave or a two step process, the ATP demand is sufficiently fast to result in an $O_2$ deficit

In humans, measurements of $\dot{V}O_2$ are normally based on breath-by-breath gas analysis at the mouth, $\dot{V}O_2$ pulmonary ($\dot{V}O_{2\,P}$). While this is an excellent way of looking at whole body exercise it makes analysis of on-kinetics slightly more complicated. This can be illustrated in Figure 2. In response to an increase in work-rate there is an initial cardiodynamic phase in which measured $\dot{V}O_2$ (measured at the mouth) increases rapidly due to increased cardiac output and an increase venous return to the lungs which does not reflect muscle $O_2$ consumption(85). The Phase I response is not normally modeled for $\dot{V}O_2$ on-kinetics. In the Phase II, referred to as the

primary response, there is monoexponential increase in $\dot{V}O_2$ to meet demand. This rise in oxygen consumption measured via pulmonary $\dot{V}O_2$ has been shown to closely reflect muscle $\dot{V}O_2$ (56, 123). Phase II is faster in aerobically trained individuals (2, 15, 137, 149) and is slower in sedentary. Also, several diseases such as cardiac failure, chronic obstructive pulmonary disease and peripheral artery disease interfere with normal $\dot{V}O_2$ on-kinetics, slowing the response to increased work-rates (1, 7, 17, 45, 88, 110).

In Phase III oxidative phosphorylation meets the vast majority of the muscle's entire ATP demand (78) and at moderate work rates levels off to a new steady state of respiration.

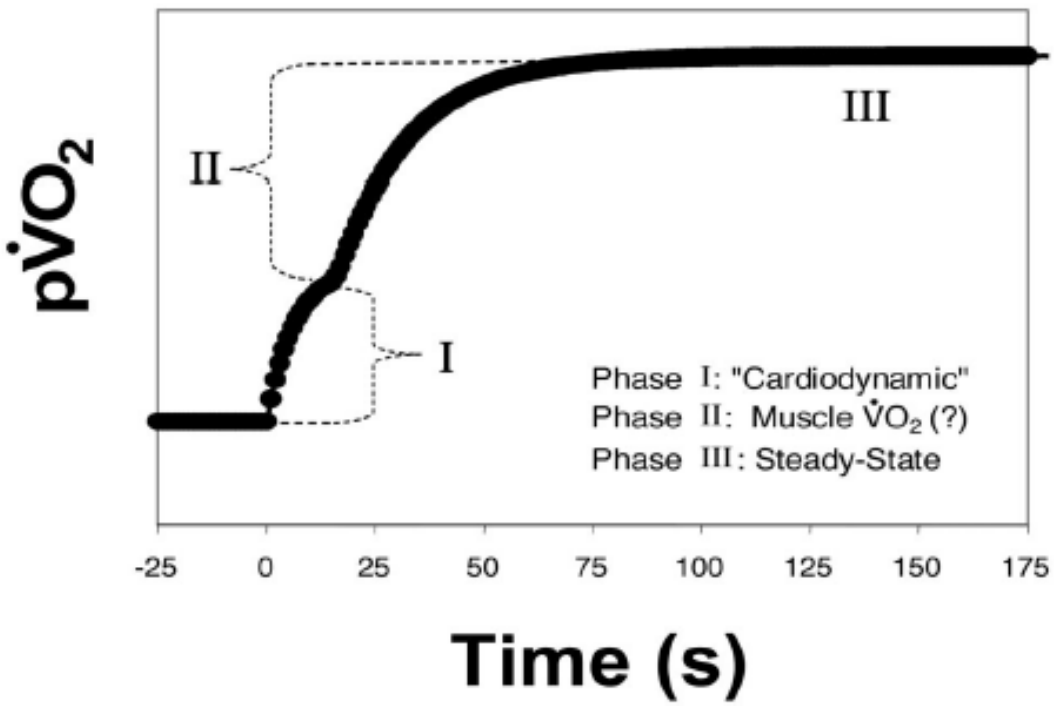


Figure 2. (88). Pulmonary $O_2$ adaptation to a step increase in work-rate to a new steady state. Phase I is usually not modeled when examining on-kinetics and Phase II kinetics closely mirror muscle $O_2$ kinetics.

At higher intensities, Phase III may exhibit a "slow component" where $VO_2$ continues to climb. When measuring $\dot{V}O_2$ on-kinetics, work rates are usually broken into three categories; 1) work rate below the lactate threshold (LT), (ventilatory threshold (LT) or gas-exchange threshold (GET) are sometimes also used interchangeably in the literature), termed moderate exercise, 2) work rates above LT but below $\dot{V}O_2$ max, often termed heavy, and 3) work rates at or above $\dot{V}O_2$ max, termed severe or extreme (88, 141). If the work-rate is below the LT, then Phase III should reach a steady state energy production and the $\dot{V}O_2$ will stabilize. Above LT, there is a "slow component" which will be seen as a delay in reaching steady state. The slow component may represent an increasing inefficiency in producing ATP (85) but other mechanisms may play a role and remain one of the key questions in $\dot{V}O_2$ on-kinetics (88). At or above $\dot{V}O_2$ max, Phase III never reaches steady state and continues to climb until exhaustion (141).

As stated above, $\dot{V}O_{2\,P}$ during Phase II on-kinetics is normally considered to reflect muscle oxygen consumption within ≈ 10% when working with humans (56, 123). In order to more closely examine muscle on-kinetics other methods have now been developed. These methods include; measuring blood $O_2$ difference across a group of muscles such as the quadriceps in humans (6, 56, 118), or measuring blood $O_2$ difference directly at the muscle in animal models with either muscles *in situ*, or in isolated muscles or using single muscle fibers *in vitro* (43, 49, 64, 112), Near-infrared spectroscopy (NIRS) allows investigators to examine the hemoglobin in the muscle to determine the extent of $O_2$ saturation during the initiation of exercise (14, 29, 36, 55). Other methods including phosphorescence quenching techniques and nuclear magnetic resonance (NMR) spectroscopy have been used to examine muscles metabolites (70, 126), myoglobin desaturation (24, 122) and partial pressure of oxygen ($PO_2$) in

capillaries and in the cell (117, 120) . These newer methods allow researchers to observe oxygen consumption in the muscle, providing a more precise way to assess on-kinetics.

When evaluating on-kinetics, the rate of Phase II change is normally described by a monoexponential mathematical model. The equation to describe this monoexponential rise in $O_2$ consumption follows:

$$\dot{V}O_2(t) = \dot{V}O_{2\,Bas} + \Delta\dot{V}O_2(1 - e^{-(t - TD)/\tau})$$

In this equation, $\dot{V}O_2$ at any time ($\dot{V}O_2(t)$) is equal to the baseline $\dot{V}O_2$, ($\dot{V}O_{2\,Bas}$) plus the amount of change in $\dot{V}O_2$ ($\Delta\dot{V}O_2$) from the baseline, also referred to as amplitude, multiplied by 1 minus the exponential function described by the negative result of time (t) minus the time delay before the $\dot{V}O_2$ starts to rise (TD) divide by a time constant tau ($\tau$). The time constant, $\tau$, is the time required for the $\dot{V}O_2$ to rise to 63% of steady-state value (123, 141). A stylized diagram of the on-kinetics with the TD and $\tau$ is shown in Figure 3. The time delay can represent the cardiodynamic phase of pulmonary measures but is also found in isolated muscle and has yet to be fully explained (51). Additionally, mean response time, (MRT), the time delay plus the time constant, is sometimes used in the literature to describe on- kinetics. Comparing tau or MRT allows for the comparison the on-kinetics of oxygen consumption under differing experimental conditions including varying work-rates, differing $O_2$ delivery levels, differing substrate levels in the muscles and differing exercise modalities, to name a few methods used to investigate the response in terms of aerobic respiration and energy production by muscle mitochondria to a increased muscle ATP demand.

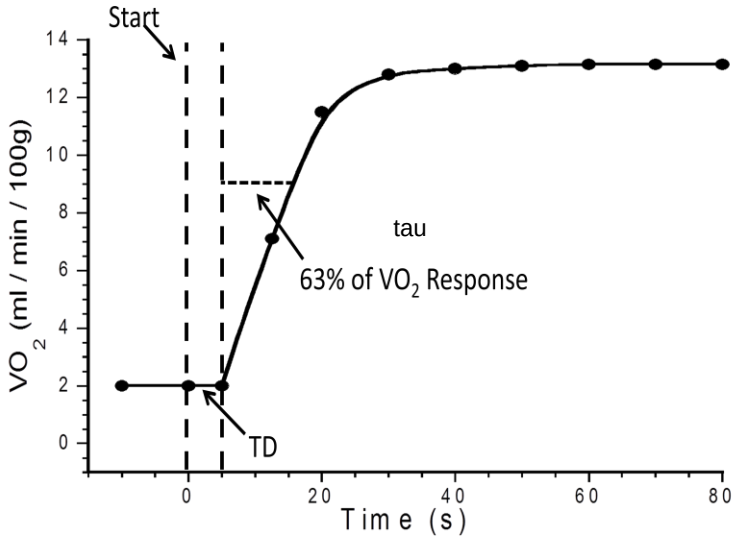


Figure 3. . Idealized from: Grassi B, Hogan MC, Kelley KM, Howlett RA, and Gladden LB. Effects of nitric oxide synthase inhibition by L-NAME on oxygen uptake kinetics in isolated canine muscle in situ. *J Physiol* 568: 1021-1033, 2005. Onset of contractions (Start); Time delay before a rise in $\dot{V}O_2$ (TD).

In the search to determine the controller/controllers of $O_2$ consumption during exercise investigators have examined; humans doing whole body and single limb exercise (8, 11-12, 21, 27, 31, 56, 81, 86, 96), animals using whole body exercise (93), isolated animal muscle *in situ* (49, 63, 66, 112, 131), isolated single muscle fibers *in vitro*(63, 91, 139) and isolated mitochondria *in vitro*(145-146). Despite increasingly sophisticated technology, there remains considerable debate over exactly what controls $\dot{V}O_2$ on-kinetics (47-48, 77-78, 83, 115, 138). The debate has focused on two main alternatives; 1) the metabolic activation hypothesis which proposes that a build up metabolic substrates within the mitochondria is required for there to be an increase in oxidative phosphorylation and thus $O_2$ consumption - a feedback system (45), and 2) $O_2$ delivery hypothesis which proposes the time required to move $O_2$ from the lungs via blood

flow to the muscle and/or the time involved in diffusion from the capillary to the mitochondria limits $\dot{V}O_2$ - a feed forward system (115).

## METABOLIC ACTIVATION

Before one can discuss the controllers of on-kinetics from a metabolic standpoint it is important to review what is known about overall control of oxidative phosphorylation. Maintaining ATP turnover during muscle contractions requires a multistep process in the mitochondria, described by the chemosmotic model first proposed by Peter Mitchell (109). The details of the reactions and mechanisms involved in this process have been thoroughly described (74, 109), however a brief review follows. The majority of ATP hydrolysis takes place in the cytosol thus ADP and $P_i$ must move from the cytosol to the mitochondria (144). The cytosol also supplies substrates (fats, pyruvate, other small carbon molecules and lactate(15)) to the mitochondria, where they are converted to reducing equivalents by β-oxidation or the tricarboxylic acid cycle (TCA). The reducing equivalents, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), donate electrons to the protein complexes in the electron transport chain (ETC) located on the inner membrane of the mitochondria. These electrons are passed between 4 complexes, I-IV. At complex I, III, and IV the energy from the electrons is used to pump $H^+$ ions from the mitochondrial matrix to the intermembrane space between the inner and outer mitochondrial membranes. This creates an electrochemical gradient and establishes a proton motive force. The passage of the $H^+$ ions from the intermembrane space through mitochondrial ATP synthase uses the supplied ADP and $P_i$ to create ATP. The reactions that occur in the ETC can be seen in Fig. 4 and can be summarized by the equation;

$$NADH + H^+ + 5ADP + 5P_i + O_2 \text{ ---> } 5ATP + NAD^+ + 2H_2O$$

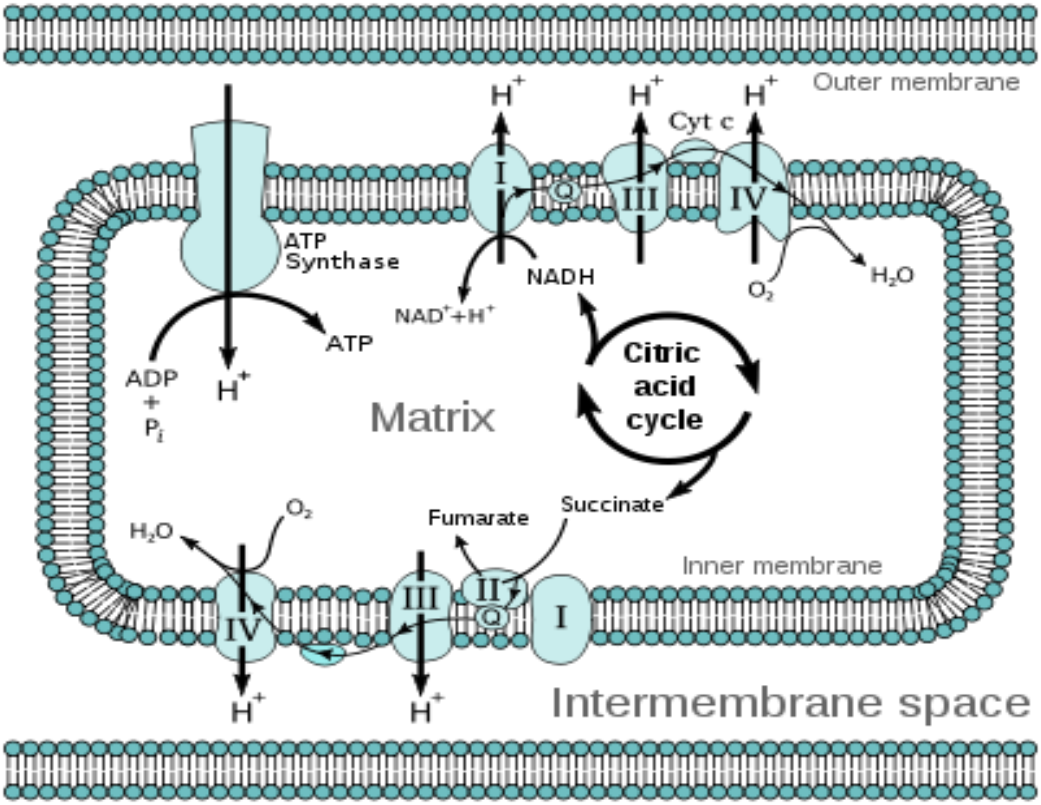


Figure 4. Oxidative phosphorylation. Stylized depiction of mitochondria with both the inner and outer phospholipid bilayer and the intermembrane space. Within the matrix the citric acid cycle (TCA) supplies reducing substrate to the electron transport chain (ETC). NADH donates two electrons at complex I which leads to a $H^+$ being pumped into the intermembrane space. Succinate donates electrons through $FADH_2$ at complex II. As electrons from complex I and II move down the chain to Complex III and IV more $H^+$ are moved into the intermembrane space. At Complex IV $O_2$ acts as the electron acceptor and forms $H_2O$. The $H^+$ ions create a proton motive force that passively re-enters the matrix through ATP synthase allowing ADP and Pi to join together and form ATP. (109).

Based on the reactions involved in mitochondrial repiration any of the following could be involved in regulation of oxidative phosphorylation; NADH, $H^+$, ADP, $P_i$, ATP and $O_2$. Additionally, because the TCA cycle supplies NADH, the regulatory steps in this cycle also may play a role in turning on oxidative phosphorylation. ADP stimulates cellular respiration and has been proposed as a regulator of oxidative phosphorylation (4, 23). The addition of ADP to isolated mitochondria *in vitro* maintained in a medium with high concentrations of $P_i$ and substrates rapidly and significantly increases the rate of oxidative phosphorylation (4, 22).

Chance et al. (23) postulate that changes in [ADP] "galvanize the function of the respiratory chain in response to energy needs….." However, Balaban (4) proposed that the regulation of oxidative phosphorylation is more complicated within the cell than a simple feedback system based on [ADP] and [$P_i$]. He proposed that a more complex model oxidative phosphorylation were three relationships; 1) movement of ATP, ADP and $P_i$ between the cytosol and the mitochondria, 2) the delivery of reducing equivalents, NADH and FADH, to the ETC, and 3) the availability of $O_2$ at Complex IV are all involved in the regulation oxidative phosphorylation (4). Wilson (144) expanded on this view outlining three hypotheses for concerning metabolic mechanisms that may control of respiration rates. The three hypotheses are: 1) The ADP hypothesis, 2) the adenine nucleotide translocase (ANT) hypothesis, and 3) the near equilibrium hypothesis. In the first hypothesis, when [ATP] drops, [ADP] increases and this stimulates respiration. This is a simple feedback model seen in isolated mitochondria as described by Chance et al., (23). Wilson points out, that the ADP hypothesis is limited to situations where mitochondrial respiration is a function of [ADP] alone. Experimental evidence has demonstrated the rate to ATP production can be dependent on [$P_i$] rather than [ADP] thus may be too simplistic (135). The ANT hypothesis suggests mitochondrial respiration cannot be dependent solely on [ADP] because ADP must move from the cytosol to the mitochondrial matrix to be converted back to ATP. This means that the ratios of [ATP]/[ADP] and the availability of ANT to move ADP into the mitochondria determine the rate of cellular respiration. As with the previous hypothesis, there is no role for [$P_i$] which has been experimental shown to affect the respiration rate, thus it cannot adequately describe the process. The near equilibrium hypothesis states that the mitochondrial respiration rate is related to the ratio of cellular [ADP]•[$P_i$]/[ATP], and intra-mitochondrial [$NAD^+$]/[NADH] and that theses substrates are at near equilibrium

normally. When exercise begins as stored ATP and PCr reserves are used the [ATP] goes down and [$P_i$] and [ADP] go up thus stimulating mitochondrial respiration. Combine with this changes in reducing substrate availability [NADH]/[$NAD^+$], "cellular energy metabolism is well controlled with respect to the rate of ATP synthesis…"(144)

The metabolic activation hypothesis for on-kinetics states that there is sufficient $O_2$ available to act as the final electron receptor for the ETC throughout the work-rate transition and that the finite time required for oxidative phosphorylation to reach steady state ATP production is limited by the same factors discussed above (48). Because the supply of NADH directly affected by the TCA cycle, allosteric ( $Ca^{++}$ ions), enzymatic or acetyl CoA availability could also limit the on-kinetics (47). There are several enzymatic steps in the TCA cycle that could be rate limiting and thus limit the production reducing equivalents, NADH and $FADH_2$. This is one of the first areas of experimentation used to evaluate metabolic activation of on-kinetics. The enzymes that can slow the TCA include; pyruvate dehydrogenase (PDH) that supplies the acetyl-CoA to the cycle, citrate synthase that converts acetyl CoA to citrate, isocitrate dehydrogenase which converts isocitrate to α-ketoglutarate and α-ketoglutarate dehydrogenase which converts α-ketoglutarate to succinyl-CoA.

Of these various limiting steps in the TCA cycle, the one that has received the most attention in terms of on-kinetics is PDH. PDH is a prime candidate for a metabolic controller based initially on experiments Timmons et al. (136). The group used dicloroacetate (DCA) infusion to raise pre-exercise PDH levels in humans. The subjects then performed leg kick exercise and $O_2$ deficit was estimated based on the rate of PCr breakdown. The results showed 50% reduction in PCr breakdown during exercise transition with increased PDH levels prior to exercise. The group interpreted this to mean that the oxygen deficit was lower due to the

increased PDH providing increased acetyl-CoA to start the TCA cycle. Following this experiment, Howlett et al.(75) treated single frog muscle fibers *in vitro* with DCA. The treatment significantly increased PDH levels and speeded muscle fiber on-kinetics. These findings indicate that an increase in PDH raised the concentration of the first acetyl-CoA increasing TCA cycle production of NADH and speeding $VO_2$ on-kinetics. Following the single muscle fiber experiment, Grassi et al., (51) used isolated canine muscle *in situ* treated with DCA to examine on-kinetics. Electrically stimulating the muscle from rest to ≈ 60% -70% of $\dot{V}O_{2peak}$, the group found that while PDH was elevated the $VO_2$ on-kinetics were not significantly different from a similar trial without DCA. They did find that the "stockpiling" of acetyl groups in the DCA treated muscle was associated with reduced fatigue(51). Other studies using DCA with humans also showed no speeding of on-kinetics with increased PDH levels or increased aceytl CoA avialability (5, 87, 97, 124). The use of prior exercise has been shown to speed $\dot{V}O_2$ on-kinetics in a subsequent bout of moderate exercise (59-60, 62). Gurd et al.(59-60), used moderate – heavy- moderate cycling protocol to compare the $\dot{V}O_2$ on-kinetics for a transition to a moderate work-rate with and without prior heavy (above LT) exercise. Between each exercise bout there was a period of 6 minutes of unloaded cycling. Following the bout of heavy exercise the $\dot{V}O_2$ on-kinetics for the 2$^{nd}$ moderate bout of exercise were faster and there was a significant increases PDH levels prior to the 2$^{nd}$ moderate bout. However, the group was unable to determine if the raised level of PDH or another factor such as increased blood flow prior to the moderate bout was responsible for the speeding of the on-kinetics (59-60). The role PDH and acetyl group availability in speeding on-kinetics, remains unclear but elevated acetyl CoA availability at the start of exercise may reduce fatigue and increase efficiency (45).

Another part of the oxidative chain which could slow the on-kinetics is found at Complex IV of the ETC where cytochrome c oxidase acts to transfer electrons to oxygen, forming water. Nitric oxide (NO) performs many functions within the body but it is also known to be a competitive inhibitor for the oxygen binding site on cytochrome c oxidase (16, 116). Experiments by Kindig et al. in horses and Jones et al. in humans used an inhibitor of NO, nitro l-arginine methyl ester (L-NAME) and found faster on-kinetics at moderate, heavy , severe and extreme exercise (89-90, 93-94, 143). All of these experiments used pulmonary measures of $\dot{V}O_2$ rather than measures of muscle $\dot{V}O_2$. There has been only one experiment to date to investigate inhibition of NO in isolated muscle. Grassi et al. using their canine model with L-NAME found inhibition of NO did not speed on-kinetics (53). Further experiments are needed to clarify the effects of NO inhibition on cellular respiration (53, 115).

As briefly described above, at the onset of contractions creatine kinase catalyzes the reversible reaction that rephosphorylates ADP to ATP. The enzyme creatine kinase has very high activity and when contractions begin PCr breakdown maintains the [ATP]/[ADP][$P_i$] ratio close to the resting levels. As discussed earlier [ADP] and [$P_i$] appear to be powerful stimulators of mitochondrial respiration, so the maintenance of [ATP]/[ADP][$P_i$] acts as temporal buffer at the start of muscle contractions. As contractions continue the reserves of PCr are rapidly used within a few contractions (144) and [ADP][Pi] rises quickly stimulating oxidative phosphorylation to meet the ATP requirements (58, 105-106, 134, 137, 139-140). The PCr system has been proposed as a controller of oxidative phosphorylation (12, 42, 57). Experimentally, Meyer working with NMR on rat gastrocnemius muscle found a linear relationship between PCr levels and ATP during muscle stimulation and proposed that creatine kinase acted as a chemical capacitor for muscle contractions (105). Later, Rossiter et al., showed

that PCr breakdown followed the same kinetics as phase II $\dot{V}O_{2P}$ on-kinetics in humans in moderate exercise and as such represented a good estimate of $\dot{V}O_{2M}$ (123). His group later went on to show that the "slow component" of $\dot{V}O_{2P}$ on-kinetics, normally seen in heavy exercise above the LT, follows the same kinetics as PCr, again estimating within 10% $\dot{V}O_{2M}$ (125). Studies *in vitro* of isolated mitochondria found a linear relationship between total creatine availability and the time required to reach a steady state in mitochondrial respiration (42).

Given this apparent buffering effect of PCr, inhibition of the reaction should result in a speeding of on-kinetics as suggested in the model by Korzeniewski and Zoladz (98). Kindig et al. (92), were the first to look at inhibiting CK in skeletal muscle using iodoacetimide (IA) in isolated frog myocytes. Their results demonstrated a faster drop in intracellular $PO_{2,}$ indicative of increased respiration with CK inhibition. They concluded that CK serves as a "temporal buffer" to [ATP]/[ADP]•[$P_i$] ratio at the transition to a higher work rate thus slowing the on-kinetics (92). In 2011, Grassi et al.(57) demonstrated that inhibition of CK speeds $\dot{V}O_2$ on-kinetics in an intact muscle *in situ*. Using isolated canine gastrocnemius complex that this group has used for several other on-kinetic experiments (49-53, 68-69), the group used IA to blocking CK and then stimulated the muscle at a rate to elicit ≈ 70% of $\dot{V}O_2$peak. The results demonstrated faster $VO_2$ on-kinetics with CK inhibition. David Poole, a leader in the field of oxygen uptake kinetics writing in the Journal of Physiology stated that the results "provide original evidence that muscle creatine kinase constitutes a locus of control for $\dot{V}O_2$ on-kinetics in mammalian muscle," (57, 113).

As presented, both pyruvate dehydrogenase and nitric oxide have had equivocal results as possible controllers of oxidative phosphorylation. There is some evidence that increasing acetyl CoA availability can reduces fatigue in a step increase in work-rate but it is unclear if it can

speed on-kinetics in either moderate or heavy-severe exercise. Experiments with humans and horses have shown faster on-kinetics with inhibition of NO but isolate muscle preparations have had opposite results. Further study measuring muscle $\dot{V}O_2$ is required to determine if indeed NO may be a metabolic controller. The recent work for Grassi et al. (57) gives direct evidence of a metabolic control of mitochondrial respiration with the inhibition of creatine kinase leading to a rapid increase in [ADP]•[$P_i$]/[ATP] and a speeding of $\dot{V}O_2$ on-kinetics. While more work remains to be done, it appears that metabolite ratios are essential to on-kinetics. However, the fact that there is a significant metabolic controller of cellular respiration does not mean that oxygen delivery is not also a regulator of the process.

## OXYGEN DELIVERY LIMITATIONS

The $O_2$ delivery limitation hypothesis postulates that the time required for oxidative phosphorylation to meet ATP demand in a transition from rest to a moderate work rate is the result of the finite time required to move of $O_2$ from the lungs to the muscle cells and/or the diffusion of $O_2$ across the cell to the mitochondria (138). The first line of support for this hypothesis comes from the fact that $\dot{V}O_2$ on-kinetics is slower in many disease states that limit cardiac output, limit blood flow or limit $O_2$ saturation. Such diseases include; heart failure (1), heart transplant (20, 54), peripheral artery disease (7), and chronic respiratory disease (110). $VO_2$ on-kinetics is also slowed in sedentary populations and with increasing age (3, 30, 128). In both these cases it appears reduced cardiac output and thus reduced muscle blood flow plays a role. However, in the disease states, and with aging and sedentary individuals there may be metabolic changes in addition to the reduce ability to deliver $O_2$. Another area where one sees slowed on-kinetics is with reduced $O_2$ availability as experienced at altitude or by breathing less than 21% $O_2$ (18-19, 130, 132-133).

In order to determine if $O_2$ delivery is the main limiting factor several different methods have been used to reduce $O_2$ delivery to the muscle including; altering cardiac output (Q) by slowing heart rate, changing the position of exercise to reduce stroke volume, chemically reducing the affinity of hemoglobin for $O_2$, and adjusting the $O_2$ content of inspired gases. β-blockers can be used to reduce Q by inhibiting the sympathetic increase in HR by blocking $\beta_1$ adrenergic receptors, restricting HR acceleration and thus cardiac output (15). At the onset of exercise, HR increases initially by the removal of parasympathetic inhibition, vagal tone, to approximately 100 beats per min. The sympathetic nervous system increases the HR above 100 bpm up to the maximum HR (127). Petersen et al.(111) had human subjects transition from unloaded cycling to a work rate of 50% of $\dot{V}O_2$max or 67% of $\dot{V}O_2$max with propranolol, a β-blocker, given intravenously and without, the control trial. Propranolol reduced HR and cardiac output and slowed $VO_2$ on-kinetics at both work rates compared to control. The steady state $VO_2$ was not significantly changed between the propranolol trial and the control. The slower HR reduced Q which reduced $O_2$ delivery to the working muscles and slowed the muscle on-kinetics. The following year Hughson (76) used an oral dose of β-blocker and a single step increase for unloaded pedaling to 100 watts and back to unloaded pedaling to look at both $O_2$ deficit and debt. He found a significant slowing of on-kinetics with the β-blocker presumably due to reduce $O_2$ delivery (76).

Another method to reduce blood flow to leg muscle in intact humans is to have the individual exercise in a supine position. When comparing upright exercise to supine exercise there is a smaller increase in SV with supine exercise (15). Convertino et al. demonstrated slower on-kinetics with supine cycling compared to upright cycling at a submaximal workload (28). In 1991 Hughson et al. (84) compared supine cycling exercise with upright cycling at

three different work rates including maximal effort. In all three cases there was a significant slowing of the $O_2$ on-kinetics in the supine position. With reduced SV in the supine position Q would be lower (Q = HR * SV) and so $O_2$ delivery to the muscle would be lower. To further examine the role of blood flow/$O_2$ delivery on oxygen uptake, Hughson et-al. (82) looked at exercise in the forearm both below and above the heart level. They found blood flow kinetics were faster with the arm below the heart and slower when the arm was above the heart level. Reflecting the changes in blood flow kinetics the $\dot{V}O_2$ on-kinetics were significantly slower with the arm above the heart level, pointing to a direct effect of blood flow on oxidative metabolism (82). These experiments demonstrated that reduced cardiac output leading to reduced blood flow does slow on-kinetics. However other experiments have demonstrated that cardiac output and muscle blood flow are usually faster than $\dot{V}O_2$ on-kinetics in healthy individuals (33, 138), implying $O_2$ delivery is faster than muscle $O_2$ consumption.

One way to examine muscle blood flow is to use thermodilution where cold saline is injected into the femoral vein at one point and then measured further along the same vein, the temperature change and the time distance between the two catheters gives the leg blood flow (LBF) (56). Grassi et al.(56) using this technique examined transitions from unloaded cycling to loaded cycling at a work-rate below LT in humans. During the trials the group also measured pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$), $VO_2$ across the leg muscles ($\dot{V}O_2leg$), LBF, and a-v$O_2$ difference across the leg. The results confirmed that $\dot{V}O_2p$ during phase II on-kinetics matches closely $\dot{V}O_2leg$ on-kinetics. The results also found that during pulmonary phase I within the first 10 to 15 seconds of onset bulk delivery of $O_2$ does not appear to limit the leg $\dot{V}O_2$ response (56). Bangsbo et al. (6) measured a-v$O_2$ difference and blood transit time across the quadriceps muscle during transition from rest to intense single leg extension exercise with similar results. They found

increased LBF and $\dot{V}O_2p$ within the first 10 -15 sec without a significant increase in $VO_{2leg}$ and a drop in a-$vO_2$ difference after which (in phase II) the on-kinetics at the leg was monoexponential (6). This increase in LBF without an increase in $\dot{V}O_2$leg demonstrates that $O_2$ delivery to the leg is faster than $O_2$ demand. In 2003, Grassi et al (55) returned to the cycling model, this time using near infrared spectroscopy (NIRS) to assess oxygen usage in the vastus lateralis muscle during transition from light cycling to a work-rate below LT and above LT. NIRS uses two infrared wavelengths to examine the oxygenation of the heme groups in the blood and muscle. The system is able to track de-oxygenation of hemoglobin and myoglobin (ΔHHbMb) as well as oxygenated hemoglobin and myoglobin ($O_2$HbMb) and total hemoglobin/myoglobin (THb/Mb). The group found a significant correlation between the mean response time of the ΔHHbMb signal from NIRS and the time constant, tau, of $\dot{V}O_2p$. During the first 6 – 10 seconds of transition there was no increase in $VO_{2P}$ neither was there a change in the ΔHHbMb signal indicating that more oxygen was available at the muscle than was needed for oxidative phosphorylation (55). This implies that here is a metabolic delay.

In an attempt to precisely examine $\dot{V}O_2$mus, Grassi et al. (49) used the canine gastrocnemius - superficial digital flexor complex (GS) *in situ* to examine the relationship between convective $O_2$ delivery and muscle on-kinetics. The experimental protocol consisted of two trials with GS complex electrically stimulated to elicit a step increase from rest to a work-rate at ≈ 60% - 70% of peak $\dot{V}O_2$. In the first trial blood flow was provided to the contacting muscles spontaneously by the animal's own heart. In the subsequent trial the blood flow was raised by pump perfusion to match the steady state level in the spontaneous trial prior to the start of contractions. This increased $O_2$ delivery to the level needed to sustain ATP production at steady state $\dot{V}O_2$ prior to the start of contractions. The a-$vO_2$ difference was measured across the

muscle to determined $\dot{V}O_{2M}$. The results showed no significant difference in the on-kinetics between the two treatments and the authors suggested that this supports the hypothesis that metabolic factors are responsible for the on-kinetics timeline (49). Later this same group duplicated these experiments with the muscle contracting at $\dot{V}O_2$peak (52). The results from these experiments showed faster on-kinetics and an ≈ 30% reduction $O_2$ deficit with speeded convective $O_2$ delivery. The group concluded that at $\dot{V}O_2$peak oxygen delivery along with metabolic factors contribute to the $VO_2$ on-kinetics.(52) Using isolated rat spinotrapezius muscle and examining the fall in muscle $O_2$ partial pressure ($PO_2$) Behnke et al. (9) found that blood flow and $O_2$ delivery were faster than the on-kinetics of $VO_{2M}$ and the group concluded that oxygen delivery is not limiting factor for on-kinetics in healthy muscle(9). These experimental results support the hypothesis that under $O_2$ delivery is faster than the $\dot{V}O_2$ on-kinetics of the muscle and that it is metabolic factors that limit on-kinetics.

Another method to test this hypothesis is to raise $O_2$ delivery by having subjects inspire a hyperoxic gas, 50% - 100% $O_2$. Hughson and Kowalchuk (80) had healthy human volunteers perform a series of rest to work transitions breathing different gas mixtures; 14% inspired $O_2$, hypoxia, 21% inspired $O_2$ normoxia and 70% inspired $O_2$, hyperoxia. They found slower on-kinetics in the hypoxia trial but no difference between normoxia and hyperoxia on-kinetics at a moderate level of exercise. Grassi et al. (50) working again with isolated canine gastrocnemius *in situ* attempted to speed $\dot{V}O_2$ on-kinetics by having the animal inspire 100% $O_2$ and in a second trial using RSR-13, a drug shown to right shift the oxyhemoglobin dissociation curve and 100% $O_2$ . This should allow for increased $O_2$ diffusion from the capillary into the working muscle. As with the previous canine experiments the muscle was stimulated to a moderate work-rate, 60% -70% of $\dot{V}O_2$peak and the Q was controlled by pump perfusion set to a level equal to a

spontaneous steady state blood flow. Comparing normoxia, hyperoxia, and hyperoxia with RSR–13 there was no difference in the $\dot{V}O_2$ on-kinetics (50). The authors concluded that enhanced peripheral diffusion does not affect $\dot{V}O_2$ on-kinetics at moderate work rates and in normoxia (50). In another experiment examining the role of hyperoxia MacDonald et al. (104) examined both $\dot{V}O_2$p and $\dot{V}O_2$leg during human leg kicking exercise. They found that hyperoxia did not speed the $O_2$ response at the alveoli or at the leg during the transition to moderate exercise. It appears that an increase in inspired $O_2$ and increased $O_2$ availability does not change $\dot{V}O_2$ on-kinetics in moderate exercise again supporting the metabolic activation hypothesis.

Another method used to speed $\dot{V}O_2$ on-kinetics is the use of "priming exercises", a bout of moderate or heavy intensity exercise followed by a short recovery at rest and then a transition to moderate exercise again with the on-kinetics of the second bout of exercise compared to a on-kinetics single transition from rest to a moderate work rate. Gerbino et al. (39) used warm-up exercises below LT and above LT to examine the subsequent exercise bout on-kinetics to a work rate below LT and above LT. The neither priming exercise had an affect on the on-kinetics of the subsequent sub LT exercise. Only supra-LT priming exercise speeded on-kinetics in a subsequent bout of supra LT exercise. The authors suggested that improved muscle perfusion and increased pH were responsible for the faster kinetics (39). MacDonald et al. (103) found similar results where only a prior exercise above LT exercise speeded the on-kinetics of a subsequent exercise bout also above LT. This group also had subject inspire a gas mixture with 70% $O_2$ in addition to priming exercise. They found even faster on-kinetics in the second bout exercise above LT but no difference in a second bout below LT. Their findings support the idea that below the lactate threshold, $VO_2$ on-kinetics are not limited by oxygen delivery however

above LT $O_2$ delivery does play a role(103). Other authors (Sheuermann et al. (128), Gurd et al(59-60), Herndandez et al. (62)) have also found speeding of on-kinetics after a set of priming exercises, however the underlying mechanisms are not clear and may be due to increased blood flow prior to the second bout caused by vasodilation, increased muscle pH, or the activation of PDH. Behnke et al. (10) examining muscle $PO_2$ following priming contractions found a shorter time delay (TD) but an unchanged the time constant, $\tau$, for the second bout of exercise. They also found that muscle $PO_2$ was no different between bouts and thus attributed the faster on-kinetics (TD + $\tau$) to metabolic changes in the muscle mitochondria.

## A NEW MODEL FOR $\dot{V}O_2$ ON-KINETICS

The above experiments appear to show that oxygen delivery is not a limiting factor in normoxia and that elevated $O_2$ delivery at the onset of contractions does not speed $\dot{V}O_2$ on-kinetics at intensities below LT. However, experiments using hypoxia and using hyperoxia in exercise above LT appear to show there are limitations caused by $O_2$ delivery. Thus it appears that $VO_2$ on-kinetics is under the control of both metabolic factors and $O_2$ delivery (24, 79, 88) . Wilson et al. (144, 147) demonstrated that in isolated mitochondrial respiration rate is sensitive to changing intracellular $PO_2$ ($P_IO_2$ or $P_{mito}O_2$) as well as metabolic factors. As discussed earlier in this paper, the metabolic mechanisms for control of respiration rates can be described by three hypotheses: 1) The ADP hypothesis, 2) the adenine nucleotide translocase (ANT) hypothesis, and 3) the near equilibrium hypothesis. The near equilibrium hypothesis appears to give the best explanation of what in occurring in the cell in vivo. The hypothesis proposes that in a steady state, the redox potential of NADH is in equilibrium with $NAD^+$ and that ATP is in equilibrium with ADP and $P_i$. It goes on to proposes that the rate determining step in respiration occurs at Complex IV in the electron transport chain where $O_2$ is converted to $H_2O$ a step that is

irreversible. This means that the [ADP]•[$P_i$]/[ATP] ratio and intramitochondrial [$NAD^+$]/[NADH] along with $PO_2$ interact to control respiration (144). Mitochondrial respiration can occur across a range of $PO_2$ depending on these two ratios. When $PO_2$ is low as might be seen in severe exercise high [ADP]•[$P_i$]/[ATP] (or intramitocondrial[$NAD^+$]/[NADH]) are required to be elevated to maintain respiration rate. At rest or low work rates with high $PO_2$ and low [ADP]•[$P_i$]/[ATP] can maintain respiration. A graphic depiction of this can bee seen in Figure 5.

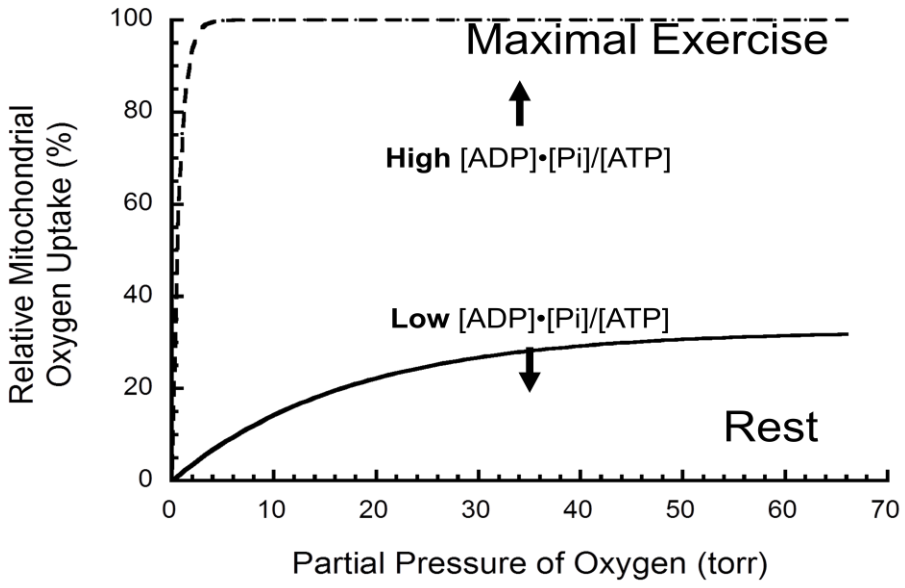


Figure 5. Predicted dependence of mitochondrial respiration on partial pressure of $O_2$ and metabolic factors. At rest based on the near equilibrium hypothesis the ration of [ADP]•[$P_i$]/[ATP] is low near equilibrium. As exercise increase [ATP] drops increasing the ration an allowing respiration to continue with very low $PO_2$. Adapted from Gladden, and Wilson et al. (145)

Hogan et al.(64), using the canine GS complex *in situ*, altered the arterial partial pressure of oxygen ($P_aO_2$) during transitions from rest to two different work-rates. The group measured

changes in [PCr], [ADP] and [ADP]•[$P_i$]/[ATP] and found that as $P_aO_2$ was reduced, there was greater [PCr] and [ADP] required to meet demand, supporting the Wilsonian model.

Applying these findings to $VO_2$ on-kinetics, Poole and Jones (114) have proposed a new model based on a continuum $O_2$ delivery. The continuum includes boht $O_2$ independent on-kinetics and $O_2$ dependent on-kinetics (Fig. 6). In $O_2$ independent zone includes the experimental findings from moderate upright cycling exercise, and various results animal studies; horse exercising in moderate and heavy domain, rat muscle stimulated in the moderate domain and canine GS at moderates rates. The $O_2$ dependent zone includes on-kinetics found in disease, supine cycling, hypoxia and slowed blood flow (114). The authors proposed that at some point on the continuum there is a transition from $O_2$ delivery independent on-kinetics to $O_2$ delivery dependent kinetics and that his constitutes a "tipping point" beyond which both metabolic and $O_2$ factors are important (114).

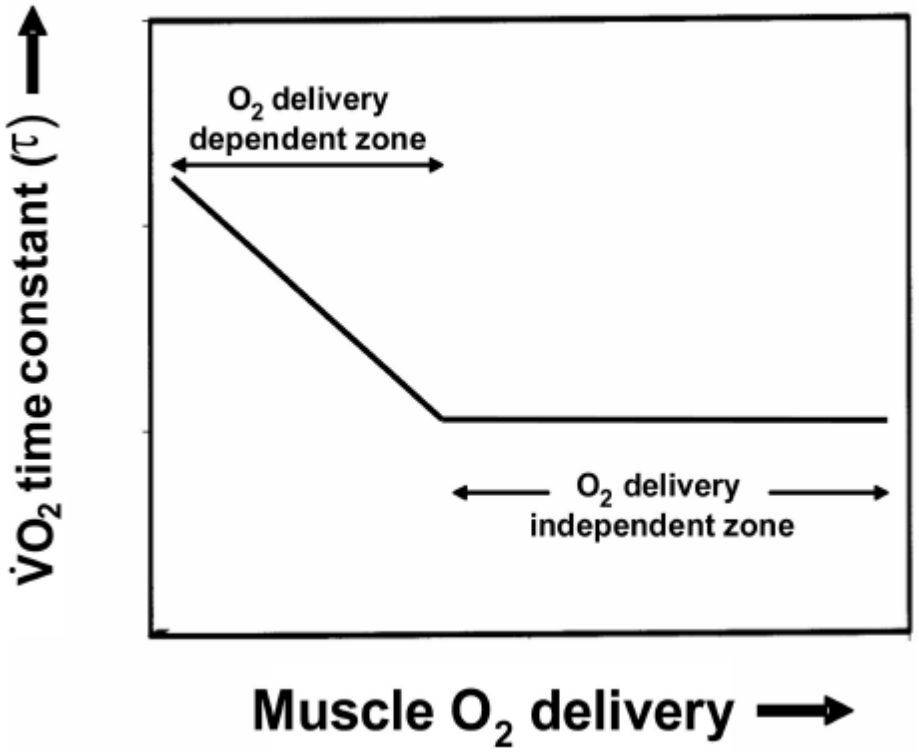


Figure 6. (88) Representation of limitation of $VO_2$ on-kinetics base on muscle $O_2$ delivery. The $O_2$ independent zone reflects normoxia in healthy humans subjects working at moderate work-rates. The $O_2$ dependent zone represents on-kinetics in individuals with disease, limited oxygen as in hypoxia and supine exercise.

Goodwin et al.(44) working in our lab and using the isolated canine GS *in situ* conducted a series of experiments to attempt to determine the point at which on-kinetics become $O_2$ dependent. Using pump perfusion, convective $O_2$ delivery was slowed by slowing blood flow on kinetics. The experiment used a control blood flow delivery rate based on spontaneous delivery determined prior to the experimental trials. The control was compared with two other treatments, blood flow kinetics slowed by 25 and blood flow kinetics slowed by 50 seconds. The resting blood flow and steady state blood flows where matched so that only the blood flow on-kinetics was different. The slower blood flow kinetics altered the convective O2 delivery. With the slower blood flow kinetics the $\dot{V}O_2$ on-kinetics were also slower. Indeed when the mean

response time for the three treatments was plotted the results were linear with an R = 0.96. The results appeared to match the predicted $O_2$ dependent model. The authors concluded that spontaneous blood flow to the canine GS operates at "the nexus of sufficient and insufficient $O_2$ delivery," in the transition to a moderate work-rate. (44).

## HYPOXIA STUDIES

Reducing the percentage of inspired $O_2$ has been used in both humans and animal models to examine the role of reduce $PO_2$ has on oxygen consumption at rest and during exercise and to examine $VO_2$ on-kinetics. Linnarsson et al., (102) were the first look at the impact of reduced inspired $O_2$ at the start of exercise. They found increased $O_2$ deficit with hypoxia. Several years later Murphy et al.(108) compared $\dot{V}O_2$ on-kinetics in normoxia and hypoxia by having eight human subjects perform a step transition test on a cycle ergometer while inspiring either a gas with 14 % oxygen and normal air. The steady state $\dot{V}O_2$ reached during both treatments was not significantly different however the on-kinetics were significantly slower with the hypoxic treatment. Because blood flow kinetics where the same in the step transitions the authors concluded that the delay was due to the lower arterial $O_2$ concentration (108). Following these findings, Hughson and Kowalchuk (80) examined $\dot{V}O_2$ on-kinetics in 6 young men performing cycle ergometer exercise at maximal and sub-maximal levels. The subjects inspired air with $O_2$ percentages of 14%, 21% and 70%. The study found that hypoxia limited maximal $\dot{V}O_2$ compared normoxia and hyperoxia. There was no difference in $\dot{V}O_2$max between the normoxia and hyperoxia treatments. The hypoxic condition also had significantly slower $VO_2$ on-kinetics compared to the other two conditions (80). Springer et al. (130) compared the changes in ventilation and heart rate response to exercise between adults and children during hypoxia and normoxia. They found that not only are $\dot{V}O_2$ on-kinetics slowed but also the heart rate response

to exercise (and thus cardiac output) was also slowed in the hypoxic condition. Engelen et al. (35) followed these experiments and examined what happens in heavy exercise with hypoxia. Again using a cycling protocol, subjects inspired air 21%, 15% and 12% oxygen. The cyclist performed work above the LT based on an incremental test to $\dot{V}O_2$peak with all three gas mixtures. As with other experiments $\dot{V}O_2$ on-kinetics was slowed in the hypoxic conditions however the steady state $\dot{V}O_2$ was not different. Additionally, the heart rate was higher at the start but had the same kinetics between treatments indicating that cardiac output could not meet the $O_2$ delivery demand in hypoxia. There was no difference in the steady state $\dot{V}O_2$ reached during in any of the treatments and no difference between treatments in the slow component. The group concluded that hypoxia only affects Phase II In 2000, MacDonald et al. (104) used had subjects inspire gas mixtures with 21%, 14%, and 70% oxygen and however rather than using a cycle ergometer they used a step transitions from rest to moderate single leg kick (weighted leg extensions) exercise. Compared to two-leg exercise (cycling), single-leg exercise has been shown to allow significantly higher leg blood flow (LBF)(15) which should increase $O_2$ delivery. The leg kick exercise also allowed the researchers to measure LBF via pulsed and echo Doppler. Additionally they measured the $O_2$ difference at the radial artery and femoral vein. Using the LBF and the radial artery $O_2$ content ($CaO_2$) and femoral vein $O_2$ content ($CvO_2$) to solve the Fick equation they determined $\dot{V}O_2$leg as well as measuring $VO_{2P}$. At rest there were no differences between $\dot{V}O_2$p or $\dot{V}O_2$leg in hypoxia, normoxia or hyperoxia, despite significantly different $CaO_2$. Once exercise began the $\dot{V}O_2$p and the $\dot{V}O_2$leg on- kinetics was not significantly different between treatments. The authors postulated that the lack of difference in on-kinetics was due to increased leg blood flow and $O_2$ extraction at the muscle compensating for lower $CaO_2$ in the case of hypoxia (104). Using a similar protocol, Delorey et al. (32), added

NIRS to the experiment using the ΔHHbMb in the vastus laterialis muscle during exercise as a surrogate for $\dot{V}O_2$mus. This group had earlier shown that ΔHHbMb accurately reflects $O_2$ delivery and consumption across the working muscle. They stated that when combined $VO_{2P}$ and leg blood flow this (ΔHHbMb) "allows for the determination of the time course for local muscle $O_2$ utilization (31). In the hypoxia experiment the subjects initially performed incremental leg kick tests while breathing air with 21% oxygen and then a second test breathing 12% oxygen. These tests established the LT and the moderate work-rate to be used in the trials. On separate days, the subjects returned to perform the step transition from unloaded leg kicks to their pre-established moderate work-rate while breathing either normal air or the hypoxic mixture. Blood flow was measured at the femoral artery and with leg $O_2$ delivery calculated based on LBF and $CaO_2$. The NIRS optical device was placed on the vastus lateralis with the optical sensors set at optimum distance. The results showed $VO_{2P}$ was significantly slower in the hypoxic condition. However, the ΔHHbMb response was not significantly different between normoxia and hypoxia. These experimental findings demonstrate that with normal blood flow in hypoxia there is slower on-kinetics. However, the results from MacDonald et al. raise the question of can increased blood flow as seen in single-leg exercise increase $O_2$ delivery sufficiently to compensate for low $CaO_2$.

Beyond human studies the impact of low $PO_2$ seen in hypoxia has been studied in muscle *in situ* and with single muscle fibers *in vitro.* The findings have helped clarify the results from human studies but the picture is not entirely clear. Hogan and Welch (73) compared hypoxic, normoxic, and hyperoxic conditions in the isolated GS during fatiguing stimulation. They found similar $\dot{V}O_2$ and fatigue in all three conditions but there was a significantly different acid-base balance values (73). King et al. (95) used the same muscle preparation and 9% $O_2$ to

examine the $O_2$-deficit with various stimulation rates using twitches. They found at using moderate twitch stimulation (2Hz) the $O_2$ deficit was not different between normoxia and hypoxia but at higher stimulation rates , there was a difference. They proposed that at the lower stimulation rates increased blood flow and that along with increased extraction of $O_2$ at the muscle compensated for lower blood $O_2$ content in hypoxia. However at higher stimulation rates these mechanisms could not keep up with demand(95). The picture that emerges from these experiments is that here may be a limitation of not just $O_2$ delivery but also a limitation in diffusion of $O_2$ from the blood to the muscle.

Diffusion at the working muscle is described by Fick's law of diffusion

$$\dot{V}O_2 = DO_2 (PcapO_2 – PmitoO_2)$$

where $DO_2$ is the lumped diffusion capacity, $PcapO_2$ is capillary $PO_2$ and $PmitoO_2$ is the $PO_2$ inside the muscle fiber (also called intracellular $PO_2$). Gayeski et al. (37-38) have found intracellular $PO_2$ ($P_iO_2$) to be uniform in the muscle fiber suggesting that $PmitoO_2$ would be the same or very close to intracellular levels. Intracellular $PO_2$ measured both in vivo and in situ have been found to be extremely low during exercise, in the range of 1-4 Torr (26, 37, 107, 122). In canine grasilis muscle flash frozen at maximum work rate to be $P_iO_2$ was found to be less than 0.5 Torr (38). However even at this low level the $PO_2$ is sufficient to allow full mitochondrial respiration which in situ is estimated to be as low as ≈ 0.5 Torr (27). Richardson et al. (117, 119-122) in a series of experiments using $H^1$NMR to examine myoglobin saturation in working human leg muscles during single-leg knee extensions have found that $PcapO_2$ is ≈ 11 Torr lower in 10% - 12% hypoxia compared to normoxia however the difference in $P_iO_2$ was only 1 Torr meaning that the pressure gradient between the capillary and the muscle remains relatively high even in hypoxia. Further they found that while $P_iO_2$ drops from rest to exercise it appears to

plateau at 50% - 60% exercise intensity and remains at the lower level up to $\dot{V}O_2$peak (120, 122). They concluded that the driving pressure from blood to cell is similar in hypoxia and normoxia (117). They went on to propose that diffusion capacity increases with hypoxia to allow for increases in $VO_2$ (117).

Hogan et al. performed a series of experiments using the canine GS (64, 67, 71-72) to examine the oxygen diffusion at $\dot{V}O_2$peak in hypoxia. The group hypothesized that the lower arterial $PO_2$ found in the capillaries under hypoxic conditions would limit $O_2$ diffusion into the muscle fiber and thus the mitochondria. The first of these experiments used three different levels of hypoxia (12%, 8% and 5% $O_2$). Blood flow was controlled with a pump and the inflow blood pressure was held constant. This resulted in slightly higher muscle blood flow (MBF) in the different hypoxia conditions which the authors attributed to vasodilation caused by the level of hypoxia. They found a linear relationship $\dot{V}O_2$peak and $O_2$ delivery at the muscle with the lowest delivery. They also found a similar linear relationship between $\dot{V}O_2$peak and $PvO_2$. They did not find a linear relation between $PcapO_2$ and peak $\dot{V}O_2$ which was predicted by Fick's law. The authors speculated that this may have been due to the increased blood flow due to vasodilation. They stated that their results were consistent with the hypothesis that $\dot{V}O_2$peak is limited by $O_2$ diffusion in the working muscle (71). In order to examine the $PcapO_2$ and $VO_2$peak relationship and the authors repeated the experiments however this time holding MBF constant (65). Here again they found linear relationship between $\dot{V}O_2$peak and both $PvO_2$ and $O_2$ delivery with the lowest inspired $O_2$ content resulting in the lowest $\dot{V}O_2$peak. They also found that there was a linear relationship between mean $PcapO_2$ and $\dot{V}O_2$peak, with the lowest $PcapO_2$ resulting in the lowest $VO_2$. They proposed that difference between the results from the two experiments was dus to increased diffusion capacity with the higher blood flow in the first

experiment. By holding blood flow stable all treatments had the same diffusion capacity and this lead to the linear relationship between $PcapO_2$ and $\dot{V}O_2peak$ (65). The group went on to examine the relationship between $PaO_2$ levels and metabolic factors that might affect mitochondrial respiration (64). Using to different stimulation rates, one designed to elicit $\dot{V}O_2peak$ and the another designed to be ≈ 60%-70% of peak, with normoxia, mild hypoxia (10% $O_2$) and severe hypoxia (7% $O_2$), they examined the oxygen consumption. They found that even at the sub-maximal work rates that $VO_2$ was significantly lower in the two hypoxic conditions. Again in these experiments blood flow was kept at a constant perfusion pressure so that $O_2$ delivery was lower in hypoxia (64).

## PURPOSE

Based on the previous discussion there may be a diffusion limitation at the muscle with low $PO_2$. However, MacDonald et al. (104) demonstrated that with increased LBF in single-leg exercise there is no difference in $\dot{V}O_2$ or $\dot{V}O_2$ on-kinetics between hypoxia and normoxia which argues against a diffusion limitation. Delorey et al. (32) using the same protocol found slower $\dot{V}O_2$ pulmonary on-kinetics however the NIRS data supports the idea that $O_2$ consumption within the muscle was the same in hypoxia and normoxia. On the other hand the canine studies of Hogan et al. support the idea that with low blood $PO_2$ associated with hypoxia leads to a diffusion limitation at least at $\dot{V}O_2$peak. However, in these studies MBF remains relatively low and thus $O_2$ delivery is significantly lower in the hypoxic condition. Given that even in hypoxia $P_iO_2$ appears to remain above the minimum required to have maximal mitochondrial respiration (117, 120) even working at $\dot{V}O_2$max. And that in hypoxia there remains a pressure gradient between the capillary and the muscle fiber that should allow diffusion (117, 121). Then increased $O_2$ delivery should be able to compensate for low $CaO_2$. My hypothesis is that at a moderate work rate muscle $\dot{V}O_2$ on-kinetics will not be different between normoxia and hypoxia if convective $O_2$ delivery to the muscle is matched, demonstrating there increased muscle blood flow can overcome any possible diffusion limitation. Therefore the purpose of this experiment is to examine $\dot{V}O_2$ on-kinetics under normoxia, hypoxia and severe hypoxia while holding convective $O_2$ delivery constant to evaluate if there are a diffusion limitations between the capillary and the muscle

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# $\dot{V}O_2$ ON-KINETICS IN SKELETAL MUSCLE IN NORMOXIA AND HYPOXIA WITH MATCHED CONVECTIVE $O_2$ DELIVERY


## ABSTRACT

The aim of this investigation was to evaluate $O_2$ uptake ($\dot{V}O_2$) on-kinetics under hypoxic conditions while holding convective $O_2$ delivery constant in isolated canine muscles *in situ*. Canine gastrocnemius muscles (n=7) were surgically isolated and then electrically stimulated via the sciatic nerve (8 V, 0.2-ms duration, 50Hz, 200-ms trains) eliciting contractions at a rate of 1 contraction every 2 seconds (about 70% of peak $\dot{V}O_2$). The dogs inspired 3 different $O_2$ fractions: 21% (normoxia), 12% (hypoxia), and 8% (severe hypoxia). Convective $O_2$ delivery [blood flow ($Q_m$) x arterial $O_2$ content ($CaO_2$)] to the muscle was held constant by elevating muscle blood flow via a perfusion pump. Blood flow was recorded with an ultrasonic flowmeter while venous $O_2$ saturation was monitored continuously in order to allow measurement of contraction-by-contraction muscle $VO_2$. Relative muscle deoxygenation (ΔHHbMb) was assessed with continuous near-infrared spectroscopy (NIRS). Arterial blood $PO_2$ (torr) was measured during each stimulation period and the mean per condition was 111 ± 11 for 21% inspired $O_2$, 41 ± 3 for 12% inspired $O_2$, and 30 ± 3 for 8% inspired $O_2$. Venous blood $PO_2$ (torr) measured prior to the end of contractions was significantly different ($P< 0.05$) among the three conditions (21% = 20 ± 5, 12% = 16 ± 5, 8% = 13 ± 5). The arterial $O_2$ content ($CaO_2$) was significantly lower in the hypoxic conditions and pump perfusion was significantly higher in the

hypoxic conditions resulting in matched convective $O_2$ delivery ($ml/Kg^{-1}/min^{-1}$) that were not different ($P > 0.05$) among the three different $O_2$ fractions, (21% = 202 ± 57, 12% = 206 ± 57 and 8% = 205 ± 59). There was also no difference in tau, the primary time constant for the $VO_2$ on-kinetics among treatments (21% = 10.9 ± 2.4s, 12% = 10.1 ± 1.8s, 8% = 9.2 ± 1.9s ). Additionally, the NIRS data showed no significant time difference in the Δ HHb time course, mean response time (MRT) (21% = 15.3 ± 2.7s, 12 % = 14.7 ± 4.4s, 8% = 13.9 ± 3.0s) for each treatment. The primary component of both $VO_2$ and ΔHHbMb on-kinetics were characterized by a monoexponential function. Thus in the presence of unchanged (or "matched") convective $O_2$ delivery with arterial hypoxemia, skeletal muscle $\dot{V}O_2$ on-kinetics were not significantly impaired indicating there is not a peripheral diffusion limitation due to lower mean capillary $PO_2$.

## INTRODUCTION

The energy demand for contracting muscles during exercise at moderate intensity can normally be met by oxidative phosphorylation. However, at the onset of exercise there is a finite time before $O_2$ consumption rises to a level necessary to support the energy demand. First discussed by Krogh and Lindhard (44), the rate of increase in oxygen consumption by the muscles (measured as $\dot{V}O_2$) is characterized by a mono-exponential rise, reaching a steady state after 2-3 minutes (9, 66-67). At exercise intensities above the lactate threshold (LT) the $\dot{V}O_2$ may continue to rise in what is described as a "slow component" (64). The underlying reasons for the relatively slow response of $\dot{V}O_2$ on-kinetics continues to be discussed and studied with focus on two hypotheses metabolic activation and $O_2$ delivery (18-19, 34, 38). These two hypotheses are not mutually exclusion giving rise to a view that there is a spectrum responses some that fall in an oxygen dependent zone, while in other circumstances there is sufficient $O_2$ present at the muscle but there is a metabolic substrate limitation (17, 39, 50).

Evidence against an oxygen availability limitation is provided by several studies that show muscle blood flow kinetics to be faster than pulmonary $\dot{V}O_2$ on-kinetics and faster than $\dot{V}O_2$ measured across the muscle (1-2, 24, 28, 37). In a series of classic experiments using isolated canine gastrocnemius (GS) *in situ*, Grassi et al., demonstrated that elevated blood flow, raised to the required steady state level prior to contractions, did not change muscle $\dot{V}O_2$ on-kinetics at moderate (60% of $\dot{V}O_2$max) contractions rates(20) The group also demonstrated that even with hyperoxia and use of a drug to right-shift the oxyhemoglobin disassociation curve, which should increase diffusion, there was no speeding of on-kinetics(21). These two experiments suggest that under conditions of normoxia and hyperoxia, there are metabolic factors that limit the speed of the on-kinetics.

Experiments that support an oxygen availability limitation to on-kinetics usually restrict $O_2$ availability to the working muscles by slowing heart rate (35, 49) or decreasing blood oxygen content via hypoxia, anemia, or carbon monoxide (10, 30-31, 61). Studies using inspired gas mixtures with less than 21% $O_2$ conducted in humans and animals and appear to demonstrate that low $CaO_2$ slows $\dot{V}O_2$ on-kinetics.

Linnarsson et al., (46) was the first to investigate the impact of reduced inspired $O_2$ at exercise onset; he found an increase in the $O_2$ deficit. Springer et al. (60) reported slower on-kinetics in both adults and children at the onset of moderate work rate exercise on a cycle ergometer with 15% inspired $O_2$. Similarly, Engelen et al. (11) reported that pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) on-kinetics were slowed in hypoxia with heavy exercise, again on a cycle ergometer. Hughson and Kowalchuk (36) also observed a slowing of $\dot{V}O_2$ on-kinetics when subjects inspired 14% $O_2$ during the transition to moderate work rate on a cycle ergometer; they speculated that the slower kinetics were due to less $O_2$ delivery to the muscle. These results taken together clearly support an oxygen availability limitation in exercise human muscle.

Similar results are seen in experiments on isolated animal muscle. Hogan and Welch (33) compared hypoxic, normoxic, and hyperoxic conditions in the isolated canine GS during fatiguing stimulation. They found $\dot{V}O_2$ rose to a similar level and fatigue was not different between the three conditions. However they did find significantly different acid-base balance values between the treatment that implies different levels of anaerobic metabolism in addition to aerobic metabolism(33). King et al. (43) used the same muscle preparation and 9% $O_2$ to examine the $O_2$-deficit with various stimulation rates. They found at using moderate twitch stimulation (2Hz) the $O_2$ deficit was not different between normoxia and hypoxia but at higher stimulation rates, there was a difference. They proposed that at the lower stimulation rates

increased blood flow and that along with increased extraction of $O_2$ at the muscle compensated for lower blood $O_2$ content in hypoxia. However at higher stimulation rates these mechanisms could not keep up with demand(43). The picture that emerges from these experiments is that there may low $CaO_2$ may not be only a delivery limitation but also a limitation in diffusion of $O_2$ from the blood to the muscle.

However the discovery by Saltin (59) that single leg exercise can significantly raise blood flow to the exercising limb above what is seen in two-legged exercise raised another opportunity to examined hypoxia and delivery versus diffusion limitation. Rowell et al., (58) found, using single leg kick exercise, that cardiac output can rise significantly increasing blood flow to meet the muscle metabolic demand in hypoxia. In 2000, MacDonald et al. (47) used this information to look at on-kinetics in single leg exercise. They had subjects inspire gas mixtures with 21%, 14%, and 70% oxygen and then used a step transitions from rest to moderate single leg kick (weighted leg extensions) exercise. The researchers measured leg blood flow (LBF) via pulsed and echo Doppler. Additionally they measured the $O_2$ difference at the radial artery and femoral vein. Using the LBF and the radial artery $O_2$ content ($CaO_2$) and femoral vein $O_2$ content ($CvO_2$) to solve the Fick equation they determined $\dot{V}O_2$leg as well as measuring $\dot{V}O_2$p. At rest there were no differences between $\dot{V}O_2$p or $\dot{V}O_2$leg in hypoxia, normoxia or hyperoxia, this despite significantly different $CaO_2$ between conditions. With the onset of exercise, LBF was significantly higher in the hypoxic condition, however $\dot{V}O_2$p and $\dot{V}O_2$leg on- kinetics were not significantly different between conditions. The authors postulated that the lack of difference in on-kinetics was due to increased leg blood flow and increased $O_2$ extraction at the muscle compensating for lower $CaO_2$ in the case of hypoxia (47). Using a similar protocol, Delorey et al. (8), examined on-kinetics examining $\dot{V}O_2$p, LBF and the change in dexoyhemoglobin

(ΔHHbMb) measured by near infrared spectroscopy (NIRS). NIRS uses infrared light absorption to measure hemoglobin and myoglobin oxygenation, which had previously been shown as an indication of the balance between $O_2$ availability and $O_2$ usage (3, 23). The Delorey group had earlier shown that ΔHHbMb accurately reflects $O_2$ delivery and consumption across the working muscle, stating that when combined $VO_{2P}$, leg blood flow, and ΔHHbMb "allows for the determination of the time course for local muscle $O_2$ utilization" (7). In the hypoxia experiment the subjects initially performed incremental leg kick tests while breathing air with 21% oxygen and then a second test breathing 12% oxygen. Blood flow was measured at the femoral artery and with leg $O_2$ delivery calculated based on LBF and $CaO_2$. .The NIRS optical device was placed on the vastus lateralis with the optical sensors set at optimum distance. The results showed LBF was 35% higher in the hypoxic condition though this was not statistically significant. $\dot{V}O_2p$ was significantly slower in the hypoxic condition. However, the ΔHHbMb response was not significantly different between normoxia and hypoxia, indicating at the muscle $O_2$ delivery and utilization were not different between the two conditions(8). The group concluded that the lower $\dot{V}O_2p$ in the hypoxic condition despite an increased blood flow and thus increased $O_2$ delivery similar to the normxoic condition was due to limited extraction as the result of a reduced diffusion gradient(8).

Diffusion at the working muscle is described by Fick's law of diffusion

$$\dot{V}O_2 = DO_2 (PcapO_2 – PmitoO_2)$$

where $DO_2$ is the lumped diffusion capacity, $PcapO_2$ is capillary $PO_2$ and $PmitoO_2$ is the $PO_2$ inside the muscle fiber (also called intracellular $PO_2$). Gayeski et al. (15-16) have found intracellular $PO_2$ ($P_iO_2$) to be uniform in the muscle fiber suggesting that $PmitoO_2$ would be the same or very close to intracellular levels. Intracellular $PO_2$ measured both *in vivo* and *in situ*

have been found to be extremely low during exercise, in the range of 1-4 Torr (5, 15, 48, 56). In canine grasilis muscle flash frozen at maximum work rate $P_iO_2$ was found to be less than 0.5 Torr (16). However even this low level the $PO_2$ is sufficient to allow full mitochondrial respiration which *in situ* is estimated to be as low as ≈ 0.5 Torr (6). Richardson et al. (52-56) in a series of experiments using $H^1$NMR to examine myoglobin saturation in working human leg muscles during single-leg knee extensions found that $PcapO_2$ to be approximately 39 Torr in normoxia and as expected lower, approximately 29 Torr in hypoxia(56). Within the muscle fiber, $P_iO_2$ was lower in both normoxia and hypoxia down to 3.1 Torr and 2.3 Torr respectively. This difference of less than 1 Torr in $P_iO_2$ allowed for a pressure gradient between the capillary and the muscle to provided diffusion even at low $PcapO_2$. Further they found that while $P_iO_2$ drops from rest to exercise it appears to plateau at 50% - 60% exercise intensity and remains at the lower level up to $\dot{V}O_2$ peak (54, 56). They concluded that the driving pressure for oxygen from blood to cell is similar in hypoxia and normoxia (52).

The differing results of MacDonald et al. (47) and Delorey et al. (8) need to be clarified. Can increased $O_2$ delivery compensate for low $CaO_2$ and result $\dot{V}O_2$ on-kinetics that are similar to normoxic conditions? Given that even in hypoxia $P_iO_2$ is appears to remain above the minimum required for maximal mitochondrial respiration (52, 55), if diffusion can be maintained with the higher blood flow and lower $CaO_2$ then on-kinetics should not change. The hypothesis to be tested in these experiments is that at a moderate work rate muscle $\dot{V}O_2$ on-kinetics will not be different between normoxia and hypoxia if convective $O_2$ delivery to the muscle is matched. Therefore the purpose of this experiment is to examine $\dot{V}O_2$ on-kinetics under normoxia, hypoxia and severe hypoxia while holding convective $O_2$ delivery constant.

## METHODS AND PROCEDURES

***Animals.*** Eight adult mongrel dogs (*canis lupus familiaris)* of both sexes were used. The Auburn University Institutional Care and Use Committee approved all procedures. The dogs had *ad libitum* access to food and water until 24 hours prior to experimental use when both food and water were withheld.

***Animal Preparation*.** All animals were housed and maintained at the Auburn University College of Veterinary Medicine prior to experimental use. The animals were anesthetized with an intravenous injection of aqueous pentobarbital sodium (dosage 30 mg per $kg^{-1}$ body weight). When each animal reached the surgical plane of anesthesia it was intubated, monitored and maintained in a deep, surgical plane of anesthesia throughout the remainder of the experiment with maintenance doses of pentobarbital sodium given as needed. Core temperature was maintained near 37° C. Each animal was ventilated with a respirator (model 613, Harvard Apparatus) at 20 ml per kg and 15- 20 breaths per min. Throughout the experiment arterial blood pH, $PCO_2$, $PO_2$, hemoglobin (Hb) concentration and $O_2$ saturation were monitored. Respirator settings, supplemental sodium bicarbonate and $O_2$, were used as necessary to maintain appropriate physiological levels of arterial blood gases and pH.

***Surgical preparation*.** For this study the gastrocnemius plus superficial digital flexor muscle complex (GS) were surgically isolated as described previously (62). Briefly, an incision was made medially in the left hindlimb going from midthigh to the ankle. The overlying

muscles (sartorious, gracilis, semiendinosus and semimembranosus) were cut at their insertions and moved aside. Venous outflow from the GS was isolated by ligating all veins draining into the popliteal vein except those from the GS. The popliteal vein was cannulated so that flow (Q) could be measured with a flow-through transit time ultrasonic probe (6NRB440, Transonic Systems, Ithaca, NY). The jugular vein was isolated, and a cannula attached to a reservoir was inserted so that blood from the popliteal vein could be returned to the animal. Once the jugular cannula/reservoir was in place the animal received heparin (3000 units per kg) administered in a bolus (half the total dosage) and then supplemented throughout the experiment. An inline oximeter probe (Opticath model no. U425C, size 4F, Hospira, Lake Forest IL) was inserted into the popliteal vein as close to the muscle as possible to allow continuous measurement of venous $O_2$ saturation. The arterial circulation to the GS was isolated by ligation of all vessels leading from the popliteal artery that did not go to the GS. The right carotid artery was isolated and cannulated. Arterial blood from the right carotid was routed via tubing and a peristaltic pump (Minipuls 3 MP2/HF, Gilson129 Incorporated, Middleton, WI) through another cannula to the isolated popliteal artery supplying the GS. A T-connector in the tubing was connected to a blood pressure transducer (model RP-1500, Narco Bio-systems, Austin, TX) for measuring systemic pressure. The calcaneus, with two tendons attached to the GS was cut from the heel and clamped into a metal bracket for connection to a load cell (Interface SM-250, Scottsdale, AZ) and then attached via a universal coupler to an isometric myograph. The universal coupler allowed the muscle to pull in a direct line with the load cell which prevented any significant torque from being produced. The head of the muscle remained attached to its origin and the femur and the tibia were fixed to the myograph with bone nails to minimize leg movement during contractions.

The sciatic nerve was isolated, exposed and the distal stump of the nerve (≈ 1.5 – 3.0 cm) was threaded through a small epoxy electrode containing two wire loops which were connected to a stimulator (Grass S48 stimulator, West Warwick, RI). A plastic holder with NIR optodes was placed over the belly of the muscle and held in place by a Velcro strap. The exposed tissue was covered with saline-soaked gauze and a thin, black opaque plastic sheet to reduce possible interference with the NIRS signal (see below). Prior to any experimental runs, optimum length of the muscle was set by progressively stretching the muscle and measuring developed tension elicited by single tetanic train stimuli. Once optimum length was determined, the muscle was allowed to recover for a minimum of 5 min.

After all experimental trials were completed the animal was euthanized with an overdose of pentobarbital sodium and saturated potassium chloride. The GS was removed, cleaned of connective tissue and weighed. The muscle wet weight (ww) was used to normalize $VO_2$ to muscle mass.

***Experimental Design*.** The experiment consisted of four trials, each separated by a minimum of 35 minutes of rest. For each trial the sciatic nerve was electrically stimulated to elicit tetanic contractions by the GS. Based on earlier experiments (20-21, 26) stimulation at 8 volts, 50 Hz, 0.2 ms pulse duration, 200 ms train duration at a rate of one contraction every 2 s evokes ≈ 60% $VO_{2peak}$ for the GS *in situ*. Using this stimulation pattern all animals performed a spontaneous exercise bout, i.e. blood flow to the GS was supplied by the animal's own cardiac output. The flow and blood pressure were measured (see below). The spontaneous run set the baseline blood flow for the normoxic condition and the calculated $O_2$ delivery from this condition was used to calculate the blood flow the tow hypoxic conditions. The three experimental trials were conducted in random order and included a normoxia trial (animal

breathing 21% $O_2$), a hypoxia trial (animal breathing 12% $O_2$) and a severe hypoxia trial (animal breathing 8% $O_2$). In each trial, blood flow to the GS was controlled via the peristaltic pump to match the convective $O_2$ delivery determined in the spontaneous trial. Five minutes prior to each trial, the breathing mixture was changed so subject inspired correct $O_2$ percentage for the trial. Arterial $CaO_2$ was checked via arterial blood sample. Immediately prior to contractions, the blood flow was adjusted to the calculated level to provided matched convective $O_2$ delivery.

***Measurements.*** Outputs from the load cell (through a strain gage coupler), ultrasonic flowmeter (T206, Transonic Systems), pressure transducer, the indwelling in-line oximeter probe and NIRS probe were directed to a computerized data acquisition system, the Oxymon MkIII (Artinis Medical systems BV, Zetten, the Netherlands). The sampling rate was 125 Hz. The load cell which was calibrated with known weights before each experiment reaches 90% of full response within 1 ms. The flowmeter was calibrated by graduated cylinder and stopwatch during each experimental trial. The Oximetrix 3 sampled Hb $SO_2$ at a rate of 244 samples per second. The samples were averaged each second resulting and output as a rolling average over 5 seconds. This output has a 90% response time of 5 seconds. The time response is mathematically de-convoluted to account for square wave changes as described by Hernandez et al.(26) Arterial and venous blood samples were drawn anaerobically into 3-ml plastic syringes, prior to commencing stimulation and at the end of stimulation. The venous blood samples were drawn from the catheter draining the muscle. All blood samples were immediately capped and stored in ice water and analyzed within 30 min of the draw on the GEM Premier 3000 (Instrumentation Laboratory, Lexington, MA). Samples were analyzed at 37$^{\circ}$C for $PO_2$, $PCO_2$, and pH. Total hemoglobin concentration ([THb]) and $SO_2$ were analyzed by a CO-Oximeter (IL-682,

Instrumentation Laboratory, Lexington, MA) set for dog blood. These instruments were calibrated before and during the experiments.

$\dot{V}O_2$ of the GS was calculated using the Fick principle: $\dot{V}O_2 = Q \bullet C(a\text{-}v)O_2$, where Q represents blood flow, and $C(a\text{-}v)O_2$ is the difference between the arterial and venous blood $O_2$ concentration [$O_2$] measured across the muscle. Contraction-by-contraction $\dot{V}O_2$ was determined using methods described by Hernandez et al. (26). Using the data from the Oximeter 3, venous blood $O_2$ concentration was rapidly determined. Arterial $O_2$ concentration was held constant. Blood flow and venous $O_2$ concentration were averaged for each contraction. Using a Microsoft Office Excel macro developed in-house, the data was analyzed for onset of each contraction and then contraction-by-contraction $VO_2$ can be determined by the Fick principle.

***Kinetics Analysis of $\dot{V}O_2$ and $\Delta$HHbMb.*** $VO_2$ and ΔHHbMb, relative muscle deoxygenation, kinetics were analyzed via the following mono-exponential function:

$$y(t) = y_{Bas} + A(1 - e^{-(t - TD)/\tau})$$

where $Y_{Bas}$ is the resting baseline value for $\dot{V}O_2$ obtained prior to the contractions (normally 5 sec), A is the amplitude of the $\dot{V}O_2$ response between the baseline and the primary component asymptote, TD is the time delay before the mono-exponential rise and τ is the time constant. Mean response time (MRT) was calculated by summing τ and the time delay (TD).

The data were fit using a nonlinear least squares program with OrginPro 8.5 (OriginLab, One Roundhouse Plaza, Northhampton, MA). Previously established criteria were used for the line of "best fit" (57, 65). In this procedure, the variables, A, TD, and τ, are allowed to "float" with the baseline $VO_2$ held constant at the experimentally measured value. The fitting window was varied to fit Phase II only. The best fit was determined on the basis of minimal values for

95% confidence interval for $\tau$, residuals and $Chi^2$. The line of "best fit" was determined by the best combination of the values above.

***Near-infrared spectroscopy.*** Microvascular $O_2$ availability at the muscle was assessed using a near-infrared spectroscopy (NIRS) system (Oxymon Mk III, Artinis Medical Systems BV, Zetten, Netherlands). The system uses two fiber-optic bundles to emit and receive two infrared light wavelengths (760 and 860 nm) which are absorbed by the heme groups in myoglobin and hemoglobin. NIRS does not distinguish between myoglobin and hemoglobin signals, although the myoglobin may account for up to a third of the signal (45). The terms for reduced heme groups (HHbMb), oxygenated heme groups ($HbMbO_2$) and total heme groups ($HbMb_{tot}$) will be used to represent the three hemoglobin/myoglobin signals. The infrared light used is maximally absorbed at different wavelengths for HHbMb and HbMbO2 and this makes it possible to measure the relative oxygenation of the heme groups. Also absolute changes cannot be calculated because the exact path length of the light cannot be determined (13), and the data are therefore reported in arbitrary units in comparison to a signal that is biased to zero immediately prior to contractions. The two optodes were locked into an epoxy holder attached directly to the belly of the GS. The optodes were held at a distance of 25 mm apart which allows a penetration depth of ≈12.5 mm. Signals were biased to zero just prior to first contraction in each experimental trial. Signals were averaged over each contraction using the same procedures outlined above for contraction-by-contraction $\dot{V}O_2$. Kinetics for ΔHHbMbMb were calculated as discussed above.

***Statistical Analysis.*** Data are presented as means ± SD. The data were analyzed using a one-way repeated measures analysis of variance (ANOVA) across the three levels of inspired $O_2$ percentage (21%, 12% and 8%). The level of significance was set at $p < 0.05$. If a main effects

difference was found, a Student-Neuman-Keuls post-hoc test was performed to identify the specific location of any differences.

## RESULTS

The wet weight of the GS muscle was 73.8 g ± 5.4 g and the dry weight was 16.1 g ± 1.1g. The water percentage was 78.1% ± 0.9%. The average water weight was slightly higher than seen in previous studies (17, 27). The higher than normal muscle water percentage was likely the result of higher than normal perfusion pressure resulting in mild edema by the end of the experiments. The additional edema in these muscles may have been the result of follow-on treatments performed for a different study, after the three experimental trials.

***Resting and steady state values.*** The resting values and values during the steady state of contractions for pertinent parameters for the study are shown in Table 1. The $PaO_2$ was significantly different among treatments both at rest and during contractions as planned on the basis of the $O_2$ concentration of the inspired gas mixtures. $PaCO_2$ was not significantly different among treatments or between rest and contractions. $PvO_2$ was significantly different among treatments both at rest and during contractions. Also arterial blood pH was not different among treatments nor was it different between resting and steady state levels. Venous pH was significantly different between rest and steady state but not different among treatments

Table 1: Blood gases, and pH at rest and during the steady state of contractions.

| Variable | Condition | | | | | |
|---|---|---|---|---|---|---|
| | Rest | | | Contractions | | |
| | 21% $O_2$ | 12% $O_2$ | 8% $O_2$ | 21% $O_2$ | 12% $O_2$ | 8% $O_2$ |
| $PaO_2$ (Torr) | 105 ± 13 | 41 ± 2† | 27 ± 1† | 111 ± 11 | 41 ± 3† | 30 ± 3† |
| $PaCO_2$ (Torr) | 31 ± 2 | 30 ± 1 | 31 ± 3 | 32 ± 3 | 30 ± 1 | 30 ± 4 |
| $PvO_2$ (Torr) | 65 ± 6 | 37 ± 3† | 27 ± 1† | 20 ± 5* | 16 ± 5*† | 13 ± 5*† |
| Arterial pH | 7.43 ± 0.04 | 7.46 ± 0.04 | 7.45 ± 0.06 | 7.42 ± 0.04 | 7.45 ± 0.00 | 7.44 ± 0.01 |
| Venous pH | 7.42 ± 0.05 | 7.44 ± 0.04 | 7.45 ± 0.06 | 7.31 ± 0.04* | 7.35 ± 0.03* | 7.36 ± 0.05* |

Data are presented as means ± SD. * indicates significance ($P < 0.05$) from rest to contractions. † indicates a significant difference between the 21% $O_2$ condition and the two hypoxic conditions.

***Oxygen Delivery.*** Values for $CaO_2$, $CvO_2$, $C(a\text{-}vO_2)$, Q, and convective $O_2$ delivery are presented in Table 2. $CaO_2$ was significantly different among treatments expected, but not different between rest and contractions. $CvO_2$ was not significantly different among treatments at rest; however, during contractions $CvO_2$ was significantly different between the normoxic trial and both the 12% hypoxia and the 8% hypoxia trials. There was no significant difference between the hypoxic treatments. $C(a\text{-}vO_2)$ difference was significantly different between treatments with the greater absolute difference occurring in normoxia, followed by the 12% hypoxia, and then 8% hypoxia. Blood flow was significantly different among treatments. The convective $O_2$ delivery ($Q \bullet CaO_2$) was not significantly different among treatments. A graphic depiction of the results can be seen in Figure 1, 2 & 3; where Fig.1 shows the comparison of the average $CaO_2$, Fig. 2 shows the average blood flow for each condition, and Fig. 3 illustrates the convective $O_2$ delivery for each condition.

Table 2. Arterial and venous $O_2$ saturation, difference, blood flow and convective $O_2$ delivery.

| Variable | Condition | | | | | |
|---|---|---|---|---|---|---|
| | Rest | | | Contractions | | |
| | 21% $O_2$ | 12% $O_2$ | 8% $O_2$ | 21% $O_2$ | 12% $O_2$ | 8% $O_2$ |
| $CaO_2$ ($mL \cdot dL^{-1}$) | 21.7 ± 1.7 | 17.4 ± 2.0 | 12.5 ± 2.8 | 21.5 ± 1.8 | 17.2 ± 2.0 | 13.3 ± 2.0 |
| $CvO_2$ ($mL \cdot dL^{-1}$) | 20.2 ± 1.9 | 16.4 ± 1.9 | 11.7 ± 2.8 | 6.8 ± 3.6‡ | 4.9 ± 2.9‡ | 3.2 ± 2.3‡ |
| $CaO_2$-$CvO_2$ ($mL \cdot dL^{-1}$) | 1.4 ± .9 | 1.4 ± 0.5 | 1.06 ± 0.7 | 14.7 ± 2.9‡ | 12.3 ± 1.8‡ | 8.8 ± 2.8‡ |
| Q ($mL \cdot kg^{-1} \cdot min^{-1}$) | 903 ± 293† | 1213 ± 325† | 1552 ± 381† | 917 ± 2.6† | 1199 ± 308† | 1555 ± 342† |
| Q x $CaO_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$) | 202 ± 58 | 210 ± 61 | 193 ± 50 | 202 ± 58 | 206 ± 57 | 205 ± 49 |

Data are presented as means ± SD. Q: blood flow; Q x $CaO_2$: convective $O_2$ delivery. * indicates significance ($P < 0.05$) from rest. † indicates significance ($P < 0.05$) between treatments. ‡ indicates significance between rest and contractions and treatments. Per kg indicates per kg wet muscle weight.

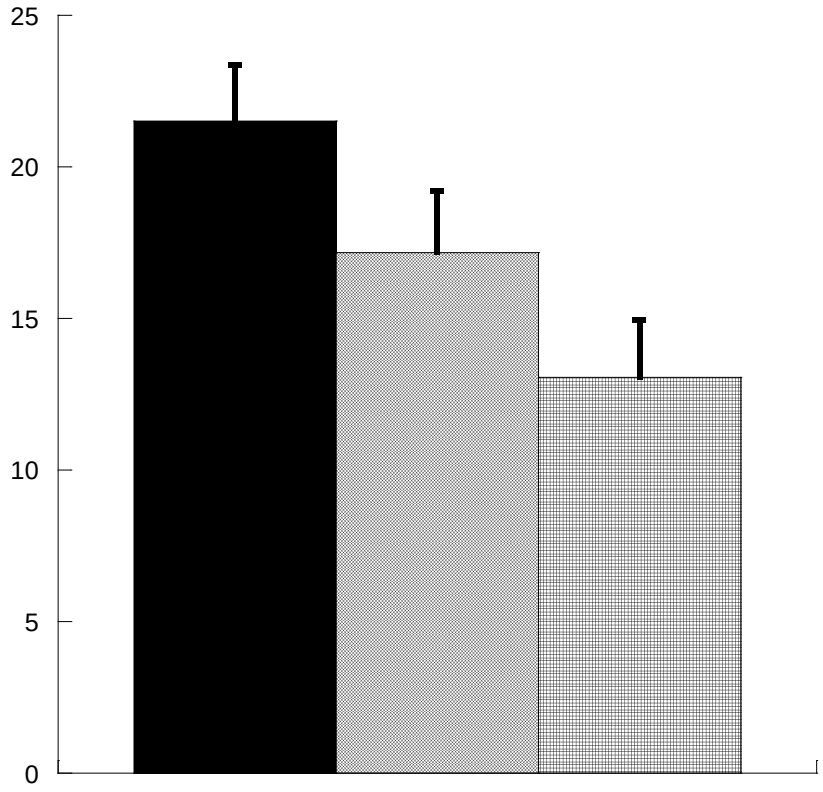


**Figure 1.** Mean $CaO_2$ by condition, filled column 21% inspired $O_2$, cross-hatch pattern 12% inspired $O_2$ and squares pattern 8% inspired $O_2$.

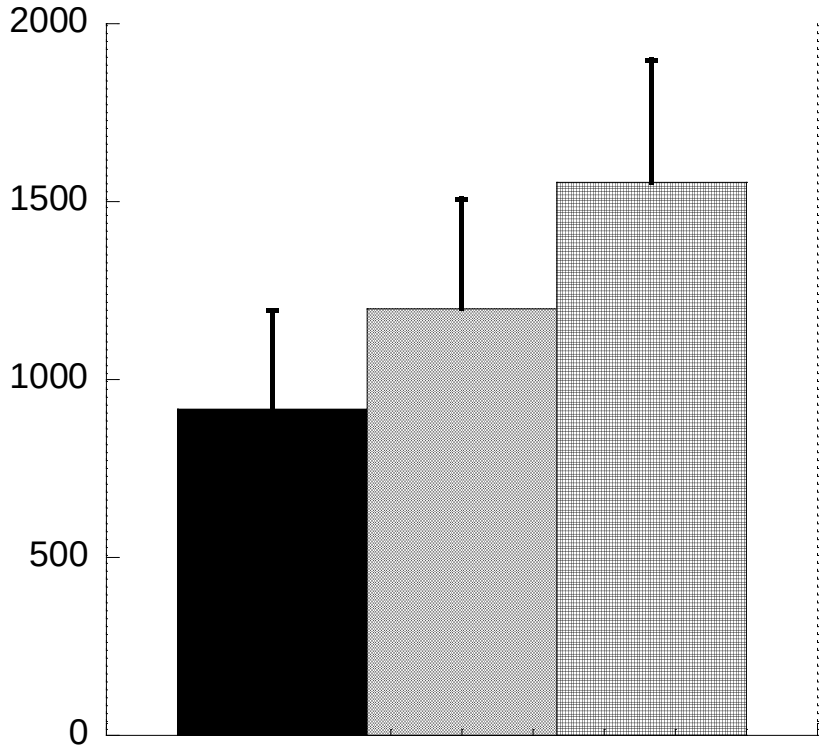


**Figure 2.** Mean blood flow (ml•kg$^{-1}$min$^{-1}$) by condition; Filled column 21% inspired oxygen, cross-hatched pattern column 12% inspired oxygen, square pattern column 8% inspired oxygen

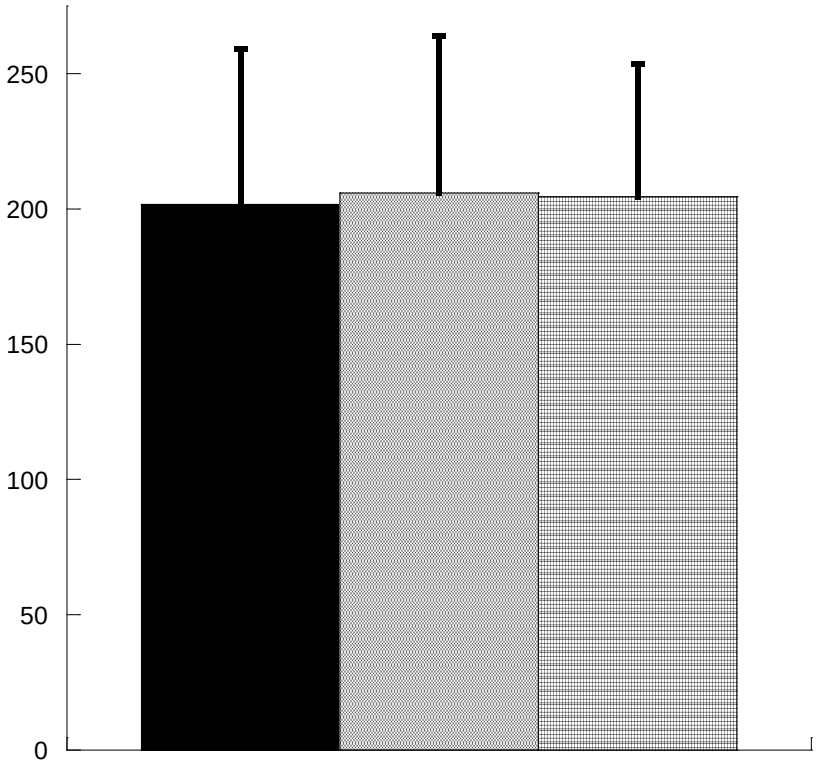


**Figure 3.** Mean convective $O_2$ delivery ($ml \bullet kg^{-1} \bullet min^{-1}$) by condition; Filled column 21% inspired oxygen, cross-hatched pattern column 12% inspired oxygen, square pattern column 8% inspired oxygen

***$\dot{V}O_2$ on-kinetics.*** Data for the primary time constant (tau), the time delay (TD) and mean response time (MRT), are shown in table 3. There was no significant difference for tau among treatments. There was a significant difference between the TD during normoxia and the two hypoxic trials. TD was not different between the two hypoxic trials. MRT in the normoxic trial was significantly slower than the 8% hypoxic trial; however there was no significant difference between the normoxic trial and the 12% hypoxic trial or between the two hypoxic trials.

The amplitude and asymptote for the primary $\dot{V}O_2$ for all three trials are presented in Table 3. There were no significant differences in either the amplitude or asymptote of the primary component among trials. There was also no difference in the highest average $\dot{V}O_2$ per

condition and no significant difference in the slow component in the three conditions. The average contraction-by-contraction $\dot{V}O_2$ data are shown in Figure 4 and normalized average $\dot{V}O_2$ is shown in Figure 5. The data was normalized by dividing the average $VO_2$ values for each condition by the highest average $\dot{V}O_2$ for that condition.

Table 3. $\dot{V}O_2$ on-kinetics data, primary amplitude, primary asymptote, $\dot{V}O_2$peak and slow component

| Variable | 21% $O_2$ | 12% $O_2$ | 8% $O_2$ |
|---|---|---|---|
| $\dot{V}O_2$ TD (s) | 7.1 ± 1.5 | 5.7 ± 1.5* | 5.3 ± 1.7* |
| $\dot{V}O_2$ Tau (s) | 10.9 ± 2.4 | 10.1 ± 1.8 | 9.2 ± 1.9 |
| $\dot{V}O_2$ MRT (s) | 17.9± 3.4 | 15.7 ± 3.1 | 14.5 ± 3.3* |
| Primary $\dot{V}O_2$ Asymptote (mL•kg $ww^{-1}$•$min^{-1}$) | 114.2 ± 11.5 | 116.1 ± 28.0 | 125.2 ± 21.4 |
| Primary $\dot{V}O_2$ Amplitude (mL•kg $ww^{-1}$ •$min^{-1}$) | 105.5 ± 13.2 | 106.0 ± 37.5 | 101.7 ± 19.8 |
| Maximum $\dot{V}O_2$ (mL•kg $ww^{-1}$•$min^{-1}$) | 137.7 ± 15.1 | 145.6 ± 32.3 | 149.0 ± 23.4 |
| Slow Component (mL•kg $ww^{-1}$• $min^{-1}$) | 23.5 ± 9.4 | 29.5 ± 15.4 | 23.8 ± 14.3 |

Data presented as means ± SD. TD - time delay; tau - time constant; MRT - mean response time: $\dot{V}O_2$, mL $O_2$ per kg ww (wet weight) per min. * indicates significant difference ($P < 0.05$) from 21% oxygen condition treatment.

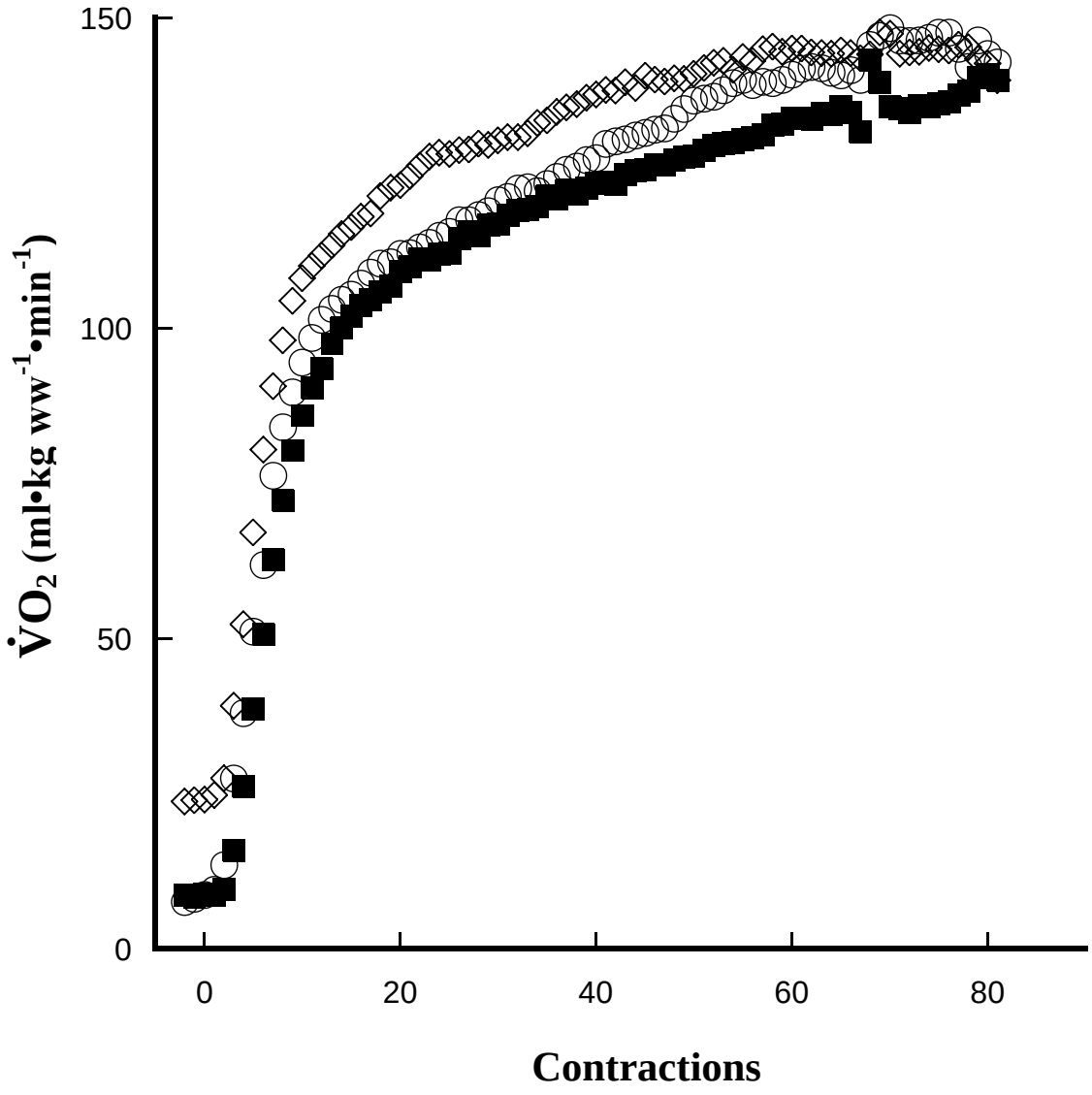


**Figure 4**. Mean contraction-by-contraction $VO_2$ data for 21% $O_2$ condition (closed square), 12% $O_2$ condition (open circles) and 8% $O_2$ condition (Open diamonds). There was no significant difference in the $\dot{V}O_2$ on-kinetic data among conditions. The mean starting $\dot{V}O_2$ was higher in the 8% $O_2$ condition but not significantly different from the 12% $O_2$ condition or the 21% $O_2$ condition.

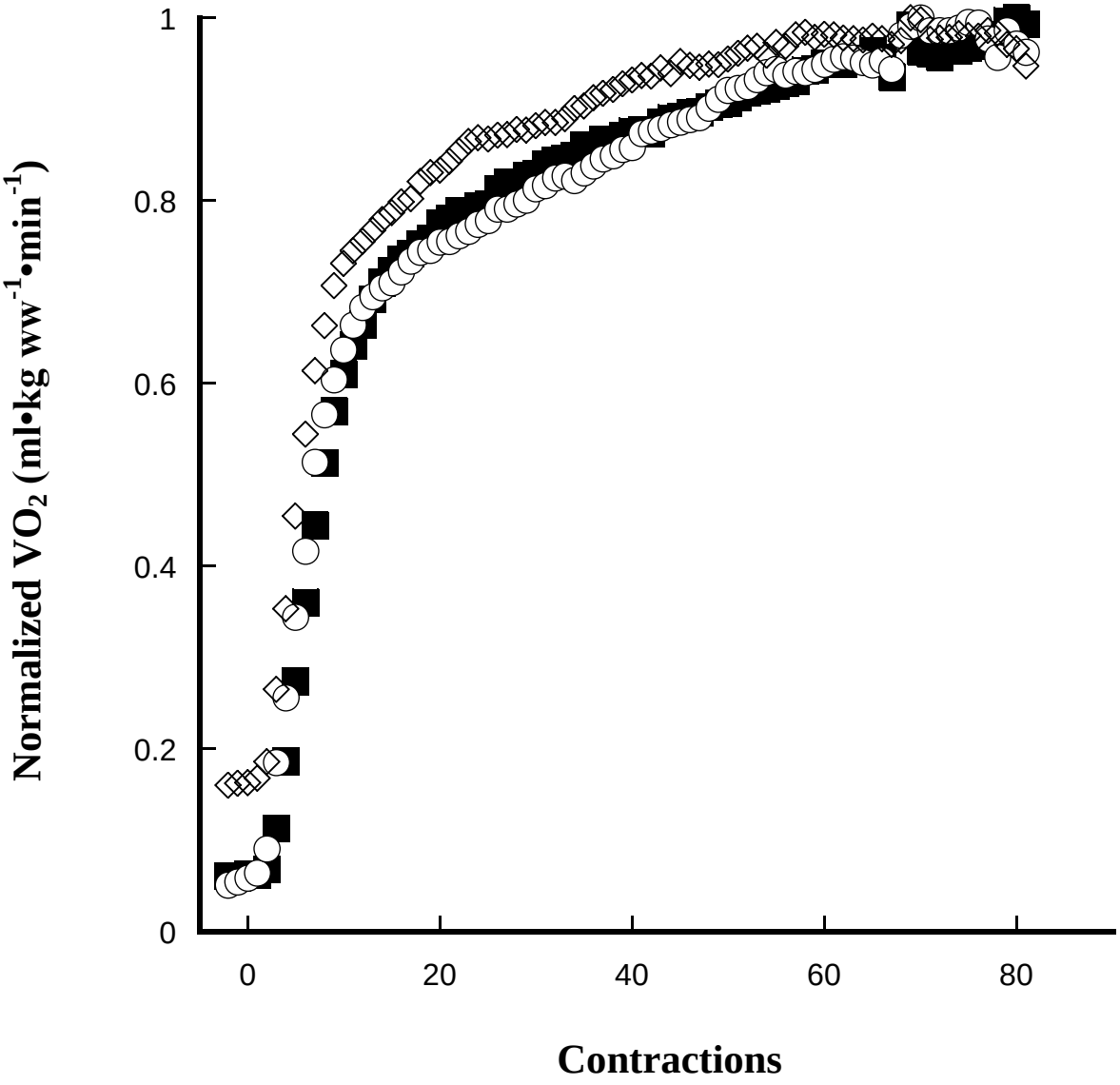


**Figure 5**. Normalized contraction-by-contraction $\dot{V}O_2$ data with shown as 21% $O_2$ condition (closed square), 12% $O_2$ condition (open circles) and 8% $O_2$ condition (Open diamonds). To normalize the data each mean contraction-by-contraction $\dot{V}O_2$ point was divided by the highest mean $\dot{V}O_2$ by condition. There was no significant difference in the normalized $\dot{V}O_2$ on-kinetic data among conditions.

***NIRS.*** The mean response of the NIRS Δ[HHbMb] signal in the different protocols is shown in Figure 4 and the tau, TD and MRT are given in Table 3. There were no significant differences between tau, TD or MRT among trials can be seen in Figure 4.

Table 3. NIRS Deoxygenated Heme Groups

| | | | |
|---|---|---|---|
| **Δ[HHbMb] TD (sec)** | **6.7 ± 0.7** | **5.7 ± 1.5** | **5.8 ± 0.6** |
| **HHb Tau (sec)** | **7.9 ± 2.4** | **8.8 ± 3.9** | **8.1 ± 2.9** |
| **HHb MRT (sec)** | **14.9 ± 2.6** | **14.5 ± 4.6** | **13.9 ± 3.0** |

Data are presented as means ± SD. There was no significant difference between conditions for time delay, TD, primary time constant, tau or mean response time, MRT

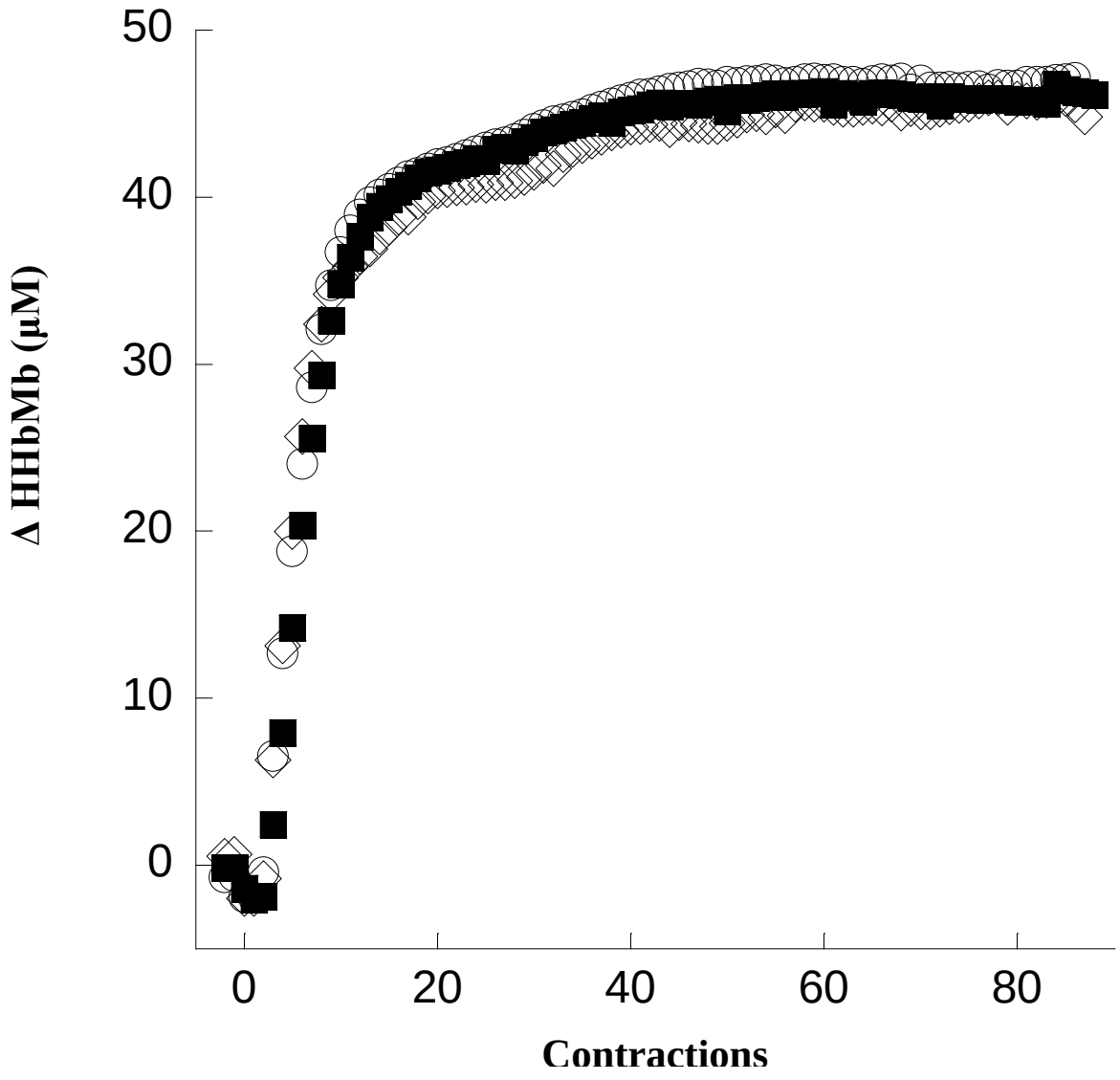


**Figure 6**. Mean NIRS data for ΔHHbMb for the three conditions; 21% $O_2$ (closed squares), 12% $O_2$ (open circles) and 8% $O_2$ (open diamonds). There was no significant difference between mean HHbMb on-kinetics.

***Fatigue.*** Table 4 contains the force and fatigue results for the three trials. There were no significant differences among treatments in peak force or final force. There was a significant difference in the fatigue index between normoxia and severe hypoxia (8% $iO_2$) but no difference between 21% and 12%, or 12% and 8% inspired $O_2$. There was also no order effect in relation to fatigue index.

Table 4. Force and Fatigue index

| Variable | Contractions | | |
|---|---|---|---|
| | **21% $O_2$** | **12% $O_2$** | **8% $O_2$** |
| **Peak Force ($kN \cdot kg^{-1}$)** | **6.2 ± 1.0** | **6.3 ± 1.1** | **6.4 ± 1.0** |
| **Final Force ($kN \cdot kg^{-1}$)** | **6.0 ± 0.8** | **6.0 ± 0.9** | **6.0 ± 1.0** |
| **Fatigue Index %** | **1.9%** | **3.4%** | **5.3%*** |

Data presented as means ± SD. There was no significant difference in peak force or final force among treatments. * indicates a significant difference from the 21% $O_2$ treatment

## DISCUSSION

The major finding of this is study is that under conditions moderate hypoxia, 12% inspired $O_2$ and severe hypoxia, 8% inspired $O_2$, normal $\dot{V}O_2$ on-kinetics can be maintained by increasing blood flow so that $O_2$ delivery to the muscle matches $O_2$ delivery with normoxia, 21% inspired $O_2$. The fact that tau, the $\dot{V}O_2$ primary amplitude and the steady state $\dot{V}O_2$ were not different among treatments supports the idea that with higher blood flow the diffusion driving pressure was sufficient to meet the muscle $O_2$ demand despite lower capillary $PO_2$. In this experiment this appears to be due to an increase in the diffusion capacitance in hypoxia.

***$\dot{V}O_2$ on-kinetics.*** In previous work from our laboratory we have studied $VO_2$ on-kinetics by modifying blood flow via pump perfusion to change convective $O_2$ delivery to an isolated muscle. The findings include; 1) under normoxic condition at a work rate less than $VO_{2peak}$ on-kinetics are not speeded by either increased $O_2$ delivery prior to commencement of contractions or increased arterial $O_2$ saturation combined with increased delivery prior to contraction (20-21), 2) With the isolated muscle working at or near $\dot{V}O_2$peak, increased blood flow and thus $O_2$ delivery prior to contractions does speed on-kinetics (22), and 3) By reducing convective $O_2$ delivery by slowing blood flow to the muscle in normoxia one can slow on-kinetics in a manner consistent with oxygen dependent zone described by Poole and Jones (17, 50). These studies led to this current research into the role of $O_2$ delivery in hypoxic conditions. Earlier work with humans and in animal models has found that under conditions of hypoxia $O_2$ deficit is increased and $\dot{V}O_2$ on kinetics are slowed (11, 36, 43, 60). Many of the human studies used upright cycling and hypoxia at various work rates less than $\dot{V}O_2$max. During upright cycling in hypoxia, $O_2$ delivery to the leg muscles is reduced compared to the normoxic condition and this results in slower pulmonary on-kinetics (11, 36, 60). However, Rowell et al., (58) found that leg blood flow is increased in hypoxia in single leg exercise when compared to upright cycling. They went on to find that in single-leg knee extension exercise under conditions of hypoxia (10% -11% $O_2$) that increased blood flow to the leg maintained $\dot{V}O_2$peak compared to normoxia. They postulated that the heart could meet $O_2$ demand by increasing blood flow along with increased muscle $O_2$ extraction. Using a similar approach Richardson et al., (53) examined a range of single leg work rates up to a peak level with inspired $O_2$ levels of 21%, normoxia and 12%, hypoxia. They found a significantly lower $VO_2$peak between normoxia and hypoxia but only a trend toward lower $\dot{V}O_2$ with work rates from 50% to 90%. Cardiac output and LBF

significantly increased in hypoxia compared to what was seen in normoxia until exercise reached ≈ 80% of $\dot{V}O_2$peak. They concluded that at 50% to 90% of $\dot{V}O_2$peak increased $O_2$ extraction allowed $\dot{V}O_2$ rise at a rate not significantly different from that of the normoxic condition. Later MacDonald et al., (47) used moderate intensity single leg kicking to examine $\dot{V}O_2$ on-kinetics in normoxia and hypoxia. They found that LBF was significantly higher in hypoxia and that muscle $\dot{V}O_2$ on-kinetics was the same between normoxia and hypoxia. They concluded that the increased blood flow and thus increased oxygen delivery along with increased extraction ameliorating the effects of low $CaO_2$ (47). DeLorey et al., (8) used a similar protocol to examine muscle de-oxygenation via NIRS while also looking at LBF and $\dot{V}O_2$p. They found that $\dot{V}O_2$p was slower in hypoxia despite an approximate 35% increase in LBF. Their NIRS results, the de-oxy hemoglobin (ΔHHbMb) showed not significant difference between conditions indicating that at the muscle the oxygen delivery and utilization were similar. They did find a tendency toward a lower ΔHHbMb in hypoxia which they suggested this was due to decrease extraction because of reduced diffusion pressure gradient. The results of the current experiment supports the idea that increased blood flow and $O_2$ delivery can compensate for low arterial $O_2$ levels and can maintain the diffusion driving pressure. We saw no difference in the $\dot{V}O_2$ muscle on-kinetics between treatments with higher blood flow and thus increased convective $O_2$ delivery. Additionally, there were no significant differences in the steady state $\dot{V}O_2$ achieved between three conditions. As can be seen in figure 4, average $\dot{V}O_2$ profiles were the similar for the three treatments and as can be seen in table 3, the is no difference in the primary time constant. There was significant difference in TD between the normoxia and the two hypoxic conditions which is probably due to the significantly higher blood flow rate prior to contractions due to increasing the pump output prior to the start of contractions. Also, the 8% hypoxia treatment had an

observably higher resting $\dot{V}O_2$ prior to contraction however; this was not significantly higher than the 21% and 12% conditions. The $O_2$ consumption for the moderate work rate was not statistically different as seen in $\dot{V}O_2$ amplitude. Taken together these findings support the results in MacDonald et al. (47) and supported the hypothesis that matched convective $O_2$ delivery can compensate for low $CaO_2$.

Hogan et al., (29), using the same isolated muscle preparation and similar levels of hypoxia without increased muscle blood flow, reported significantly different $\dot{V}O_2$ between the treatments both at moderate work rates and at a work rate designed to elicit $\dot{V}O_{2PEAK}$. However blood flow was held constant in these experiments and thus $O_2$ delivery was significantly different even at a moderate work rate.. Hogan et.al, postulated that in hypoxia the lower $\dot{V}O_2$ was due to a diffusion limitation as a result of low capillary $PO_2$ The findings in this experiment at 60% -70% $VO_2$ peak found no difference in $\dot{V}O_2$ with increased $O_2$ delivery and that diffusion was not limited with the higher flow despite the low $PcapO_2$. As Richardson et al. point out in the human leg kick studies "a major cause of increased $\dot{V}O_2$ is an increase in $DO_2$ not a change in $PO_2$" (52). Interestingly there was lower absolute $O_2$ extraction, $C(a\text{-}vO_2)$ difference between normoxia, hypoxia 12% and hypoxia 8% with greater extraction occurring in normoxia and lower in both hypoxia treatments. This is similar to what has been seen in human single leg kick preparations (8). The fact that there was no significant difference in tau for either hypoxic treatment though there was different extraction appears to support the idea of increased diffusion capacity in hypoxia.

***NIRS***. As mentioned in the introduction the NIRS ΔHHbMb signal is a composite of both myoglobin and hemoglobin de-oxygenation and estimates of the role of myoglobin in the signal during exercise continue to be debated (14, 45) however there is general agreement that

the ΔHHbMb reflects the balance between $O_2$ delivery and $O_2$ extraction (7, 12, 23). In this study ΔHHbMb and muscle $\dot{V}O_2$ on- kinetics were essentially the same confirming that in our model NIRS data is an excellent substitute for muscle oxygen consumption. As can be seen in Table 4 there was no significant difference in the TD between ΔHHbMb and $\dot{V}O_2$m. The time delay for $\dot{V}O_2$m is consistent with previous experiments using the canine GS (20, 27) and with the delay in the fall of $PO_2$ at the onset of contraction in single muscle fibers in vitro (40, 63). This supports the hypothesis that there is a matching of $O_2$ delivery and utilization during the first few seconds of an increase in work.(25). There was no difference in the tau ΔHHbMb between the normoxic and either hypoxic condition again demonstrating that $O_2$ availability and use were similar.

***Diffusion limitation.*** Gayeski et al. (15-16) have found intracellular $PO_2$ to be uniform in the muscle fiber suggesting that $PmitoO_2$ would be the same or very close to $P_iO_2$. Intracellular $PO_2$ measured both in vivo and in situ have been found to be extremely low during contractions, in the range of 1-4 Torr (5, 15, 48, 56) and in canine grasilis muscle flash frozen at maximum work rate to be as low as 0.5 Torr (16). However even at this low level the $PO_2$ is sufficient to allow full mitochondrial respiration which in situ is estimated to be > 0.5 Torr (6). Richardson et al., (52-56) in a series of experiments using $H^1$NMR to examined myoglobin saturation in working human leg muscles during single-leg knee extensions has found that $PcapO_2$ is ≈ 11 Torr, from 39 Torr in normoxia to 29 Torr in 12% hypoxia. While the drop in $P_iO_2$ difference in the muscle and thus $PmitoO_2$ was only 1 Torr, 3.1 Torr in normoxia to 2.3 Torr in hypoxia. Further they found that while $PmitoO_2$ drops from rest to exercise it appears to plateau at 50% - 60% exercise intensity and remains at the lower level (≈ 3 Torr) up to $VO_2$ peak (54, 56). They concluded that the driving pressure from capillary to muscle cell is similar in hypoxia and normoxia and thus diffusion capacity must increases with hypoxia to allow for

similar $\dot{V}O_2$ despite lower $PcapO_2$ (52). In this experiment we did not measure $PcapO_{2,\ however}$ using $PvO_2$ as a surrogate, we saw a significant lower $PvO_2$ in both hypoxic conditions. However, the results, similar $\dot{V}O_{2,}$ and similar $\dot{V}O_2$ on-kinetics indicate that diffusion driving pressure was maintained in all conditions. Using Fick's law as mentioned above for $\dot{V}O_2$ to remain the same between conditions despite apparently significantly lower $PvO_2$ ($\approx PcapO_2$) in the hypoxic conditions, the diffusing capacity would need to increase. This is similar to what has been seen in human single leg kick preparations (8).

***Force and Fatigue:*** There was no difference between peak force and final force between treatments. The fatigue index for the 8% hypoxia was significantly greater than for normoxia but not different from 12% hypoxia and the 12% hypoxia did not have a significantly greater fatigue index compared to normoxia. There was a significant difference between the venous pH at rest and at the end of exercise in all three treatments (normoxia - 7.42 ± 0.05 rest, 7.31 ± 0.04 end, 12% hypoxia - 7.44 ± 0.04 rest, 7.35 ± 0.03 end, 8% hypoxia - 7.45 ± 0.06 rest, 7.36 ± 0.05 end) but final pH was not different between treatments. Lactate was not significantly different between treatments. These data taken together demonstrate that in each treatment mitochondrial respiration was able to meet the work demand with the majority of energy coming from oxidative sources.

***Limitations***. The major limitation of this study was that we did not explore $VO_2$peak along with the moderate stimulation protocol. In earlier study Hogan et al. (32) found a dissociation of $VO_2$peak with matched convective $O_2$ delivery in normoxia and hypoxia. It would have been interesting to compare data but in the Hogan study the blood flow and thus $O_2$ delivery was at slightly lower than spontaneous rate in normoxia resulting in a slightly lower $O_2$ delivery to the muscle and the hypoxic treatment used a blood flow to match the lower normoxic

delivery rate. In this model $O_2$ delivery was calculated to match spontaneous delivery in normoxia in two hypoxic condtions. A further study at $VO_2$ peak using a similar protocol would help clarify the question of $O_2$ diffusion verses delivery as the limiting factor.

The canine GS model offers many advantages in the study of on-kinetics but it also has limitations. The muscle is highly oxidative and homogeneous so any conclusions need to be interpreted with caution particularly if being applied to a human leg model. The muscle is performing isometric contractions with all motor units firing synchronously unlike exercise where recruitment of fibers is required and work is done.

Additionally, while we were able to control bulk blood flow via the perfusion pump we were not able to measure what was happening with capillary blood flow in the three different conditions. While I do not suspect that there was additional capillary recruitment at the higher blood flow based on the work of Kindig et al.,(41-42), the increased blood flow may have increased the red blood cell to capillary surface contact as suggested by Poole et al., (51). This would account for increased diffusion from capillary to muscle fiber and might alleviate the need for a change in the diffusion capacity at the interface between capillary and muscle. In either case there would be increased $O_2$ available to meet demand.

***Conclusions.*** For the first time this study has demonstrated that increase $O_2$ delivery can maintain $VO_2$ on-kinetics under conditions of moderate and severe hypoxia. In the presence of unchanged ("matched") convective $O_2$ delivery with arterial and venous hypoxemia, skeletal muscle $VO_2$ on-kinetics were not significantly altered. These data suggest that during hypoxia there is an increase in diffusion capacity, $DO_2$, such that the $VO_2$ follows Fick's principle. The higher blood flow provided sufficient $O_2$ delivery to maintain the $O_2$ diffusion driving pressure and allow normal aerobic function. These data support other findings where increased cardiac output

during hypoxia can compensate for low $CaO_2$ (4, 47). Additionally, the results showing $VO_2$ muscle to be reflected the NIRS ΔHHbMb gives further credence to proposition that NIRS provides a very close approximation of oxygen consumption at the muscle.

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