SKELETAL MUSCLE MITOCHONDRIAL FUNCTION IN ENDURANCE ATHLETES MEASURED WITH NEAR INFRARED SPECTROSCOPY

by

JARED TODD BRIZENDINE

(Under the Direction of Kevin McCully)

ABSTRACT

The purpose of this experiment was to determine if near-infrared spectroscopy (NIRS) measurements of muscle oxidative capacity could detect the known differences between endurance trained athletes (N=6) and inactive subjects (N=7). Muscle oxygen consumption (mVO_2) was measured during brief arterial occlusions. The recovery of mVO_2 after electrical stimulation was fit to an exponential curve, with the time constant (Tc) used as an index of mitochondrial capacity. Tc values for endurance trained were 18.3 ± 2.3 and 19.2 ± 1.6 seconds, whereas inactive controls were 31.6 ± 5.1 and 33.1 ± 3.5 seconds, for the shallow and deep channels respectively ($p < 0.001$ for comparison between groups). The magnitude of the differences in muscle oxidative capacity between endurance trained and inactive subjects matched literature values, supporting the use of the NIRS method to measure muscle oxidative capacity.

INDEX WORDS: NIRS, mitochondrial capacity, electrical stimulation, oxidative metabolism, endurance training, maximal oxygen uptake, competitive cyclists

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by

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DEDICATION

I would like to dedicate my work to my parents, Jack and Pamela Brizendine, my twin brother Chad Brizendine, and my grandmother Vivien Kuntz. Dad, as I grew up you taught me by example that hard work and passion will surmount any challenge. Mom, your smile and love always shined through the hardest of times and thus by your actions taught me perseverance. Chaddy, I couldn't have asked for any greater blessing than walking the journey of life with you always by my side, reminding me that one is never too old to imagine and dream the impossible. And last but never the least, Grammy, who without your support and love, I would not be the person I am today. I love you all so dearly.

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CHAPTER 1

INTRODUCTION

Endurance training modifies the response to prolonged exercise through a multitude of biochemical mechanisms that augment both oxygen transport capacity and peripheral oxidative potential (14, 42). Well known peripheral adaptations are increases in mitochondrial density (19, 20, 38), mitochondrial enzyme activity (19, 20), and oxygen utilization (19, 20, 37). These adaptations have pronounced effect on the oxidative capacity of skeletal muscle. The regions of muscle participating in locomotor activity are the most profoundly influenced by endurance training - strongly implicating a local rather than systemic signal for mitochondrial adaptation (13, 35). It has been illustrated in past studies that a positive linear relationship exists between maximal mitochondrial oxidative power ($mVO_{2\ Max}$) and whole-body maximal oxygen uptake (VO_{2Max}) in humans (37, 42, 43). Endurance trained athletes have a predominance of type I fibers that in comparison to type II fibers have both higher capillary density and oxidative potential (6, 13). Potentially the most critical of these adaptations is the increase in mitochondria volume and density with an increase in respiratory capacity.

Needle-biopsy of human skeletal muscles has allowed the evaluation of skeletal muscle tissue and isolated mitochondria through histochemical and enzymatic analysis (8, 20, 42). Longitudinal and cross-sectional literature of endurance trained and inactive humans and animals have evaluated the extensive differences induced through physical activity between these two groups (2, 13, 18, 43). Though a fundamental tool in our understanding of

bioenergetics of skeletal muscle, needle-biopsy is invasive and potentially damaging. Non-invasive methodologies such as magnetic-resonance spectroscopy (MRS) and near-infrared spectroscopy (NIRS) have recently been developed and offer several advantages over the muscle biopsy technique in the study of muscle bioenergetics (5, 15). These advantages include measurement of a larger sample of whole muscle and allowance for repetitive measurement in the same muscle without inducing injury or scarring. Mitochondrial capacity can be assessed with MRS by measuring the kinetics of phosphocreatine (PCr) resynthesis after exercise (26, 30). Cross-sectional studies of endurance athletes and inactive controls have measured a twofold difference in the rate of phosphocreatine recovery in endurance athletes of different training modality (22, 25, 29). Despite that MRS has become the gold-standard for measurement of mitochondrial capacity; there are potential limitations such as cost, technical expertise, and portability to its use. NIRS is an affordable, portable, and relatively less complicated technology that has been used to evaluate skeletal muscle oxygenation and hemodynamics in vivo. The application of arterial or venous occlusion allows for the assessment of muscle oxygen consumption (mVO_2) using NIRS (1, 15, 31, 41). NIRS measurements have also been validated with MRS metabolic measurements of phosphocreatine breakdown and recovery (15, 28, 34). Thus, NIRS has the potential to be an accurate and reliable methodology to quantify mitochondrial function.

Statement of the Problem:

Mitochondrial function is tightly coupled with health and performance. Oxidative metabolism is the major biochemical process for synthesizing adenosine triphosphate (ATP) during activities of daily living. Improvements in the oxidative capacity of the body translate to higher performance in sport and amelioration of functional deterioration in health. Our novel

technique has the potential to measure mitochondrial capacity using NIRS but has yet to be validated.

Specific Aims:

To evaluate a NIRS technique that uses repeated arterial occlusions to assess skeletal muscle mitochondrial function by comparing endurance trained athletes with college-age inactive individuals.

Hypotheses:

(1) Endurance trained humans will have faster mitochondrial time constants than the inactive humans.

(2) The magnitude of the difference between the endurance trained and untrained individuals will be consistent with the literature for measurements of mitochondrial capacity from measurements of muscle biopsy and magnetic resonance spectroscopy.

Significance of the Study:

Muscle oxidative function is vital to the understanding of health and disease; improvements translate to performance in athletic events whereas decline leads to deterioration in health. In order to bolster our knowledge of skeletal muscle oxidative capacity non-invasive and novel methods need to be developed. Our technique utilizes near-infrared spectroscopy, a non-invasive, affordable, and simple technology whose portability is ideally matched to use in both research and clinical perspectives. Nevertheless, this technique needs to be validated and comparisons need to be made to established methods. The biochemical and oxidative differences between endurance trained athletes and inactive individuals are well known. This study will utilize these differences to validate our measurements of mitochondrial capacity.

CHAPTER 2

REVIEW OF THE RELATED LITERATURE

Near-Infrared Spectroscopy and Skeletal Muscle

Near-infrared spectroscopy (NIRS) has become a useful tool for the evaluation of skeletal muscle oxygenation and oxidative energy metabolism in a multitude of fields including sport, health, and clinical sciences (16). Light within the near-infrared range (700-1000 nm) penetrates skin, subcutaneous fat, and skeletal muscle. Light will either be absorbed or scattered within the tissue. Attenuation of NIR light in tissue is due to: (a) O2 dependent absorption from chromophores of variable concentration (hemoglobin, myoglobin, and cytochrome oxidase); (b) absorption from chromophores of fixed concentration (skin melanin); or (c) light scattering (9). The application of NIRS to skeletal muscle principally depends on the strong reliance of skeletal muscle to oxidative metabolism, which is the major biochemical process for synthesizing adenosine triphosphate (ATP) during activities of daily living. Improvements in the oxidative capacity of the body translate to higher performance in sport and amelioration of functional deterioration in health.

Different NIRS devices have been developed in the past few decades and differ in characteristics based on the NIRS method applied. The penetration depth into the tissue is approximately equal to half the source-detector separation and that the region of muscle sensitivity is ‘banana-shaped’ due to the pattern of the light path through tissue (9, 16). The amount of subcutaneous adipose tissue greatly influences the NIR signal intensity from the

muscle tissue below (27, 39). The three different NIRS devices approach the issue that, due to scattering effects of different tissue layers, the length light travels through the medium (optical pathlength) is longer than the distance between the source and the detector through specific illumination techniques. These NIRS techniques include: (a) continuous-wave (CW) that based on constant illumination of the tissue, measures the attenuation of light through the tissue; (b) frequency-domain (FD), which illuminates the tissues with intensity-modulated light and enables the measurement of both attenuation and phase shift of the emerging light; and (c) time-domain (TD) that illuminates the tissues with short pulses of light and then detects the shape of the pulse after propagation through the tissues (10). The most commercially available and least expensive NIRS devices are the continuous-wave spatially resolved spectroscopy (CW-SRS) systems. CW and CW-SRS devices use a modified form of the Beer-Lambert law in order to account for the previously stated limitation that tissues are generally highly scattering media and scattering will increase the pathlength of light, augmenting the probability of light absorption and loss of light.

$$A = \log\left(\frac{I_O}{I}\right) = \in \times\ (C)\ \times L\ \times DPF + G$$

In the above modified Beer-Lambert law A is light attenuation; I_O is incident light (original light transmitted into the tissue); I is the emergent light; $\in$ is the specific extinction coefficient; (C) is the chromophore concentration; and L is the light pathlength (distance between points where light enters and leaves the medium); differential pathlength factor (DPF) is included to account for the extended pathlength due to scattering; and G represents the losses due to scattering (11). Values for DPF have been published for several muscles and the assumption that DPF and G are always constant is made in CW NIRS (10). Changes in light attenuation are then used to calculate changes in (C) from an arbitrary baseline. These systems allow you

to characterize the relative concentration changes oxygenated hemoglobin (O_2Hb), deoxygenated hemoglobin (HHb), and a scaled absolute value for total hemoglobin (tHB) concentrations. Nevertheless, only FD and TD technique based systems offer the absolute characterization of the tissue optical properties that allow absolute measurement of O_2Hb, HHb, and tHB concentrations.

To date only about 20 out of the approximately 600 skeletal muscles have been investigated by NIRS. Of these the lower limb muscles (i.e. biceps femoris, gastrocnemius, rectus femoris, tibialis anterior, vastus lateralis, vastus medialis) and upper limb muscles (i.e. biceps brachii, brachioradialis, deltoid, forearm flexors, triceps brachii) have been extensively evaluated over a range of conditions (10). Constant and incremental work rate on a bicycle ergometer has been used to investigate skeletal muscle oxygenation of the quadriceps muscle with NIRS (3, 4). The measurement of skeletal muscle oxygenation with NIRS is not limited to the modality of cycle ergometry, the effect of treadmill speed and slope on quadriceps oxygenation as well as during weight-lifting exercise have been assessed with NIRS (33, 36). For example, Chance and colleagues (4) determined that in rowers the rate of O2 re-saturation post-exercise is faster than in sedentary controls.

Muscle metabolism and oxygenation have also been simultaneously assessed by the in concert use of ^{31}P magnetic resonance spectroscopy (MRS) and NIRS. In 1994 McCully *et al*. demonstrated that the kinetics of re-oxygenation paralleled those for regeneration of the high-energy compound phosphocreatine after submaximal exercise as well as that re-oxygenation was not influenced by intensity (28).

Simultaneous application of strain-gauge plethysmography with NIRS to measure blood flow has shown excellent agreement, though flow was two to threefold higher with

plethysmography (40). Muscle oxygen consumption (mVO_2) can be measured with NIRS with the application of venous and arterial occlusions (1, 15, 31, 41). Van Beekvelt and colleagues (41) successfully demonstrated that local muscle oxygen consumption can be measured reliably by NIRS, both at rest and during exercise, through the application of arterial occlusion and linear regression analysis of the rate of desaturation post rhythmic isometric handgrip exercise.

Invasive Methods of Assessing Mitochondrial Function

Mitochondrial function remains the root to our understanding of bioenergetics. Demands of physical activity can increase skeletal muscle energy turnover 400 fold that of rest, and muscle oxygen consumption may increase by more than 100 fold (37). The close matching of mitochondrial ATP production to ATP demand during steady state demonstrates the existence of efficient cellular mechanisms to control mitochondrial energy flux in a wide dynamic range. Prior to the development of non-invasive technologies such as MRS and NIRS, analysis of mitochondrial function was performed through histochemical and enzymatic analyses of needle biopsy samples of skeletal muscle tissue and isolated mitochondria. These methods are still used frequently as despite being invasive are one of the few methods to evaluate cellular markers of mitochondrial function. Endurance training modifies the response to prolonged exercise through a multitude of biochemical mechanisms that augment both oxygen transport capacity and peripheral oxidative potential (14, 42). Principal amongst these peripheral adaptations are increases in mitochondrial density (19, 20, 38), mitochondrial enzyme activity(19, 20), and oxygen utilization (19, 20, 37).

Cross-sectional studies of endurance trained athletes with untrained controls allows the evaluation of adaptations in skeletal muscle mitochondrial content to physical training over pronounced periods of time. Gollnick *et al*. investigated groups of subjects who had been engaged in various types and intensities of training and observed profound differences in succinate dehydrogenase (SDH) activity between trained and untrained muscle groups (13). Notably, the oxidative capacity of the leg muscle of cyclists and arm muscle of swimmers were 2 – 2.5 fold greater than that of untrained subjects. These findings are coherent with the SDH activity differences observed in the gastrocnemius muscle of elite runners and untrained individuals (7). Blomstrand and colleagues (2) observed in well-trained and untrained individuals that aerobic training increases the activity of the mitochondrial enzyme oxoglutarate dehydrogenase, which is a key mitochondrial enzyme. The mean percentage difference in the values of $VO_{2\ Max}$ and oxoglutarate dehydrogenase between untrained and well trained was 49 and 90%, respectively. Also determined was the activity of 6-phosphofructokinase that along with oxoglutarate dehydrogenase permitted the calculation of the maximum rate of ATP generation via aerobic and anaerobic metabolism. The ability to generate ATP from aerobic metabolism was twofold in the muscle of the well trained subjects. These findings are in agreement with each other and previous literature that mitochondrial concentration and density are markedly different in trained and untrained humans (2, 12, 13, 20, 21).

Adaptation to endurance training in skeletal muscle mitochondria involves an alteration in composition; some enzymes increase two to threefold, others increasing 30 – 60%, and some not increasing at all (19). Holloszy and colleagues (18) were the first to

show in young rats that endurance exercise training induces an increase in the mitochondrial content of skeletal muscle. For the trained rats run time to exhaustion averaged 186 ± 18 minutes compared with 29 ± 3 minutes for control rats who were exposed to a maintenance program of 10 minutes of running 5 days per week. The capacity of the mitochondrial fraction extracted from the gastrocnemius muscle of a trained rat to oxidize pyruvate doubled. Also observed in the hindlimb was a twofold increase in activities per gram of muscle in succinate dehydrogenase, NADH dehydrogenase, NADH cytochrome-c reductase (SCR), and cytochrome oxidase (COX). Total protein content of the mitochondrial fraction increased 60% and cytochrome-c concentration increased twofold. After 6 weeks of endurance training in humans, Wibom and colleagues (43) demonstrated the well-known pattern of changes in oxidative enzymes induced by training: glutamate dehydrogenase activity increased 51 ± 21%, citrate synthase increased 46 ± 38%, SCR increased 21 ± 21%, and COX increased 76 ± 38%. Mitochondrial fraction increased by roughly 80% post training, which is consistent with the animal training literature (8, 18). Also observed with endurance training is an increase in fiber respiratory capacity, mitochondrial ATP production rates (MAPR), has been demonstrated to increase by 38 – 70% (42, 43). It has been illustrated in past studies that a positive linear relationship exists between maximal mitochondrial oxidative power ($mVO_{2\ Max}$) and whole-body maximal oxygen uptake ($VO_{2\ Max}$) in humans (37, 42, 43). Endurance trained athletes have a predominance of type I fibers that in comparison to type II fibers have both higher capillary density and oxidative potential (6, 13). Nevertheless, likely the most critical of these endurance training adaptations is the increase in mitochondria volume and density with an increase in respiratory capacity.

Non-Invasive Methods of Assessing Mitochondrial Function

In the past two decades noninvasive methodologies have strengthened the ability to evaluate skeletal muscle oxidative metabolism (32). Magnetic resonance spectroscopy (MRS) is the gold standard of non-invasive detection of skeletal muscle bioenergetics; principally because of the ability to measure in vivo active forms of high-energy phosphorus metabolites and intramuscular pH (5, 23, 25, 29). With MRS mitochondrial capacity can be assessed through the use of the kinetics of phosphocreatine (PCr) resynthesis after exercise (26, 30). McCully and colleagues (29) determined the time constant (T_c) of phosphocreatine recovery after submaximal exercise in endurance trained and untrained runners to be 17.5 ± 2.7 and 31.4 ± 6.8 seconds, respectively. These results are coherent to the differences observed in other studies which evaluated endurance trained athletes and untrained controls with MRS (22, 25). However, MRS is costly and requires a high level of technical expertise and careful maintenance. Near-infrared spectroscopy (NIRS) is an optical method, which utilizes wavelengths in the range of 700 – 900 nm, for the measurement of skeletal muscle tissue oxygenation and haemodynamics (9, 10, 17). The most portable, widespread, and commercially available NIR device uses continuous-wave spatially resolved spectroscopy (CW-SRS) and provides information about the relative changes in binding state of heme. Oxygen delivery and consumption must be separated in order to evaluate skeletal muscle oxidative metabolism using NIRS; which has been accomplished previously using both venous and arterial occlusions (1, 15, 24, 31). Motobe and colleagues originally described a transient arterial occlusion technique with NIRS in the forearm to assess mitochondrial capacity (31). This technique used repeated arterial occlusion to fit a

mono-exponential curve from which a time constant was calculated as a measure of mitochondrial function. Hamoaka *et al*. (15) utilized another technique, simultaneously measuring muscle oxygen consumption and PCr breakdown during arterial occlusion it was determined that the rate of decline in oxygenated hemoglobin and both PCr and ADP were strongly correlated. These results are similar to that of Sako and colleagues (34) and suggest the validity to measure muscle oxygen consumption with NIRS.

CHAPTER 3

SKELETAL MUSCLE MITOCHONDRIAL FUNCTION IN ENDURANCE ATHLETES MEASURED WITH NEAR INFRARED SPECTROSCOPY

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Abstract

The purpose of this experiment was to use near-infrared spectroscopy (NIRS) with the application of arterial occlusions to assess potential differences between elite cyclists and sedentary subjects in muscle oxidative capacity. The vastus lateralis muscle of six elite cyclists and 7 sedentary controls were tested using NIRS. Whole-body maximal oxygen uptake was determined by indirect calorimetry during a continuous ramp protocol on a cycle ergometer. The recovery of oxygen consumption ($mVO_{2\ Max}$) after 15 seconds of electrical stimulation at 4 Hz was fit to an exponential curve from which a time constant was determined that represents mitochondrial capacity. Time constants (Tc) for the recovery of $mVO_{2\ Max}$ in endurance trained cyclists were 18.3 ± 2.3 seconds and 19.2 ± 1.6 seconds for shallow and deep channels respectively. In the sedentary group the recovery time constants were 31.6 ± 5.1 seconds and 33.1 ± 3.5 seconds for shallow and deep channels. The differences between the endurance trained cyclist and inactive group were statistically significant for both channels ($p < 0.001$). We also observed a positive correlation between recovery time constants and $VO_{2\ Max}$ for all participants ($r = 0.9$, $F(1,12) = 36.4$, $p = 0.001$). Resting mVO_2 was 0.52 ± 0.29 $\%\cdot s^{-1}$ (CV = 12%) for elite cyclists and 0.6 ± 0.48 $\%\cdot s^{-1}$ (CV = 20%) for sedentary participants ($p = 0.69$). The recovery rates of elite cyclists were almost two-fold faster than sedentary subjects measured with NIRS, which are consistent with the expected differences in muscle metabolism between these groups and suggest that elite cyclists have greater blood flow and/or oxidative capacity.

Keywords: NIRS, mitochondrial capacity, electrical stimulation, oxidative metabolism, endurance training, maximal oxygen uptake

Introduction

Muscle oxidative function is vital to the understanding of health and disease and improvements translate to performance in athletic events (15, 20, 27). In the past two decades noninvasive methodologies have strengthened the ability to evaluate skeletal muscle oxidative metabolism (27). Magnetic resonance spectroscopy (MRS) is the cardinal method of non-invasive detection of skeletal muscle bioenergetics; principally because of the ability to measure in vivo active forms of high-energy phosphorus metabolites and intramuscular pH (5). With MRS mitochondrial capacity can be assessed through the use of the kinetics of phosphocreatine (PCr) resynthesis after exercise (23, 25). However, MRS is costly and requires a high level of technical expertise and careful maintenance. Near-infrared spectroscopy (NIRS) is an optical method, which utilizes wavelengths in the range of 700 – 900 nm, for the measurement of skeletal muscle tissue oxygenation and haemodynamics (8, 10, 16). The most portable, widespread, and commercially available NIR device uses continuous-wave spectroscopy (CWS) and provides information about the relative changes in binding state of heme. Muscle oxygen consumption has been measured previously using both venous and arterial occlusions (1, 14, 21, 26). Motobe and colleagues originally described a transient arterial occlusion technique with NIRS in the forearm to assess mitochondrial capacity (26). Arterial occlusion has been suggested to be complicated by blood volume changes during occlusion, which could confound the slope measurements for oxygen consumption (33). In order for this technique to be valid potential blood volume changes during arterial occlusion must be corrected. Ryan and colleagues developed and evaluated several methods for blood volume correction in NIRS and provided a reliable and reproducible

measure of muscle oxygen consumption during arterial occlusion (28). To support the validity of NIRS measurements in mitochondrial function, comparisons need to be made between groups which are known to differ in mitochondrial function.

The primary aim of endurance training is to improve the rate and efficiency at which chemical energy can be converted into mechanical energy for skeletal muscle contraction; that in turn translates to a heightened ability to sustain higher average power outputs or speed of movement for a given distance or time (17). Adaptation induced by endurance training is most pronounced in regions of muscle participating in locomotor activity; whereas in inactive muscle changes in mitochondrial function are little to none - strongly implicating a local rather than systemic signal for mitochondrial adaptation (12, 29). Endurance training modifies the response to prolonged exercise through a multitude of biochemical mechanisms that augment both oxygen transport capacity and peripheral oxidative potential (13, 34). Principal amongst these peripheral adaptations are increases in mitochondrial density (18, 19, 31), mitochondrial enzyme activity(18, 19), and oxygen utilization (18, 19, 30). It has been illustrated in past studies that a positive linear relationship exists between maximal mitochondrial oxidative power ($mVO_{2\ Max}$) and whole-body maximal oxygen uptake ($VO_{2\ Peak}$) in humans (30, 34, 35). Endurance trained athletes have a predominance of type I fibers that in comparison to type II fibers have both higher capillary density and oxidative potential (6, 12). Nevertheless, likely the most critical of these is the increase in mitochondria volume and density with an increase in respiratory capacity.

The purpose of this experiment is to validate a NIRS technique that uses repeated arterial occlusions to assess skeletal muscle mitochondrial function by comparing endurance trained athletes with college-age inactive individuals. We hypothesized that the physiological adaptations to endurance training would result in faster rates of recovery of muscle oxygen consumption (i.e. the time constant for $mVO_{2\ Max}$) in endurance trained individuals compared to inactive individuals.

Materials and Methods

Subjects

Thirteen subjects (10 male, 3 female) were tested in this study. Subjects were chosen to represent a wide disparity of muscle oxidative capacity. The endurance trained group consisted of college-aged cyclists, all of whom were in the middle of their competitive season and category 1 riders. Untrained individuals did not perform regular exercise more than once per week. The study was conducted with the approval of the Institutional Review Board at the University of Georgia (Athens, GA), and all subjects gave written, informed consent before testing.

Experimental Procedures

Testing occurred on one visit to the laboratory. Subjects were instructed not to consume caffeine or tobacco on the day of the test or to use alcohol or perform moderate or heavy physical activity for at least 24 hours before testing. NIRS testing was performed prior to measuring maximal oxidative capacity ($VO_{2\ Peak}$).

Near-infrared Spectroscopy Testing

Near-infrared testing was performed as previously described (28). Each subject was

placed on padded table, supine, with both legs extended (0° of flexion). The right foot was placed into a home-built isometric exercise device to limit motion artifact during data collection. The foot was strapped firmly to the exercise device using non-elastic Velcro® straps proximal to the base of the fifth digit, with the knee supported. The NIRS optode was placed on the vastus lateralis, approximately two third's the way down from its origin (greater trochanter) to its insertion (patella), and secured with Velcro® straps and biadhesive tape. NIRS signals were obtained using a continuous wave NIRS device (Oxymon MK III, Artinis Medical Systems, The Netherlands), which consists of 2 channels (2 equivalent pulsed light sources, 2 avalanche photodiode detectors, shielding from ambient light), uses intensity-modulated light at a frequency of 1 MHz and laser diodes at 3 wavelengths (905, 850, and 770 nm) corresponding to the absorption wavelengths of oxyhemoglobin (O_2HB) and deoxyhemoglobin (HHB), with an autosensing power supply (approximately 40 W at 110-240 V). The probe was set for two source-detector separation distances after measurement of adipose tissue thickness. Adipose tissue thickness (ATT) was measured at the site of the NIRS optode using B-mode ultrasound (LOGIQ e; GE HealthCare, USA). The deeper source-detector pair separation distance was always 1 cm greater than the shallow. NIRS data was collected at 10 Hz.

Four aluminum foil electrodes (2" x 2") attached to a Theratouch 4.7 stimulator (Rich-mar, Inola, OK) were placed on the skin, two proximal and two distal to the NIRS optode (Figure 3.1). A blood pressure cuff (Hokanson E20 cuff inflator; Bellevue, WA) was placed proximal to the NIRS optode.

Resting measurements of muscle oxygenation (as a percentage of an ischemic

calibration) were calculated as the average over the first minute of NIRS data collection. Following the measurement of muscle oxygenation, resting muscle oxygen consumption was assessed by inflation of a blood pressure cuff (250~300 mmHg). Twitch electrical stimulation was performed using 15-seconds of continuous electrical stimulation (biphasic pulse: duration/interval = 200/50 μs) and was administered at 4.0 Hz. The current intensity was adjusted for each individual to produce twitch contractions at the maximal tolerable level. Immediately following each bout of electrical stimulation a series of 10-18 brief (3-10 seconds) arterial occlusions utilizing a blood pressure cuff were applied to measure the rate of recovery of mVO_2 back to resting levels. Finally, to normalize the NIRS signal, a 3-5 minute arterial occlusion was applied to completely deoxygenate the tissue under the optode (i.e. 0% oxygenation) and the peak hyperemic response upon release of the cuff was used to indicate 100% oxygenation (Figure 3.2).

Calculation of Muscle Oxygen Consumption

mVO_2 was calculated as the slope of change in O_2Hb and HHb during the arterial occlusion using simple linear regression. This measurement was made at rest and repeated a number of times after exercise. The post-exercise repeated measurements of mVO_2 were fit to a mono-exponential curve according to the formula below:

$$y = End - Delta * e^{-1/Tc} \qquad \text{(Equation 1)}$$

For this equation, *y* represents relative mVO_2 during the arterial occlusion, *End* is the mVO_2 immediately after the cessation of exercise, *Delta* is the change in mVO_2 from rest to end exercise, and *Tc* is the fitting time constant.

Whole-Body Maximal Oxygen Uptake

Whole-body maximal oxygen uptake ($VO_{2\ Peak}$) was determined by indirect calorimetry during a continuous ramp protocol to either exhaustion or inability to maintain a pedaling rate of 50 rpm. Participants pedaled on an electrically-braked cycle ergometer (Lode BE, Netherlands) beginning at a low workload (50-200 Watts, depending on fitness level) for three minutes at a self-selected cadence (RPM). After the first three minutes, workload was then increased by one Watt every two seconds until exhaustion. This protocol was selected to avoid large and unequal increments in workload. Oxygen consumption and carbon dioxide production were measured continuously by open-circuit spirometry and analyzed using a Parvo Medics metabolic measurement system (model TrueMax 2400, Sandy, UT) that was calibrated before each experimental run. Heart rate was monitored by a Polar heart rate monitor (Polar beat, Port Washington, NY). Ratings of perceived exertion (RPE) were assessed during the last ten seconds of every minute during the ramp protocol using Gunnar Borg's 6-20 RPE scale (4). For the test to be considered an acceptable measurement of physiological VO_{2Peak}, two of the following criteria had to be met: (1) a leveling or plateauing of VO_2 (defined as an increase of VO_2 of <2 ml/kg/min with increased workload); (2) respiratory exchange ratio (RER) >1.1; (3) maximal heart rate within 10 beats of age-predicted maximum; and (4) rate of perceived exertion (RPE) ≥18.

Statistical Analysis

Data are presented as means ± SD. Statistical analyses were performed using SPSS 19.0 (IBM®, Armonk, NY). Comparison between endurance trained and inactive

subjects were made with Student's unpaired t-test for measurements of oxidative recovery and $VO_{2\ Peak}$. The relationship between two variables was analyzed by least squares regression analysis. Significance was accepted when $p < 0.05$.

Results

All subjects were able to complete testing with no adverse events. The physical characteristics of participants are shown in Table 3.1.

Near-Infrared Spectroscopy Measurements

Resting oxygen consumption was measured by NIRS during arterial occlusion (~270 mmHg). Resting mVO_2 was 0.52 ± 0.29 $\%\cdot s^{-1}$ (CV = 12%) for endurance trained athletes and 0.6 ± 0.48 $\%\cdot s^{-1}$ (CV = 20%) for inactive participants. The difference in resting oxygen consumption between endurance trained and inactive participants was not statistically significant ($p = 0.69$). We also compared resting mVO_2 from the long resting arterial occlusion (~30 seconds) with the average of three 10-second arterial occlusions. There were no differences in either group for resting mVO_2. We have previously reported that short repeated cuffs do not influence resting metabolic rate and saw no evidence for such here as well (28).

End-exercise recovery of mVO_2

Time constants (Tc) representing $mVO_{2\ Max}$ in endurance trained athletes following electrical stimulation were 18.3 ± 2.3 seconds and 19.2 ± 1.6 seconds for shallow and deep channels respectively. In the inactive group the recovery time constants were 31.6 ± 5.1 seconds and 33.1 ± 3.5 seconds for shallow and deep channels. The differences between the endurance trained group and inactive group were statistically

significant for both channels ($p < 0.01$). Figure 3.5 illustrates the average measurements of mitochondrial capacity with NIRS for both endurance trained and inactive humans. Representative $mVO_{2\ Max}$ recovery curves from a single subject for each group are shown in Figure 3.3.

Whole-Body Oxygen Uptake and Measurement of Mitochondrial Function

Table 3.2 contains the average variables related to $VO_{2\ Peak}$, such as $VO_{2\ Peak}$ expressed as the traditional $VO_{2\ Peak}$: body mass ratio ($ml \cdot kg^{-1} \cdot min^{-1}$), in absolute terms ($L \cdot min^{-1}$), RER, RPE, heart rate (HR), and average end work level for both endurance trained athletes and inactive controls. Figure 3.4 shows the typical work capacity of an endurance trained human and inactive human. We also observed a positive correlation between recovery time constants and $VO_{2\ Peak}$ for all participants ($r = 0.9$, $F(1,12) = 36.4$, $p = 0.001$), which are shown in Figure 3.6. However, no correlation between recovery time constant and VO_{2Peak} was observed in either the endurance trained group ($r = 0.118$, $F(1,5) = 0.043$, $p = 0.85$) or inactive group ($r = 0.46$, $F(1,6) = 1.36$, $p = 0.3$).

Influence of Depth

All NIRS testing was performed using two inter-optode distances. For endurance trained athletes the average distance for the shallow and deep channels were 3 and 4 cm respectively; whereas, the average distances for the inactive group were 3.2 ± 0.27 and 4.2 ± 0.27. We compared the agreement between the physiological range and ATT in Figure 6.

Effects of ATT on mVO_2

Adipose tissue thickness (ATT) was 4 ± 0.7 mm over the vastus lateralis muscle for endurance trained group and 7.2 ± 1.8 mm for the inactive group. ATT ranged from

3.1 to 9.2 mm in our participants. Furthermore, we found a significant relationship between the physiological range from ischemic calibration and ATT ($r = 0.91$, $F(1,12) = 50.1$, $p < 0.01$).

Discussion

The principal finding of this study was that endurance trained athletes have approximately twice the mitochondrial capacity compared with inactive controls as measured with near infrared spectroscopy. These results are similar in magnitude to studies of oxidative enzyme activity in endurance trained athletes compared to inactive subjects with biochemical measurements of muscle biopsy tissue (2, 7, 12, 35). Previous studies have also well documented a relative increase of about 40% in mitochondrial oxygen consumption in response to endurance training that was matched by a similar increase in muscle citrate synthase (CS) activity (30, 34). Assessment of oxidative capacity using the rate of phosphocreatine (PCr) recovery measured with MRS has also showed consistent results. Both track athletes and competitive rowers have been observed to have PCr recovery rates twice as fast as untrained controls in their respective active muscle tissue (22, 24). Thus the difference in time constants observed between endurance trained athletes and inactive subjects agreed well with the known differences between endurance trained and untrained muscle. Furthermore the time constants in this study are similar to those using phosphorus magnetic resonance spectroscopy (24).

The subjects in the present study were classified into different groups based on their $VO_{2\ Peak}$. Endurance trained athletes were individuals classified with $VO_{2\ Peak}$ measurement higher than 60 $ml \cdot kg^{-1} \cdot min^{-1}$ and inactive subjects were defined as those

less than 40 $ml \cdot kg^{-1} \cdot min^{-1}$. The two groups therefore represent quite disparate degrees of endurance capacity as observed in Table 3.2 and Figure 3.6, though no less than observed in similar cross-sectional studies comparing oxidative capacity (2, 22, 24). In addition, it should be noted that the average age and BMI for the cyclists and inactive subjects were similar. However, the endurance trained were all at racing weight at the time of the measurement.

The limitations to continuous wave NIRS includes a handful of potentially confounding factors that include unknown optical pathlength, absorption, and scattering coefficients, as well as the interaction of adipose tissue with NIR light (15, 27, 28). Measurement of pathlength, absorption, and scattering coefficients has been facilitated through the development of frequency-and time-domain NIRS devices (3, 9, 11). Despite new developments in NIRS devices if ATT is not taken into consideration the monitored muscle oxygenation changes are under estimated. The estimated depth of penetration with continuous wave NIRS is one half the inter-optode distance. In order to account for this we measured ATT at the site of measurement prior to commencement and then set the inter-optode distance on our device accordingly. With the large range of adipose tissue thickness in our participants (3.2 – 9.2 mm) we used a physiological ischemic calibration for the calculation of muscle oxygen saturation and consumption in each subject. This allowed us to report our measurements of mVO_2 as a percentage change per unit time as well as compare individuals by reducing the influence of adipose tissue and skin overlying individual muscle. In previous studies by Van Beekvelt *et al.* (32) and Ryan *et al.* (28) quantification of mVO_2 using the differential pathlength factor (DPF) was influenced by ATT, with the application of an ischemic calibration this influence was

abolished. In addition, this study utilized two inter-optode separation distances, which enabled us to compare measurements of muscle oxygen consumption at a shallow and deep penetration depth. As in our previous study we did not find a significant influence of optode distance (sample depth) on muscle metabolic measurements.

In summary, the recovery rates of endurance trained athletes were almost two-fold faster than inactive subjects measured with NIRS. These results are consistent with the expected differences in muscle oxidative capacity between these groups, and support the use of NIRS and repeated arterial occlusion to measure mitochondrial function. The ease of use of the method provides the potential to be used in studies of different human subjects.

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Conflict of interest statement

The authors report no conflicts of interest.

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Figure Legends

Fig 3-1: Experimental setup for measurements on the vastus lateralis muscle.

Fig 3-2: Muscle oxygenated hemoglobin/myoglobin during rest, resting arterial occlusions, and a 15-s electrical stimulation exercise followed by a series of transient arterial occlusions after exercise. The final 3-5 minutes are an ischemic calibration used to determine a relative concentration.

Fig 3-3: Sample recovery curves from an endurance trained and inactive participant. Raw, blood volume corrected data are represented by blue-squares (endurance trained) and red-triangles (inactive control).

Fig 3-4: Work capacity of a typical endurance trained human and inactive human.

Fig 3-5: Comparison between the average recovery time constant for oxygenated hemoglobin/myoglobin (O_2Hb) at the shallow and deep channels in both groups.

Fig 3-6: Relationship between the recovery time constant calculated following 15 seconds of electrical stimulation and whole-body maximal oxygen uptake. Endurance trained individuals are represented by blue squares and inactive controls by red triangles.

Fig 3-7: Relationship between ranges calculated during the physiological calibration and adipose tissue thickness. Endurance trained individuals are represented by blue squares and inactive controls by red triangles.

Fig 3-8: Comparison of the results in the current study (Brizendine) to that of previously discussed results: Blomstrand *et al. (2)*, Gollnick *et al.* (13),

Holloszy *et al.* (20), and McCully *et al.* (29). For each study the mitochondrial assay or rate data was adapted from the literature by normalizing the average value for the endurance trained group to a value of 1. The value of the untrained group was then a fraction of the trained group.

Table 3.1. Physical Characteristics of Participants

Group	**Height** *(cm)*	**Weight** *(kg)*	**BMI**	**Age** *(yr)*	**Gender** *(M/F)*	**ATT** *(mm)*
Elite Cyclist (n = 6)	179.9 ± 6.2	71.2 ± 4.6	22 ± 1.4	24.3 ± 1.4	6 M	4 ± 0.7
Sedentary (n = 7)	173.8 ± 13.4	68 ± 16.3	22.4 ± 4.1	21.3 ± 1.7	4 M/ 3 F	7.2 ± 1.8

Values are expressed as mean ± SD. ATT = adipose tissue thickness.

Table 3.2. Maximal Oxygen Uptake Variables

Group	**$VO_{2\ Peak}$** *(*$ml \cdot kg^{-1} \cdot min^{-1}$*)*	**$VO_{2\ Peak}$** *(*$L \cdot min^{-1}$*)*	**RER**	**RPE**	**HR** *(BPM)*	**End Work Level** *(Watts)*
Elite Cyclist (n = 6)	76.8 ± 7.6	5.5 ± 0.6	1.13 ± 0.1	18.8 ± 0.8	190.8 ± 7.6	476.8 ± 47.6
Sedentary (n = 7)	32.8 ± 4.1	2.3 ± 0.7	1.3 ± 0.1	18.7 ± 1	195.8 ± 10.9	204 ± 55.2

Values are expressed as mean ± SD.

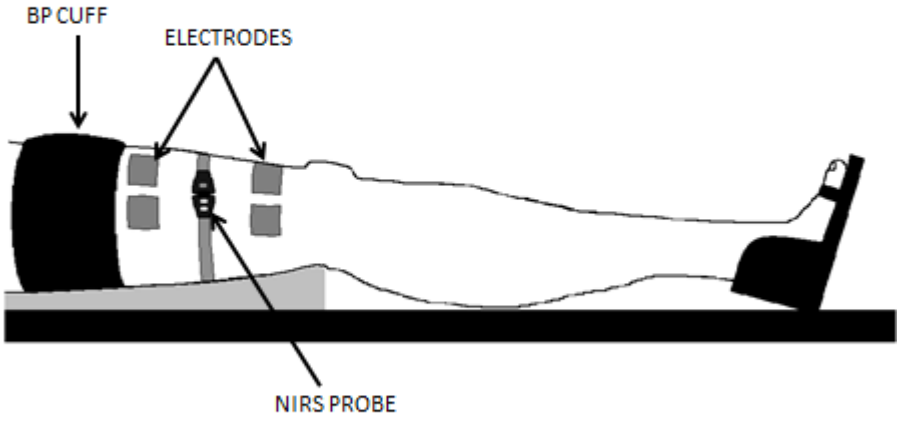

Figure 3.1

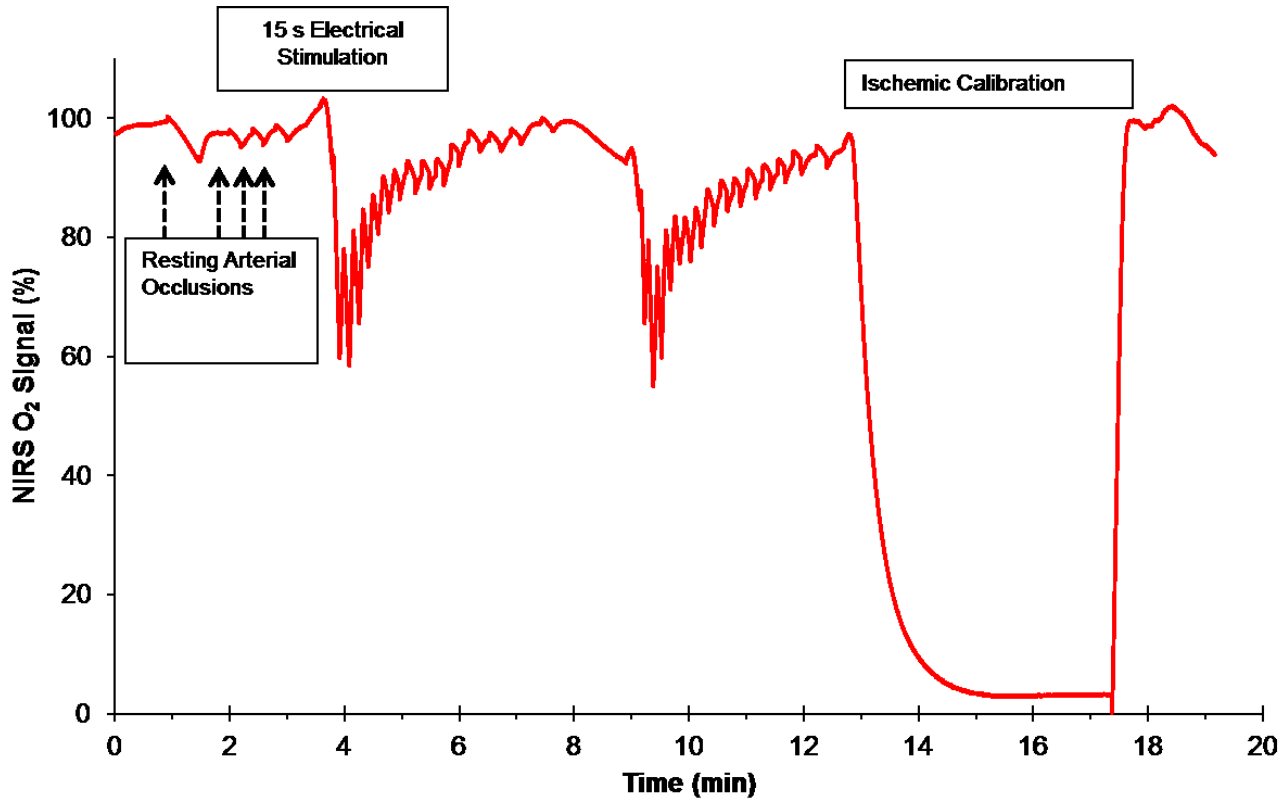
15 s Electrical Stimulation
Ischemic Calibration
Resting Arterial Occlusions
NIRS O_2 Signal (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Time (min)

Figure 3.2

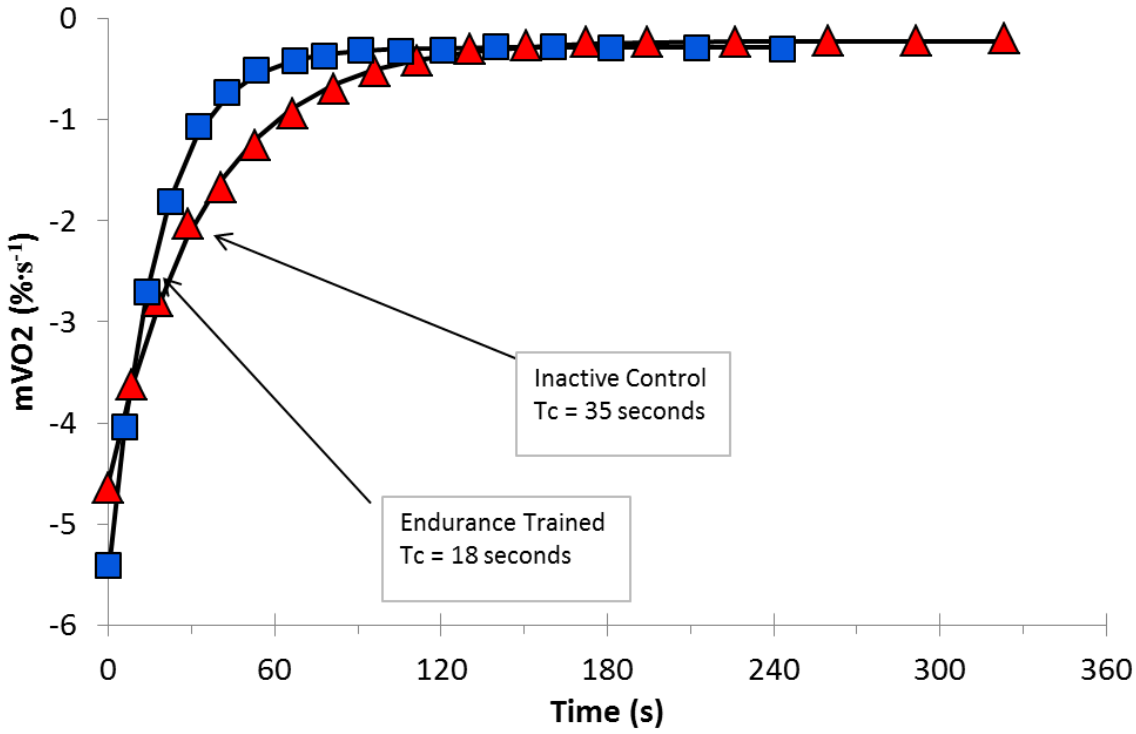
0
-1
-2
-3
-4
-5
-6
mVO2 (%·s-1)
0
60
120
180
240
300
360
Time (s)
Inactive Control
Tc = 35 seconds
Endurance Trained
Tc = 18 seconds

Figure 3.3

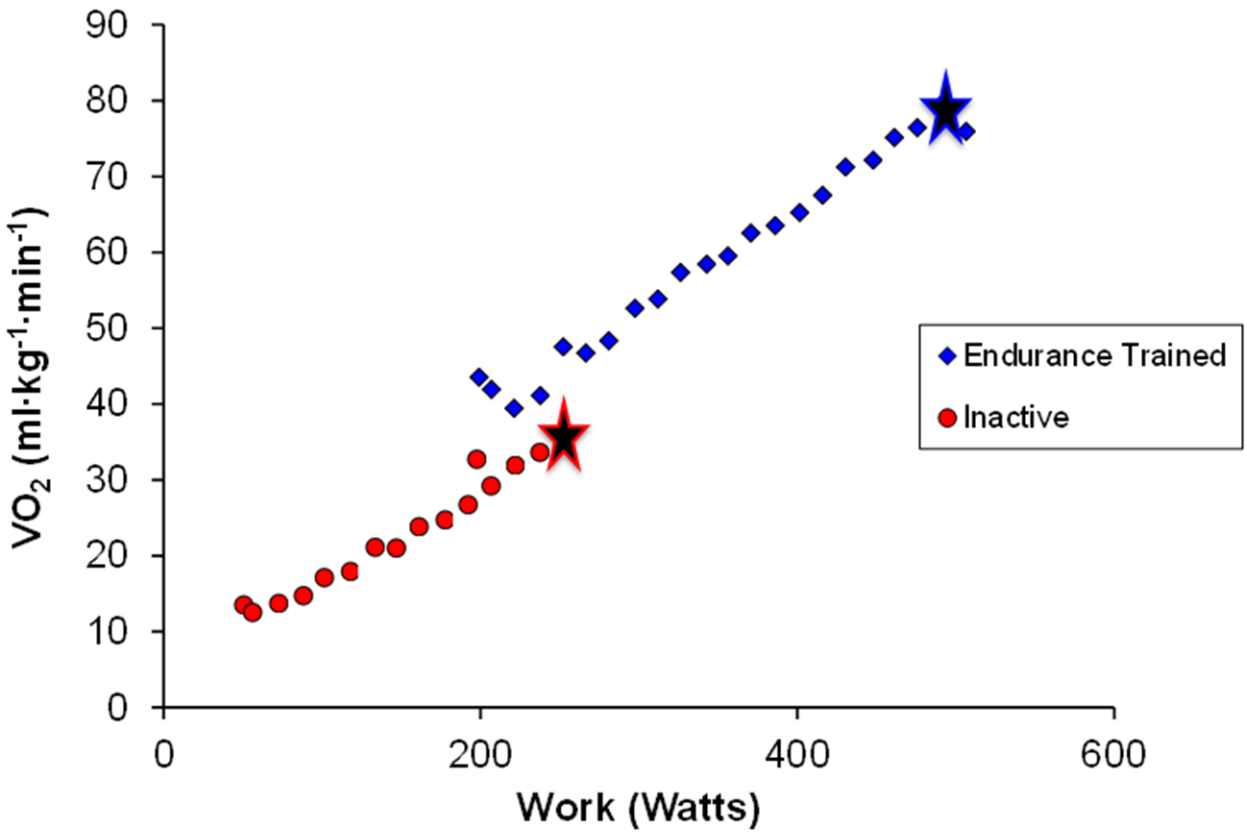
90
80
70
60
50
40
30
20
10
0
VO_2 (ml·kg^{-1}·min^{-1})
0
200
400
600
Work (Watts)
Endurance Trained
Inactive

Figure 3.4

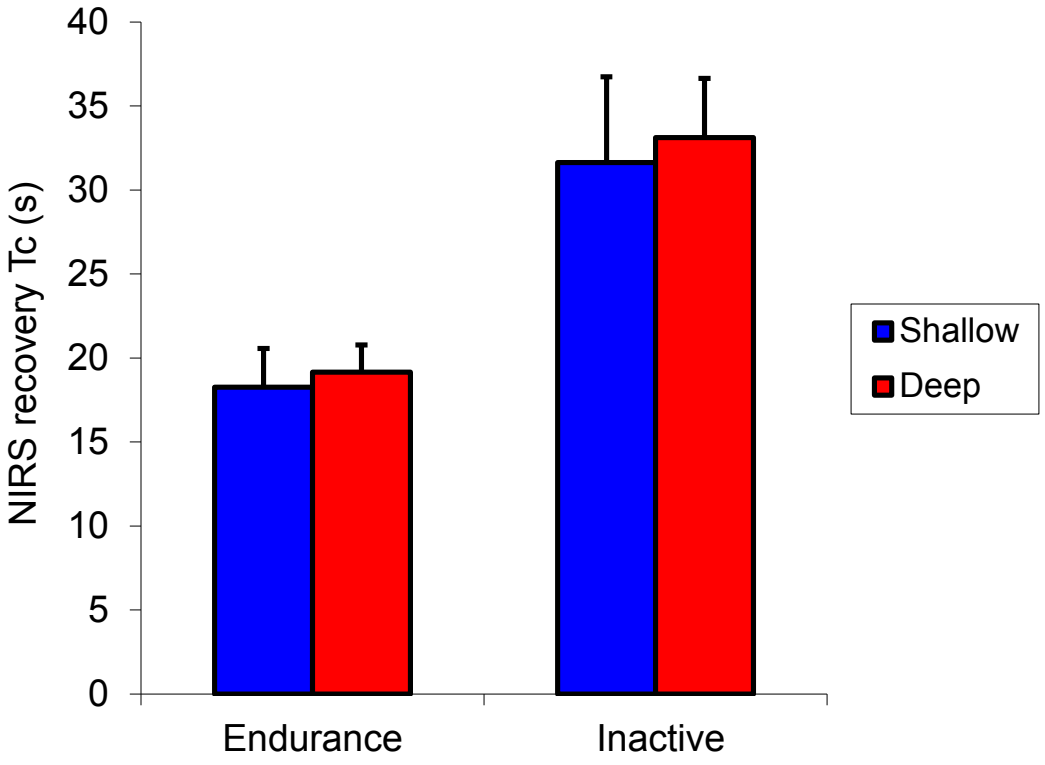
40
35
30
25
20
15
10
5
0
NIRS recovery Tc (s)
Endurance
Inactive
Shallow
Deep

Figure 3.5

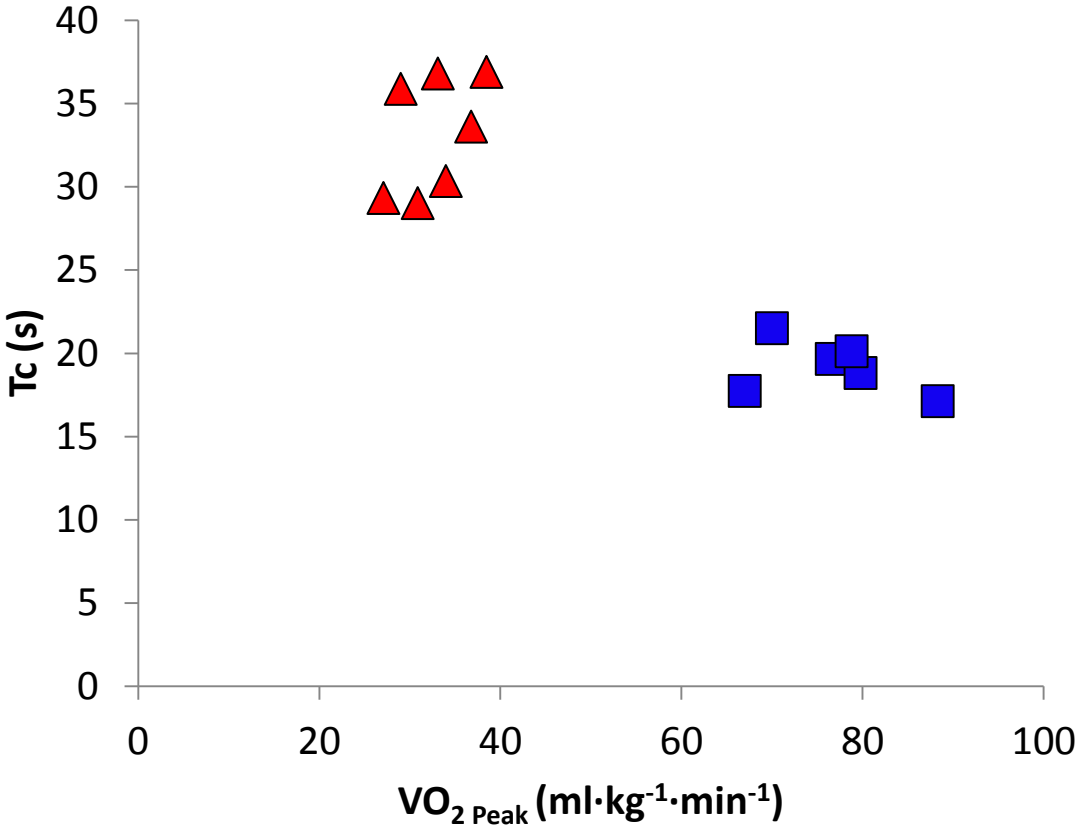

Figure 3.6

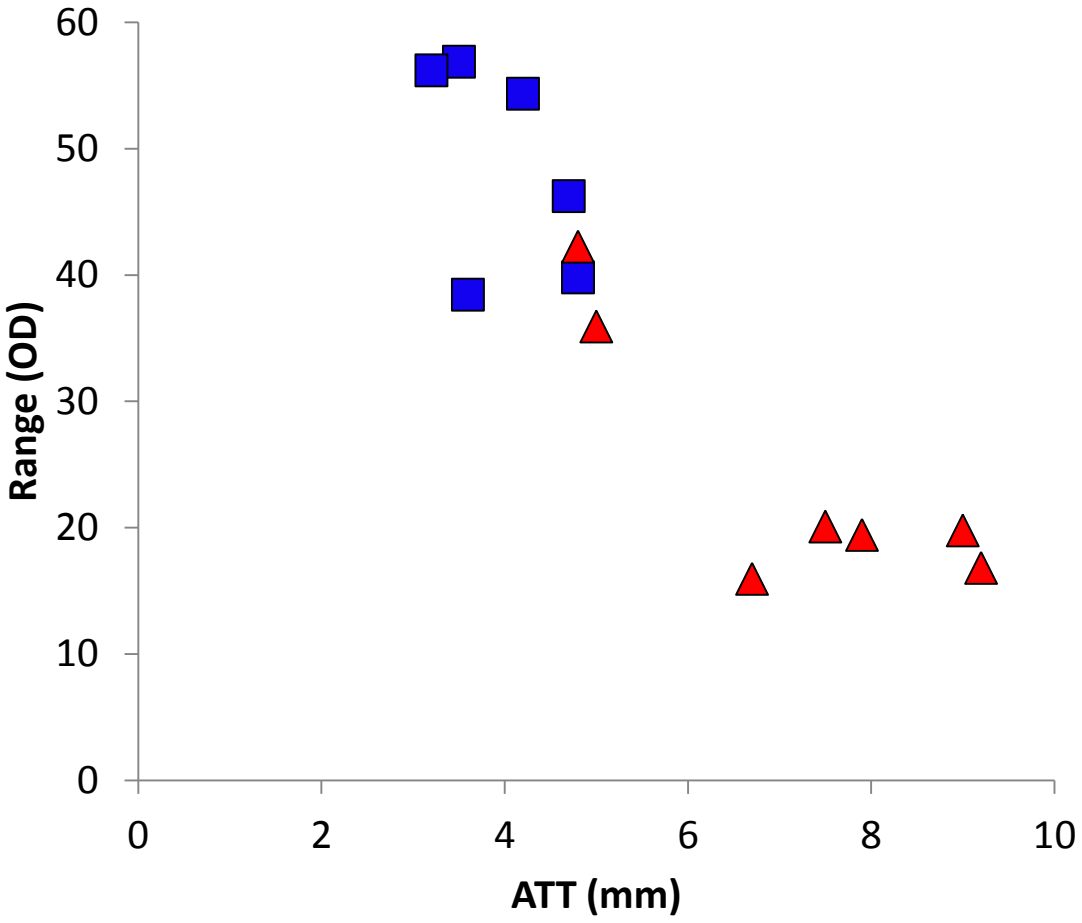

Figure 3.7

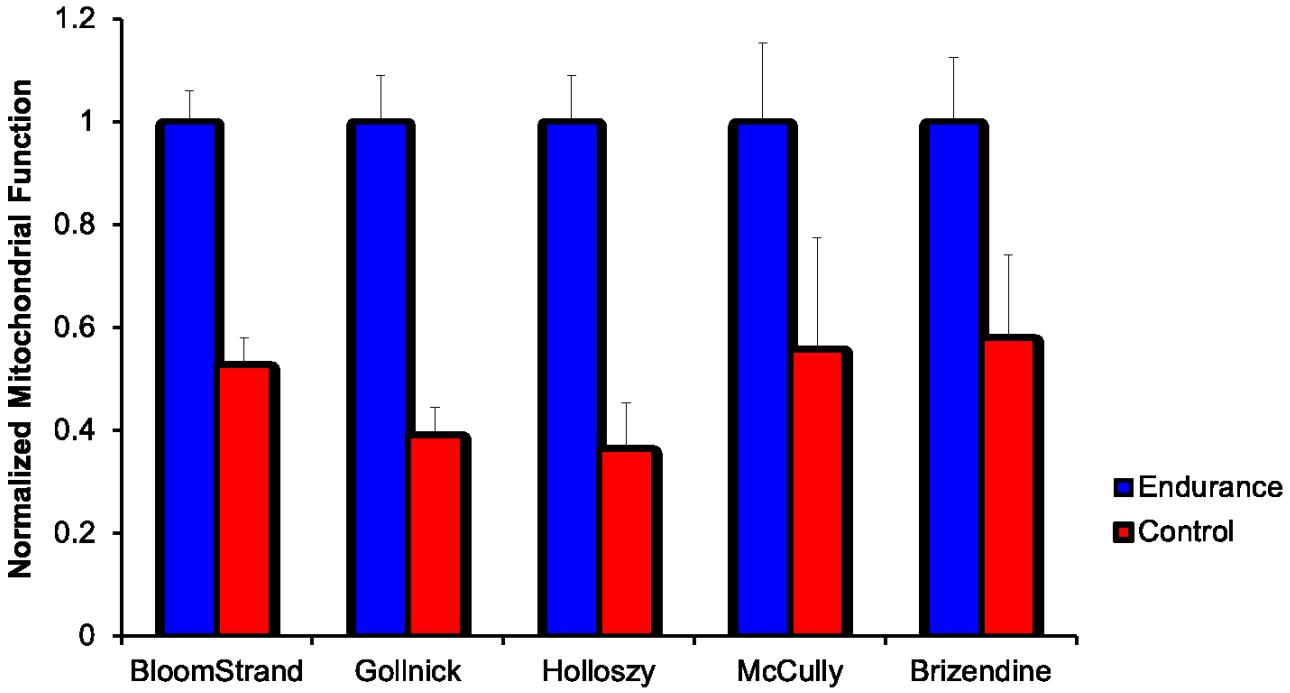
Normalized Mitochondrial Function
1.2
1
0.8
0.6
0.4
0.2
0
BloomStrand
Gollnick
Holloszy
McCully
Brizendine
Endurance
Control

Figure 3.8

CHAPTER 4

SUMMARY AND CONCLUSION

Major Findings

The primary finding in this study was that endurance trained athletes (Tc = 18.3 ± 2.3 s and 19.2 ± 1.6 s) had recovery rates approximately 73% faster than inactive controls (Tc = 31.6 ± 5.1 s and 33.1 ± 3.5 s). These results are consistent with the twofold higher activities of oxidative enzymes such as succinate dehydrogenase and oxoglutarate dehydrogenase in cross-sectional muscle biopsy studies (2, 13). In addition they compare directly to the absolute recovery time constants as well as magnitude observed in the recovery of phosphocreatine measured with magnetic resonance spectroscopy (MRS) in elite runners (29). Therefore, our technique to measure muscle oxygen consumption and determine mitochondrial function with near-infrared spectroscopy (NIRS) is valid as a tool to evaluate skeletal muscle oxidative capacity.

Significance of Our NIRS Technique

Muscle biopsy and magnetic resonance spectroscopy have extended the study of muscle bioenergetics for decades. Yet each suffer their own limitations, biopsy is invasive and potentially damaging while MRS is non-invasive but expensive and highly technical. There exists a need for techniques that can measure mitochondrial capacity both efficiently and affordably. NIRS devices are inexpensive (~$15,000 - 90,000),

portable, and applicable to use in both sports and disease. The techniques performed in this study with NIRS are safe, cost-efficient, and reliable. The total cost to acquire the equipment necessary to perform these measurements of oxidative capacity would amount to around $36,000, which is quite inexpensive compared to the cost just to acquire a 3 Tesla magnet (1.5 million). Also, with the use of Matlab software and our customized analysis routines data analysis is relatively simple and immediate. Thus, these techniques have tremendous potential for use in both a research setting as well as clinical setting where questions of cost and invasiveness are becoming more prevalent.

NIRS Testing Limitations

It is important to define a test failure and for all purposes of the matter in this study it was defined as inaccurate or un-interpretable data. There are a handful of reasons this can occur. Continuous-wave NIRS has inherent limitations based on assumptions made to account for the scattering of light through tissues with this modality. Thus it is difficult to account for the path of light through tissue as the amount of subcutaneous adipose tissue greatly influences the NIR signal intensity from the site of interest – the muscle below. Though this was not especially an issue in this study as both groups were relatively lean and the device used is capable of changing separation distance from 2.5 to 5.5 cm, a few tests (ATT = 12 and 18 mm) were excluded due to excessive contribution to the heme signals from inactive tissue. I feel confident these two tests could have had better signal to noise if the separation distance had been widened and intensity of electrical stimulation increased, though the ladder would have required the participants to have a higher tolerance of electrical stimulation. This brings forth another limitation of this test, the use of electrical stimulation to induce muscle activity. Most individuals

tolerated stimulation to a level of intensity adequate for the acquisition of quality data but some individuals who in combination with larger subcutaneous fat measurements did not. The combination of the limitation of excessive adipose tissue and intolerance to stimulation will make accurate measurement of muscle metabolism difficult, if not impossible. There are two options to work around these issues. One, measure a muscle that normally has less adipose tissue above it (i.e. gastrocnemius or wrist-flexors). Two, construct a protocol that uses voluntary contraction and a level of oxygenation as the stimulus to measure mitochondrial capacity.

An interesting limitation that is likely unique to the population measured in this study is that the air compressor driving cuff occlusion can be limiting in very endurance trained individuals. In a few cyclists, the recovery from exercise was so fast ($Tc \leq 14$ s) that the compressor could not drive adequate inflation after the first two to three cuffs to measure oxygen consumption. Though it should be noted in these tests an adequate number of points before the time constant was still captured but a few more might have improved the preciseness of measurement.

How to Perform Successful NIRS Measurements

There are a few aspects to performing this test that were vital to success. Emphasis must be given to the participant that NIRS is very sensitive to movement and thus is to remain as still as possible throughout testing, especially during arterial occlusion. Construction of a stereotaxic device that will hold the test limb or area in place is suggested and will improve success. Placement of the NIRS device, blood pressure cuff, and electrodes are important as well. Knowledge of human anatomy will help with the correct placement of the NIRS probe on the muscle of interest; misplacement could

result in evaluation of the wrong muscle or inactive tissue. If evaluating a quadriceps muscle the blood pressure cuff needs to be positioned as high on the thigh as possible to avoid unnecessary discomfort and allow optimal space for placement of the electrodes and NIRS probe in order to avoid motion artifact.

Set-up is not the only consideration to a successful test. Before commencement of the measurement protocol it is absolutely necessary to check the amount of light the device is receiving. For testing muscle the ideal range of light reception is 2-8%, though slightly higher values in the range of 10-18% are not necessarily devastating to your test. I would recommend shutting off the device and changing the separation distance accordingly if manipulation of the intensity and gain does not provide the necessary reception of light. The last consideration to be made for a successful test is arterial occlusion and electrical stimulation familiarization. At some point in a protocol, either before measurement or at a break in measurement the participant needs to be familiarized with the cuff by a series of progressively lengthening (1-10 seconds) occlusions. Electrically stimulating the subject at the intensity of their tolerance for a brief (~5s) period of time is not necessary but recommended. The combination of these two familiarizations reduces the likelihood of the participant moving from discomfort or surprise during the measurement portions of testing.

CHAPTER 5

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