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Simultaneous Sampling of Tissue Oxygenation and Oxygen Consumption in Skeletal Muscle

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Abstract

Under physiologic conditions, microvascular oxygen delivery appears to be well matched to oxygen consumption in respiring tissues. We present a technique to measure interstitial oxygen tension ($P_{\text{ISF}O_2}$) and oxygen consumption (VO_2) under steady-state conditions, as well as during the transitions from rest to activity and back. Phosphorescence Quenching Microscopy (PQM) was employed with pneumatic compression cycling to achieve 1 to 10 Hz sampling rates of interstitial PO_2 and simultaneous recurrent sampling of VO_2 (3/min) in the exteriorized rat spinotrapezius muscle. The compression pressure was optimized to 120–130 mmHg without adverse effect on the tissue preparation. A cycle of 5 s compression followed by 15 s recovery yielded a resting VO_2 of $0.98 \pm 0.03 \text{ ml O}_2/100\text{cm}^3 \text{ min}$ while preserving microvascular oxygen delivery. The measurement system was then used to assess VO_2 dependence on $P_{\text{ISF}O_2}$ at rest and further tested under conditions of isometric muscle contraction to demonstrate a robust ability to monitor the on-kinetics of tissue respiration and the compensatory changes in $P_{\text{ISF}O_2}$ during contraction and recovery. The temporal and spatial resolution of this approach is well suited to studies seeking to characterize microvascular oxygen supply and demand in thin tissues.

Keywords

Oxygen Consumption (VO_2); Phosphorescence Quenching Microscopy (PQM); Skeletal Muscle; Rat Spinotrapezius Muscle; Microvasculature; Oxygen Measurement; Muscle Stimulation; Tissue Compression

INTRODUCTION

The oxygen (O_2) supply and demand of skeletal muscle exists in a dynamic and responsive equilibrium within the interstitial fluid providing a key focal point of observation (Ferreira, Poole et al. 2005). At rest, O_2 diffuses according to its partial pressure gradient from hemoglobin binding sites within red blood cells (RBCs) through the interstitium to match

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overall rates of mitochondrial oxygen consumption (VO_2) within cells (Sarelius and Pohl 2010). The partial pressure of O_2 in the interstitial space ($\text{P}_{\text{ISF}\text{O}_2}$), which is dissociated from red blood cell (RBC) hemoglobin and free to diffuse to intracellular mitochondrial sinks without appreciable interference from myoglobin desaturation, is sensitive to rapid changes in the balance between supply and demand. Different approaches ranging from whole animals (Hoyt, Wickler et al. 2006, Rodrigues, Figueroa et al. 2007), isolated muscles (Goodwin, Hernandez et al. 2012); (McDonough, Behnke et al. 2005), to single fibers (Hogan 2001); (Kindig, Howlett et al. 2003); (Wust, van der Laarse et al. 2013) have sought to characterize the relationship of the partial pressure of O_2 (PO_2) and VO_2 . The description of $\text{P}_{\text{ISF}\text{O}_2}$ supply/demand coupling for intact muscle preparations *in vivo*, however, remains incomplete.

When assessing the VO_2 of a particular microvascular bed under physiological conditions, the difference in PO_2 between the input arteries/arterioles and output veins/venules can be related to blood flow through Fick's principle to provide a good description of O_2 utilization. With regards to isolated, contracting skeletal muscle operating below the lactate threshold - an oft-studied model of PO_2 and VO_2 dynamics - it is acceptable to say that changes in VO_2 are proportionate to energy demand (Gutierrez, Pohil et al. 1989, Poole and Richardson 1997). VO_2 calculated by Fick's principle is regarded as a metric of tissue/organ respiration and has yielded values in mammalian skeletal muscles at rest ranging from 0.054 to 3.1 ml O_2 /100 g*min (Edmunds and Marshall 2001); (McDonough, Behnke et al. 2005, Hoy, Peoples et al. 2009); (MacInnes and Timmons 2005). While fully practical for determining the amount of O_2 consumed by a particular organ or tissue, Fick's principle cannot directly deconstruct fiber/tissue-specific VO_2 rates from these heterogeneous preparations nor indicate what spatial relations exist between VO_2 and the gradients of O_2 supply. Thus, for a variety of different muscle compositions a wide range of VO_2 values have been reported. Additionally, there is limited temporal resolution since the hemoglobin O_2 capacitance can act as a buffer between cellular respiration and changes in vascular O_2 depletion.

Another approach to determining the rate of O_2 extraction from a particular region of tissue is to arrest blood flow and chart the local oxygen disappearance curve (ODC). One approach uses PO_2 microelectrodes (Buerk, Nair et al. 1986), but has limited spatial sensitivity and may cause tissue damage. A less invasive technique makes use of Near-Infrared Spectroscopy (NIRS) (for review see: (Ferrari, Muthalib et al. 2011). Briefly, NIRS works by illuminating the target tissue with wavelength pairs of 700–805 nm and 830–805 nm to assess hemoglobin and myoglobin oxygenation, blood volume dynamics (relative measurements of absorption at ~805 nm over time) and mitochondrial cytochrome aa3 oxygenation to assess both vascular O_2 supply and tissue metabolic rate. For measurements of VO_2 , blood flow is arrested and the rate of oxyhemoglobin conversion to deoxyhemoglobin is related to a change in PO_2 over time, thus providing indirect information on VO_2 kinetics. This non-invasive approach has yielded resting VO_2 values in human calf muscles around 0.20 ml O_2 /100 g*min (Cheatle, Potter et al. 1991); (De Blasi, Luciani et al. 2009), which fall into the lower range found using Fick's principle, but have greater spatial and temporal resolution, as well as an assessment of mitochondrial respiration by which to compare the measured ODC. A lingering problem with these measurements is

the muting of the ODC resulting from the buffering actions of hemoglobin and the sigmoidal shape of the ODC.

Phosphorescence Quenching Microscopy (PQM) is a well-established technique (Rumsey, Vanderkooi et al. 1988); (Smith, Golub et al. 2002) which associates the phosphorescence emission of a porphyrin dye to the PO_2 of biological preparations with a high degree of spatial ($\sim 10 \mu m$) and temporal ($< 0.1 s$) resolution (Wilson, Vanderkooi et al. 1987). PQM, by virtue of its unintrusive nature, has been applied to measure the distribution of O_2 in a number of microvascular preparations (Torres Filho and Intaglietta 1993); (Golub, Popel et al. 1997); (Behnke, Kindig et al. 2001, McDonough, Behnke et al. 2005) (Copp, Hirai et al. 2011). Similarly to NIRS, but without a dependence on hemoglobin, PQM it has been found to be a useful tool in making discrete, single measurements of VO_2 by means of mechanically isolating small regions of tissue from the blood supply and observing the ODC (Golub, Tevald et al. 2011). Here we demonstrate a method of VO_2 sampling utilizing PQM that, in addition to providing continuous readings of $P_{ISF}O_2$, also allows for simultaneous, recurrent measurements of VO_2 in a variety of metabolic states without interfering with microvascular function. This new technique allows for extended measurements that can capture many systemic and local events that might influence tissue oxygenation with a simultaneous assessment of energy demand.

METHODS

Animals

The following protocols and experimental procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and are consistent with the National Institutes of Health guidelines for the humane treatment of laboratory animals, as well as the American Physiological Society's Guiding Principles in the Care and Use of Animals. Fifty male Sprague Dawley rats (250–300 g; Harlan, Indianapolis, IN) were given an initial dose of ketamine/acepromazine (75 mg/kg and 2.5 mg/kg, respectively; i.p.). A femoral vein cannula was inserted to maintain a continuous plane of anesthesia (assessed to have heel response with minimal toe pinch response) with Alfaxalone acetate (Alfaxan, Schering-Plough Animal Health, Welwyn Garden City, UK) at 0.1 mg/kg/min. A carotid artery catheter with attached pressure transducer was inserted to provide continuous measurement of mean arterial pressure (MAP) (MP-150, BIOPAC Systems, Goleta, CA). A tracheal cannula was inserted to maintain airway patency. After experimentation, animals were euthanized with an overdose of Euthasol (150 mg/kg, pentobarbital component, intravenously; Delmarva, Midlothian, Virginia).

Spinotrapezius Preparation

The rat spinotrapezius muscle was exteriorized as described by Gray (Gray 1973) with some modification to accommodate measurement of $P_{ISF}O_2$ and VO_2 , on a thermostatic animal platform adapted for intravital microscopy (Golub and Pittman 2003). A longitudinal, midline incision beginning between the shoulder blades exposed the muscle, which was then separated from underlying fascia and connective tissue by blunt dissection. Careful attention was given to maintaining tissue hydration with phosphate-buffered saline (PBS) and to avoid

stretching the muscle as it was mounted on the transparent, thermostatic pedestal of the animal platform. The preparation was then covered with a transparent, gas impermeable film (Krehalon, CB-100; Krehalon Limited, Japan) to isolate it from the atmosphere.

Intravital Microscopy with Phosphorescence Quenching

Measurements of $P_{ISF}O_2$ and VO_2 in the exteriorized spinotrapezius preparation were performed using an intravital microscope (Axioimager2m, Carl Zeiss, Germany) configured for PQM via epi-illumination through a 20×/0.8 objective (Plan-APOCHROMATE, Zeiss, Germany). The phosphor (Oxyphor R2; Oxygen Enterprises, Philadelphia, PA) was topically applied to the tissue and allowed to diffuse into the interstitium. The phosphor probe was excited by a xenon flash lamp (Model FX249, EG&G Electro-optics Co., Salem, MA), which delivered 0.5 J/flash for 4 μ s (full width at one third maximum pulse height) to an octagonal region 300 μ m in diameter at a frequency of 1 or 10 Hz. The excitation light pulse was passed through a filter cube consisting of a narrow-band filter (INTOR 525/70/75%, Intor Inc. Socorro, NM), which was specifically designed to excite the probe at 525 nm (Q-band), a dichroic mirror (Chroma 565 DCLP, Chroma Technology Corp. Bellows Falls, VT), and a wide-band filter (Oriel Cut-on >650nm, Newport Corp, Stratford, CT) for selective collection of phosphorescence emission. The phosphorescence signal was collected by a photomultiplier tube and sent to a modified amplifier (OP37EP, Analog Devices, Norwood, MA) which functioned as a current-to-voltage converter outfitted with a precision analog switch (ADG419BN, Norwood, MA) allowing for a gating time of 12 μ s. The 12 μ s delay between pulse initiation and data collection covered the 5 μ s propagation delay from the plasma arc to the flash, the 4 μ s light pulse, and 4–5 μ s of any residual thermal tail from the flash lamp and lingering fluorescence signal from tissue or probe. Thus, the data collected were optimized to contain the highest contribution from the short phosphorescence lifetimes (i.e., highest PO_2) without interference from fluorescent artifact, for reliable measurements of $P_{ISF}O_2$. These signals were visually monitored in real-time with an oscilloscope (72-3060, Tenma, Springboro, OH) before they were passed to a 12-bit analog-to-digital converter (PC-MIO-16E-4, National Instruments, Austin, TX), and saved digitally on a Dell Optiplex PC (Round Rock, TX). The tissue's microvasculature was visualized under transillumination with a light emitting diode (Luxeon V Star white, Quadica Developments Inc., Brantford, Ontario) that was fed through the microscope's condenser. The image, under 20× magnification, was captured in real-time by a color CCD camera (KP-D20BU, Hitachi, Tokyo, Japan) and displayed on a flat-screen color video monitor (Model LN19A450C1D, Samsung, Japan). Transillumination was used for measurement site acquisition and to verify tissue flow and arrest during air bag compressions.

Phosphorescent Probe

Pd-meso-tetra-(4-carboxyphenyl)porphyrin (Oxyphor R2) was obtained from Oxygen Enterprises (Philadelphia, PA) and conjugated to human serum albumin in PBS. The probe was then topically applied to the spinotrapezius muscle at a concentration of 10 mg/ml for 30 minutes. This allowed for deep, uniform diffusion of the probe into the muscle's interstitium from where the phosphorescence signal exclusively emanated (Golub, Tevald et al. 2011). The preparation was then rinsed with PBS to remove unloaded probe. Probe

preparation, loading, and measurements were all performed under dark conditions to minimize photobleaching.

Analysis of phosphorescence decay

A nonlinear fitting procedure (Levenberg-Marquardt) for the individual phosphorescence decay curves was based on the rectangular P_{ISF}O₂ distribution model (Golub, Popel et al. 1997) and yielded the following fitting equation for the phosphorescence time course:

$$I(t) = I_0 \exp[-(K_0 + K_q M)t] \sinh(K_q \delta t) / (K_q \delta t) + B \quad [1]$$

where t (μ s) is the time from the beginning of the phosphorescence decay, $I(t)$ (volts) is the phosphorescence decay curve, I_0 (volts) is the magnitude of the phosphorescence signal at $t = 0$, M (mmHg) is the mean PO₂ in the volume of detection, δ (mmHg) is the half-width of the PO₂ distribution, and B (volts) is the baseline offset of the amplifier. The constants for Oxyphor R2 are $K_0 = 1.53 \times 10^{-4} \mu\text{s}^{-1}$ and $K_q = 4.3 \times 10^{-4} \mu\text{s}^{-1} \text{ mmHg}^{-1}$.

Pneumatic Tissue Compression

Based on a previously described technique (Golub, Tevald et al. 2011), an objective-mounted air bag was inflated to produce a focal compression of the underlying tissue that resulted in immediate blood flow arrest and blood extrusion from the measurement region. Prior to compression, the air bag was pressurized to 5 mmHg to preserve gentle contact between the Krehalon barrier and the tissue to extrude any excess fluid on the surface of the tissue that might interfere with phosphorescent lifetime measurements. Next, following baseline measurements, the bag was pressurized to 120–130 mmHg. The rapid arrest of blood flow (<1 s), RBC extrusion, and any lateral shifting was observed under transillumination for quality assurance. Following the required compression duration (see below), the pressure in the air bag was decreased to 5 mmHg and flow was restored. Compression cycling was controlled by a custom-built automatic dual-stage pressure regulator, which allowed for appropriately timed alternation between two pressure levels, high (120–130 mmHg) and low (~ 5 mmHg), accommodating the repetitive 5 seconds of compression and 15 seconds of recovery cycle (denoted “5 s $\times$ 15 s cycle” below). The “5 s $\times$ 15 s cycle” was based on pilot experiments where 5 s of compression were needed to obtain five descending PO₂ values for accurate plotting of the ODC and calculation of VO₂ from the linear component. 15 s of decompression then permitted P_{ISF}O₂ recovery to P₀ prior to compression. A digital display reported the current pressure inside the air bag and a toggle switch allowed for the selection of manual pressure delivery for longer durations of both high and low pressure.

VO₂ Analysis

From the moment of flow arrest, the O₂ supply was stopped and the P_{ISF}O₂ began to decrease in a linear fashion. This ODC was recorded and used to compute the VO₂ of the underlying tissue as previously described (Golub, Tevald et al. 2011). Briefly, the rate at which P_{ISF}O₂ changes under conditions of flow arrest depends on three factors which appear on the right hand side of Eq. 2 below: 1) the rate at which O₂ is consumed through respiration in the excitation region; 2) the rate at which O₂ is consumed by the method itself

(i.e., photoconsumption); and 3) the rate at which O₂ diffuses into the region of excitation from the surrounding tissue (i.e., “refill” or replenishment of consumed O₂). Each flash of the excitation source gives rise to a phosphorescence decay curve and each curve can be analyzed by Eq. 1 to yield a best-fit value for P_{ISF}O₂ (i.e., M in Eq. 1) in the excitation region. On a per flash basis, the change in P_{ISF}O₂ can be described by Eq. 2:

$$dP_n/dn = -V_n - KP_n + Z(p_n - P_n) \quad [2]$$

where dP_n/dn is the rate per flash of O₂ disappearance from the excited region, P_n is the average P_{ISF}O₂ within the 300 μm diameter measurement area after flash n , V_n is the contribution of cellular respiration per flash, K is the coefficient of O₂ consumption by the PQM method, and Z is the coefficient of O₂ inflow (refill) by passive diffusion at the periphery of the measurement area. The interaction of an excited phosphor molecule with O₂ (collisional quenching) causes the conversion to singlet oxygen and thereby reduces the local PO₂. O₂ consumption by the method depends on concentration of phosphor, intensity of excitation light and PO₂. Since measurements are typically made with constant phosphor concentration and excitation intensity (incorporated in the factor K), the contribution of O₂ consumption by the method is expressed as the term $-KP_n$, proportional to local PO₂. Refill decreases as the radius of the illuminated measurement region increases. With a large excitation area of 221,841 μm² and a small KP_n , 0.5% per flash, the coefficient of refill (Z) was found to be insignificant for the initial, linear portion of the ODC and Eq. 2 can be simplified to:

$$dP_n/dn = -V_0 - KP_0 \quad [3]$$

V_0 (mmHg/flash) can then be converted to VO₂ by accounting for both flash rate (F) and the solubility of O₂ in the interstitial fluid ($\alpha = 39$ nl O₂/(cm³·mmHg))(Mahler, Louy et al. 1985) to yield:

$$VO_2 = V_0 F \alpha \quad [4]$$

Rearranging Eq. 3, the expression for transforming dP_n/dn into a measurement of tissue oxygen consumption is as follows:

$$VO_2 = (-dP_n/dn - KP_0) F \alpha \quad [5]$$

Determination of PQM's Photoconsumption Rate

Phosphorescence photoconsumption is directly proportional to the amount of light injected into the system per unit time. Therefore, to assess the impact of these measurements on the observed ODC, two separate excitation frequencies were used to measure dP_n/dn at random sites in intact spinotrapezius preparations. Using the 5 s × 15 s high/low pressure cycling method, a site was selected in the R2-loaded muscle and measured for 100 s at either 1 or 10 Hz excitation. Immediately following this a second measurement was made using the other frequency for 100 s. The differential ODCs were then compared as follows:

$$K = [(dP_n/dn)_1 - (dP_n/dn)_2] / [(FP_0)_2 - (FP_0)_1] \quad [6]$$

Where F is the flash rate for each respective measurement (1 or 10 Hz here) and dP_n/dn is the rate of oxygen disappearance per flash.

Pressure Tests

The spinotrapezius muscle was prepared and mounted as described above. A site was selected that contained well-perfused vessels with diameters $< 15 \mu\text{m}$. Based on preliminary and concurrent measurements, a compression recovery profile of 5 s high pressure and 15 s low pressure was selected to assess the influence of increasing pressure on both dP_n/dn and the PO_2 recovery time course back to baseline. After a brief baseline measurement using PQM at low pressure ($\sim 5 \text{ mmHg}$) the sequence of high and low pressures was initiated. The first high-pressure compression was set to 40 mmHg and subsequently increased by 10 mmHg per measurement to a maximum of 130 mmHg.

Muscle Contraction

PQM was used to monitor the changes in $\text{P}_{\text{ISF}}\text{O}_2$ and VO_2 between rest and contraction in the rat spinotrapezius muscle. Two silver wire electrodes were placed in parallel along the length of the muscle at its edges. This positioning was found to maximize the passage of current into the tissue without creating air pockets or other morphological distortions that might interfere with both PQM and/or VO_2 sampling. Next, appropriate sites for measurement, as defined by a proximity of $\sim 200 \mu\text{m}$ to a sufficiently large ($40\text{--}100 \mu\text{m}$ diameter) arteriole and possessing sufficient capillary perfusion, were selected using transillumination at $10\times$ magnification. The $20\times$ objective with attached air bag was then used with transillumination to verify flow arrest under a brief ($\sim 2 \text{ s}$) compression at high pressure. Next, a short succession of PQM measurements at 1 Hz was performed to confirm that $\text{P}_{\text{ISF}}\text{O}_2$ corresponded to the observed perfusion rates. The protocol for contraction measurements consisted of the following sequence: 60 s rest, 120 s of contraction (2 Hz stimulation train at 10 volts with 20 ms duration) and 120 s of recovery. PQM (1 Hz) and VO_2 sampling ($5 \text{ s} \times 15 \text{ s}$ cycle) were run throughout the 5-minute time course. The concurrent profiles of $\text{P}_{\text{ISF}}\text{O}_2$ and VO_2 were then plotted for analysis.

Statistical Analysis

Data within these experiments are shown as mean $\pm$ standard error unless otherwise noted in figures and tables. One-way ANOVA was conducted using JMP software version 8.0 (SAS Institute Inc., Cary NC) followed by Tukey's multiple comparison test to determine significant differences between points in the time course of measurements. Statistical significance was taken to be $P < 0.05$.

RESULTS

Parallel Frequency Analysis of Photoconsumption

Four spinotrapezius muscles at rest (11 sites each) were assessed for the differential rates of oxygen disappearance during compression at 1 and 10 Hz phosphorescence excitation rates. The change in $-dP_n/dn$ (Fig. 1) was then used to calculate the coefficient of photoconsumption (K) per the PO_2 . K was found to be $7.7 \pm 0.6 \times 10^{-3}$ ($n=44$), which indicated that approximately 0.5% of the observed O_2 consumption per flash during resting conditions was due to O_2 consumption by PQM itself.

Assessment of Compression Pressure on Microvascular Oxygenation and VO_2

Nine resting state spinotrapezius muscles were subjected to stepwise 10 mmHg increases in air bag pressure from 40 to 130 mmHg and assessed for changes in dP_n/dn (Fig. 2A) and the restoration of $P_{ISF}O_2$ (see Fig. 2B) during the $5\text{ s} \times 15\text{ s}$ compression/recovery protocol. A baseline measurement of $P_{ISF}O_2$ at the standard low pressure of 5 mmHg preceded initiation of the $5\text{ s} \times 15\text{ s}$ VO_2 sampling protocol. $P_{ISF}O_2$ recovery did not vary significantly over the range of increasing compression pressures. The linear drop in $P_{ISF}O_2$ (dP_n/dn) that occurred with each compression was reported as an absolute value in terms of mmHg/sec as shown in Figure 2B. There was a progressive rise in $-dP_n/dn$ from 3.2 ± 0.2 mmHg/sec at 40 mmHg pressure to 4.4 ± 0.2 mmHg/sec at 80 mmHg, above which $-dP_n/dn$ leveled off. There was no statistical difference in $-dP_n/dn$ between groups from 80 mmHg to 130 mmHg. There was a slight, but insignificant, elevation in $-dP_n/dn$ at 120 mmHg. To accommodate variable tissue thickness and ensure that both blood flow-arrest and RBC extrusion took place rapidly and effectively, a range of 120–130 mmHg was chosen as optimal for the $5\text{ s} \times 15\text{ s}$ VO_2 sampling protocol. Values of $-dP_n/dn$ were not converted to VO_2 because some lower pressure measurements had incomplete blood flow-arrest and RBC extrusion.

Determination of $5\text{ s} \times 15\text{ s}$ Compression/Recovery Cycle on Microvascular Oxygenation and VO_2

The VO_2 method necessitated a temporary reduction in local $P_{ISF}O_2$ which introduced the potential for these brief compressions to create a cumulative disruption in microvascular O_2 supply/demand dynamics. To address this, preliminary studies (data not shown) were first carried out under resting conditions to produce an optimal VO_2 sampling rate that did not produce noticeable changes in microvascular oxygenation or tissue VO_2 . As a result, a 5 s compression duration was determined to be a sufficient amount of time for an accurate assessment of the rate of O_2 disappearance while still remaining in a physiological time course of brief ischemia. Figure 3A shows the averaged, $P_{ISF}O_2$ curves from eight animals (~ 14 sites per animal) after 15 s of recovery following 5 s of compression of the spinotrapezius muscle at rest. The 15 s profile of recovery showed no significant differences between baseline and subsequent measurements following compression in terms of $P_{ISF}O_2$ and VO_2 (see Fig. 3).

VO₂ Dependence on P_{ISF}O₂ to determine normoxic, resting VO₂

Early data analysis of randomized sites showed that for various levels of perfusion, as indicated by the P_{ISF}O₂ prior to the rapid onset of compression (P₀), there existed a range of P_{ISF}O₂ where VO₂ was dependent on PO₂. Fourteen resting state spinotrapezius muscles (~10 sites per animal) using a 10 hz PQM excitation frequency were used to study the range of VO₂ dependence on P_{ISF}O₂ under resting conditions (Fig. 4). VO₂ rose from 0.24±0.09 ml O₂/100 cm³ min at a P_{ISF}O₂ of 12.2±3 mmHg to 1.07±0.16 O₂/100 cm³ min at a P_{ISF}O₂ of 27.1±0.5 mmHg, which then remained within a range of 1.07 to 1.33 ml O₂/100 cm³ min to a P_{ISF}O₂ of 62 mmHg. At higher P_{ISF}O₂ levels, VO₂ appeared to drop off slightly. Statistical tests were run against the VO₂ measurement at a P_{ISF}O₂ of 12.2 mmHg and trend towards a critical point for VO₂ dependence on P_{ISF}O₂ between 25 and 30 mmHg. To limit any sort of edge effect, a 10 mmHg buffer was placed between the high end of this critical point so that only measurements of VO₂ where P₀>40 mmHg were included in the analysis of resting VO₂. Occasions of apparent atmospheric contamination (P₀>70 mmHg) were also excluded from analysis to guard against artifactually high PO₂. For the range of P₀ values above 40 mmHg (Fig 3A; P₀= 59.8 ± 1.1 mmHg; n= 125) where the VO₂ data could be reliably considered insensitive to P_{ISF}O₂, the mean VO₂ was measured to be 0.98 ± 0.03 ml O₂/100 cm³ min (Fig. 3B; n= 102).

Muscle Contraction

Electrical stimulation was applied to the spinotrapezius muscles of seven animals to assess VO₂ and P_{ISF}O₂ under dynamic conditions. Measurements consisted of the following series: 60 s baseline (allowing 3 measurements of VO₂ and P_{ISF}O₂) to provide an assessment of the resting state, 120 s of electrical stimulation, and a final 120 s of recovery without stimulation (Fig. 5). Contractile activity was isometric and confined to the period of electrical stimulation. Measurements of P_{ISF}O₂ made during the 60 s baseline under resting conditions were found to be consistent with other measurements of resting skeletal muscle conditions reported here. The onset of electrical stimulation produced an immediate decline in P_{ISF}O₂ (as assessed during the low pressure recovery period immediately prior to each high pressure compression), which significantly decreased from 67.7±2.0 mmHg (n=25) to 37.2±2.7 mmHg (n=25; p<0.0001) within 20 s. This reduction continued for the next 20 s and then began to rise slowly; however, the rise in P_{ISF}O₂ above the minimum value at 40 s of stimulation was not statistically significant. P_{ISF}O₂ remained near 30 mmHg for the duration of electrical stimulation. Following muscle stimulation, P_{ISF}O₂ rose from the average value during stimulation of 35.1±3.3 mmHg (n=25) back toward the baseline value (Fig. 5A). The onset of electrical stimulation caused a significant increase in VO₂ from 1.1±0.1 ml O₂/100cm³ min (n=28) to 3.1±0.2 ml O₂/100cm³ min (n=28) within 20 s. This increased consumption persisted for the duration of stimulation. VO₂ did not change significantly between the final data point taken during stimulation (3.0±0.2 ml O₂/100cm³ min; n=25) and the first time point of recovery (i.e., 20 s into the recovery period; 3.2±0.2 ml O₂/100 cm³ min; n=28). Following this, however, the recovery phase saw a progressive decrease in VO₂ back towards baseline with a final measured value of 1.6±0.1 ml O₂/100 cm³ min over 120 s (Fig. 5B).

DISCUSSION

A new technique for concurrent measurements of microvascular O_2 supply (related to $P_{ISF}O_2$) and demand (related to VO_2) dynamics under various metabolic conditions was developed and employed in the rat spinotrapezius muscle. This VO_2 method was adapted from a previously described technique that utilized the pressurization of an airbag to rapidly arrest blood flow while simultaneously observing the resultant drop in $P_{ISF}O_2$ with PQM (Golub, Tevald et al. 2011). The system, which was originally designed to record a single, protracted ODC, was extended to include an automatic dual-pressure regulator protocol that allowed for brief, recurrent ODC collection. The early part of the ODC was found to be linear reflective of tissue VO_2 . When corrected for a 0.5 or 5% photoconsumption per second of O_2 at 1 and 10 Hz flash rates respectively, resting VO_2 values corresponded to the range of 0.5 to 3.1 ml O_2 /100 cm³ min reported by other investigators of mammalian skeletal muscle respiration (MacInnes and Timmons 2005, McDonough, Behnke et al. 2005, Hoy, Peoples et al. 2009). The use of PQM to continuously sample $P_{ISF}O_2$ in conjunction with the automatic compression/decompression cycling method to make serial assessments of VO_2 afforded excellent temporal and spatial resolution that was able to track $P_{ISF}O_2$ dynamics in a muscle during electrically stimulated isometric contractions.

Two confounding variables in localized VO_2 measurements of intact organs are the O_2 buffering capacity of hemoglobin-bound O_2 in RBCs and dissolved O_2 in the interstitium. By extruding RBCs from the measurement site and measuring PO_2 directly in the interstitial space, the ODC can be used to determine local VO_2 without interference from luminal O_2 capacitance. In oxidative muscles the contribution of intracellular myoglobin to the O_2 buffering capacity of cells cannot be ignored at intracellular PO_2 values near and below the P_{50} of myoglobin (~ 5 mmHg); however, the PO_2 range over which normoxic VO_2 was determined in the studies presented here was well above this level. To mitigate the influences of RBCs and interstitial buffering, a transparent air bag mounted on a 20× microscope objective was chosen to provide focal pressure to the region of interest, while retaining the objective's ability to perform both intravital and phosphorescence microscopic measurements. The necessary pressure to arrest flow and extrude RBCs for early studies was initially based on the rat's mean arterial pressure – approximately 100 mmHg for 250 g male Sprague Dawley using this protocol (Song, Nugent et al. 2014)) - which dictated the air bag's high-pressure setting to be 120 mmHg. Once applied to the tissue, compression was observed, both through transillumination and PQM, to cause rapid flow-arrest (< 1 s) and RBC extrusion. PQM was used to record the resultant ODC, whose initial component was linear. During extremely hypoxic conditions, a mono-exponential decay could be observed for the last 1–2 seconds of compression. In the case of lingering RBCs, the early part of the ODC would have registered as an exponential decay instead of a linear drop in the normoxic region. At the lowest excitation frequency of 1 Hz, 5 s of compression covered the linear portion of the ODC (P_0 = ~60 mmHg) and provided up to 5 data points, which were sufficient to produce an accurate linear fit of $-dP_n/dn$ for eventual conversion to VO_2 .

A cycling protocol of 5 s of high pressure followed by 15 s of recovery was found to: 1) restore $P_{ISF}O_2$ back to baseline; 2) prevent cumulative effects of serial VO_2 measurements from altering the skeletal muscle's oxygen supply and demand interplay; and 3) provide a

reasonable number (3) of VO_2 measurements per minute. Given the rapid flow arrest and extrusion of RBCs brought on by pneumatic compression, a sufficiently linear portion of the ODC can be captured during shorter compressions at higher $\text{P}_{\text{ISF}}\text{O}_2$ sampling rates. The 5 s duration of compression was chosen to provide the capability of measuring VO_2 under conditions where the changes were not too rapid. Shorter compression durations down to ~2 s are likely possible in future refinements of the technique as 2–10 Hz $\text{P}_{\text{ISF}}\text{O}_2$ sampling would be sufficient to obtain a linear trend of the early ODC without reducing the signal-to-noise ratio. Figure 4 shows data captured using a 10 Hz $\text{P}_{\text{ISF}}\text{O}_2$ sampling rate and lies in good agreement with the 1 Hz data. 10 Hz was found useful during hypoxia when consumption rate shortened the linear ODC component.

To fully describe the effects of pneumatic compression on the microvasculature, a range of pressures from 40 to 130 mmHg was tested using the 5 s $\times$ 15 s sampling protocol for the recovery of $\text{P}_{\text{ISF}}\text{O}_2$ (see Fig. 2B) and the rate of O_2 disappearance during compression ($-\text{dP}_n/\text{dn}$; see Fig. 2A). No significant difference was found for the recovery of $\text{P}_{\text{ISF}}\text{O}_2$ over this range of compression pressures. It was expected that at some point, recovery would either show a pronounced hyperemic response or begin to show protracted recovery times if compression pressure brought about vascular damage. Neither situation was observed, though there did appear to be a statistically insignificant trend in the elevation of $\text{P}_{\text{ISF}}\text{O}_2$ during recovery around 70 mmHg of pressure. Likely, the detrimental effects of compression would have occurred at higher pressures, but these were not tested, as pressures over 130 mmHg greatly increased the probability of air bag rupture.

Stepwise increases in compression pressure of 10 mmHg starting with 40 and ending with 130 mmHg (5 mmHg was considered low pressure for the purpose of extrusion of any surface fluid layer that might confound PQM measurements) showed the corresponding rise in $-\text{dP}_n/\text{dn}$ that one might expect as flow-arrest and RBC extrusion becomes increasingly complete until reaching a plateau. Compressions from 80 mmHg to 130 mmHg did not yield a significant change in $-\text{dP}_n/\text{dn}$ as shown in Figure 2A, which indicated that maximal flow arrest and RBC extrusion had been achieved. Measurements at 90 and 100 mmHg appeared to be slightly elevated over measurements made from 110 to 130 mmHg, but this difference was not statistically significant. As higher-end pressures did not appear to cause any adverse effects in either VO_2 or the recovery of $\text{P}_{\text{ISF}}\text{O}_2$, and the initial VO_2 sampling interval was determined using 130 mmHg, the decision to use a range of compression pressures from 120 to 130 was deemed appropriate. This allowed for a justifiable adjustment in compression pressure to accommodate a range of tissue sizes and microvascular architectures. The effectiveness of flow arrest was always visualized prior to measurement and 120 to 130 mmHg had a consistently high success rate over the course of experimentation.

Another point of interest was the relevance of the $-\text{dP}_n/\text{dn}$ versus pressure profile on the microvasculature below complete flow arrest. As pressure rose, perfusion dropped with an apparent linear progression. If these pressures had been continued beyond 5 s, an eventual point where the reduction in perfusion could no longer keep up with O_2 demand would be reached - inevitably resulting in cellular hypoxia. This sort of behavior has been found to occur in Compartment Syndrome (de Laet and Malbrain 2007) and a study looking into the

pressure needed to surpass this “tipping point” in the development of hypoxic tissues using this method would be useful.

Photoconsumption of O_2 by PQM is a documented phenomenon (Golub and Pittman 2012) and most readily observed at high excitation frequencies or in cases where microvascular O_2 delivery was purposely stopped to observe the ODC for VO_2 measurements. Use of PQM to determine local PO_2 relies on the ability of O_2 to quench the phosphorescence of an excited probe (Oxyphor R2 in this case). In doing so, O_2 is converted to singlet oxygen, thereby consuming the original O_2 molecule. The consumption rate for the linear portion of the ODC is constant, however, and easily included in the equation for VO_2 . When O_2 flux, probe concentration, and measurement region are held constant, photoconsumption becomes dependent on light intensity. Using this fact, we obtained an optimal measurement for photoconsumption in the spinotrapezius preparation by varying the flash rate during flow arrest and accounting for the contribution of photoconsumption to the measured $-dP_n/dn$. Other probe-loaded media (i.e., gels, dead muscle tissue, microcapillary tubes) proved too difficult to translate to the physiologically intact situation, despite being excellent models of the photoconsumption effect. In the case of the R2 probe at 10 mg/ml which diffused into the spinotrapezius muscle, our reported value of K to account for photoconsumption of oxygen by PQM was $7.7 \pm 0.6 \times 10^{-3}$ (see Eqs. 3 and 6). New values for K need to be determined for different experimental conditions. In cases where PQM is used to measure a well-perfused region at low excitation frequencies (< 1 Hz), the value of K will be small and unlikely to affect the measured $P_{ISF}O_2$.

VO_2 determined by the procedure described above predominantly represents mitochondrial respiration where O_2 functions as the terminal electron acceptor in the mitochondrial electron transport chain. In addition to energy production (ATP and heat), mitochondria produce small quantities of reactive oxygen species, reported as anywhere from 0.15 to 2% of mitochondrial VO_2 under physiological conditions (St-Pierre, Buckingham et al. 2002); (Misra, Sarwat et al. 2009); (Tahara, Navarete et al. 2009). This percentage may rise in times of mitochondrial dysfunction, as seen during ischemia/reperfusion injury.

Measurements included in this study were performed under normoxic conditions (P_0 40 mmHg) where the ODC was observed to remain independent from $P_{ISF}O_2$ during compression. Our definition of normoxia was a conservative limit based on measurements of VO_2 vs $P_{ISF}O_2$ (Fig. 4), which suggest a critical point somewhere between 25 and 30 mmHg for the interstitial space. We added an additional 10 mmHg buffer to avoid any edge effect from reducing our assessment of VO_2 . We then used electrical stimulation of the spinotrapezius muscle as a model to demonstrate the utility of PQM in simultaneous recording of VO_2 - $P_{ISF}O_2$ kinetics. Stimulation of the muscle showed an immediate decrease in $P_{ISF}O_2$ which leveled off by the 20 s time point, in good agreement with the rapid adjustment of blood flow to meet sudden increases in metabolic demand (Naik, Valic et al. 1999); (Clifford and Hellsten 2004); (Mihok and Murrant 2004). VO_2 increased 3-fold over baseline 20 s following the onset of contraction, a time course consistent with modeled and published data (Kindig, Richardson et al. 2002, Jones, Grassi et al. 2011). Following the termination of stimulation, VO_2 slowly returned to baseline over 120 s reflecting the accumulated oxygen debt from the 120 s of stimulation.

Several limitations exist when utilizing this technique to study VO_2 on kinetics in whole muscle: 1) As VO_2 significantly increases, $\text{P}_{\text{ISF}}\text{O}_2$ may fall below the 40 mmHg threshold, which could decouple measured VO_2 from energy production as glycolysis and lactate production increase, and 2) Measurements of VO_2 lose accuracy as $\text{P}_{\text{ISF}}\text{O}_2$ drops below 10 mmHg due to reduced collection of the linear ODC component. VO_2 measurements during isometric contraction, as presented here for demonstration of the technique's capacity to track VO_2 and $\text{P}_{\text{ISF}}\text{O}_2$ dynamics, need to be interpreted with their corresponding $\text{P}_{\text{ISF}}\text{O}_2$ data, which may impose a physiologically valid restraint on the increase in VO_2 . An increase of the sampling rate to 2–10 Hz would greatly increase hypoxic accuracy after correction for photobleaching and is suggested for adapting this technique to various tissues and experimental designs. Additionally, for measurements where VO_2 is limited by $\text{P}_{\text{ISF}}\text{O}_2$, the 5 s $\times$ 15 s compression cycling might produce a cumulative effect of reducing O_2 supply versus a situation where $\text{P}_{\text{ISF}}\text{O}_2$ were recorded without compression. This may manifest itself as increased lactate production and/or decreases in $\text{P}_{\text{ISF}}\text{O}_2$ values over time. Since the total amount of $\text{P}_{\text{ISF}}\text{O}_2$ delivery interruption can be extracted from the tracing and recovery intervals, the impact of compression can be calculated and measurements corrected accordingly.

In conclusion, serial measurements of VO_2 represent a robust method for assessing oxygen supply and delivery dynamics in thin tissues with minimal intrusiveness. It satisfies the need for better spatial and temporal resolution in animal models and is adaptable to applications such as: exercise on-kinetics, ischemia/reperfusion injury, compartment syndrome, and the impact of pharmacologic agents on microvascular performance and tissue respiration.

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ABBREVIATIONS

$\text{P}_{\text{ISF}}\text{O}_2$	Interstitial Partial Pressure of Oxygen
PO_2	Partial Pressure of Oxygen
P_0	First measurement of Partial Pressure of Oxygen
ODC	Oxygen Disappearance Curve
dP_n/dn	Disappearance rate of oxygen
VO_2	Oxygen Consumption by tissues
K	Photo-consumption of Oxygen by Method
Z	Coefficient of Oxygen Refill into Excitation Region
PQM	Phosphorescence Quenching Microscopy
α	Solubility of oxygen in skeletal muscle

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Highlights

- New method for simultaneous measurement of $P_{\text{ISF}O_2}$ and tissue VO_2 in skeletal muscle
- Intermittent compressions and PQM are minimally disruptive to microvasculature
- Serial measurements detail transitions from rest to altered metabolic states
- Applications in exercise, compartment syndrome, ischemia/reperfusion & therapeutics

Figure 1.

Representative same site comparison of $P_{\text{ISF}}\text{O}_2$ sampling between 1 and 10 Hz excitation frequencies in spinotrapezius muscle at rest. Consumption by PQM is illustrated here in a single site assessment using the high/low pressure cycling to assess VO_2 and $P_{\text{ISF}}\text{O}_2$. The differences between the baseline $P_{\text{ISF}}\text{O}_2$ s were perfusion-dependent, while the differences in the oxygen disappearance rates were perfusion independent due to complete flow arrest. Therefore, they provided the best evaluation of PQM's contribution to oxygen consumption. N=44 sites over 4 animals.

Figure 2.

P_{ISFO_2} and O_2 disappearance rate versus air bag pressurization during $5\text{ s} \times 15\text{ s}$ protocol in the spinotrapezius at rest. A) O_2 disappearance rate. Each measurement represents the linear portion of ODC observed during a 5-s compression at the pressure indicated. Data were not converted to VO_2 because in the cases of lower pressure, flow did not appear to be fully arrested and therefore no valid comparison of VO_2 could be made. Oneway ANOVA showed that dp/dt plateaued at 100 mmHg and above. B) P_{ISFO_2} recovery. Following a baseline measurement at 5 mmHg (standard low pressure), the high pressure component of the high/low cycle was varied from 40 to 130 mmHg to assess the impact of pressure on microvascular oxygenation. Measurements were made using the $5\text{ s} \times 15\text{ s}$ VO_2 sampling protocol with each compression receiving a 10 mmHg increment above the previous one from 40 to 130 mmHg. There was no significant effect of compression on recovery P_{ISFO_2} over the measured range when compared to BL. $N=29$ replicates per pressure over 9 animals.

Figure 3.

P_{ISFO_2} and VO_2 sampling with the 5 s $\times$ 15 s protocol. A) P_{ISFO_2} . P_{ISFO_2} was sampled at a rate of 1 Hz over a 100 s time course of high and low pressure compression/decompression cycling. 5 s averages prior to each compression are reported here and show no statistical differences between baseline (P_{ISFO_2} at 20 s) and measurements following the next four compression/decompression cycles ($P_{\text{ISFO}_2_n}$, where $n=1-4$). B) Calculated VO_2 . With P_{ISFO_2} sampling at 1 Hz, a 100 s time course of high and low pressure compression cycling (5 s $\times$ 15 s) was conducted. Each period of high pressure lasted 5 s and generated an ODC. The linear slope of each ODC was then used to calculate VO_2 . Baseline VO_2 (compression at 20 s) was compared to subsequent measurements at 40, 60, and 80 s to assess the impact of recurrent compressions on VO_2 . No statistical differences between baseline and subsequent measurements were found. $N=115$ sites over 8 animals.

Figure 4.

VO₂ vs P_{ISF}O₂. VO₂ was measured across the physiological range of P_{ISF}O₂ for the resting state spinotrapezius muscle above 10 mmHg. P_{ISF}O₂ values were obtained using a 10 hz sampling rate and the 5 s of data before compression were averaged. VO₂ was not found to be significantly different between P_{ISF}O₂ values of ~16 mmHg (the second data point) and ~68 mmHg although there is a trend to suggest that VO₂ levels out above 25 mmHg. All comparisons were made to the first group at ~12 mmHg for ease of presentation. Interestingly, VO₂ for high P_{ISF}O₂ began to trend downward. Statistical significance is indicated by * p< 0.05 and ** p< 0.01. N= 14 animals with approximately 10 replicates each. N values varied between different P₀ groups.

Figure 5.

Rest-stimulation-recovery time course of spinotrapezius muscle. A) P_{ISF}O₂ averages. The average P_{ISF}O₂ for the 5 s prior to each compression were recorded for 60 s at rest, 120 s with electrical stimulation and for 120 s of recovery. Statistical comparisons were made against baseline measurements and a significant drop in P_{ISF}O₂ was observed during stimulation and persisted for the first 60 s of recovery. B) VO₂. Baseline (BL) measurements were considered to be the first three compressions while the muscle was at rest. A sharp rise in VO₂ was detected within 20 s of the onset of electrical stimulation. After 120 s, stimulation was stopped and VO₂ gradually fell back to baseline over and additional 120 s. Statistical differences versus BL measurements are indicated by *. VO₂ measurements during stimulation did not vary significantly. N=25 sites over 7 animals.