Spike of interstitial PO$_2$ produced by a twitch in rhythmically contracted muscle

Aleksander S. Golub | William H. Nugent | Bjorn K. Song

Abstract
Oxygen (O$_2$) exchange between capillaries and muscle cells in exercising muscles is of great interest for physiology and kinesiology. However, methodical limitations prevent O$_2$ measurements on the millisecond scale. To bypass the constraints of quasi-continuous recording, progressive measurements of O$_2$ partial pressure (PO$_2$) in rhythmically contracting skeletal muscle were compiled to describe the O$_2$ kinetics surrounding and including a single muscle contraction. Phosphorescence quenching microscopy measured PO$_2$ in the interstitium of the rat spinotrapezius muscle. Measurements were triggered by contraction-inducing electrical pulses. For the first 60 seconds, measurement preceded stimulation. After 60, measurement followed with a progressive 20 ms increment. Thus, the first 60 measurements describe the overall PO$_2$ response to electrical stimulation initiated after a 10 second rest period, while 61–100 (stroboscopic mode) were compiled into a single 800 ms profile of the PO$_2$ transient surrounding muscle contraction. Thirty seconds of stimulated contractions decreased interstitial PO$_2$ from a baseline of 71 ± 1.4 mmHg to an “active” steady-state of 43 ± 1.5 mmHg. The stroboscopic mode compilation revealed an unexpected post-contractile rise in PO$_2$ as a 205 ms spike with a maximum amplitude of 58 ± 3.8 mmHg at 68 ms, which restored 58% of the PO$_2$ drop from baseline. Interpretation of this phenomenon is based on classical experiments by G.V. Anrep (1935), who discovered the rapid thrust of blood flow associated with muscle contraction. In addition to the metabolic implications during exercise, the physiological impact of these PO$_2$ spikes may grow with an increased rate of rhythmical contractions in muscle or heart.

New&Noteworthy: The principal finding is a spike of interstitial PO$_2$, produced by a twitch in a rhythmically contracting muscle. A possible mechanism is flushing capillaries with arterial blood by mechanical forces. A technical novelty is the PO$_2$ measurement with a “stroboscopic mode” and progressively increasing delay between stimulator pulse and PO$_2$ measuring. That permitted a 20 ms time resolution for a 205 ms spike duration, using an excitation flash rate one per second.

KEYWORDS
exercise, O$_2$, phosphorescence quenching, PO$_2$, rhythmical contraction, skeletal muscle
1 | INTRODUCTION

Muscle cell oxygen \( (O_2) \) consumption increases substantially at the transition from rest to exercise (Behnke et al., 2002; Behnke et al., 2001; Golub et al., 2011), leading to a decrease in the partial pressure of \( O_2 \) (\( \text{PO}_2 \)) in the interstitial space on the surface of muscle cells and adjacent microvessels (Hirai et al., 2018; Nugent et al., 2016). Blood flow rises to meet this demand on the order of seconds extending to minutes, but the immediate, millisecond responses surrounding contraction have yet to be characterized.

The study of \( \text{PO}_2 \) levels associated with variable respiration in muscle cells at rest and exercise became possible with the invention of the phosphorescent quenching method (PQM) for measuring oxygen (Vanderkooi et al., 1987). In recent decades, \( \text{PO}_2 \) dynamics in the microvessels responding to rhythmic contractions of the rat spinotrapezius muscle have been characterized (Behnke et al., 2002; Nugent et al., 2016). Because of these works, the rat spinotrapezius muscle has become a standard for studying \( O_2 \) transport in organs with a controlled change in the respiration rate of myocytes. PQM can localize the \( \text{PO}_2 \) signal in various physiological compartments of the organ. With intravascular administration of an oxygen probe, \( \text{PO}_2 \) can be measured in the plasma of microvessels, locally or in the mass of vessels (Behnke et al., 2001; Golub & Pittman, 2008; Smith et al., 2002; Torres Filho et al., 1996). Situated between the dense capillary network that delivers \( O_2 \) and the skeletal muscle fibers that consume it, the interstitial space is a prime focal point for measuring \( O_2 \) availability.

The rat spinotrapezius muscle is a thin planar organ, which is convenient for bio-microscopy, thermal stabilization, and isolation from ambient air. The loading of a phosphorescent oxygen probe into the interstitium of a surgically exposed muscle occurs quickly and evenly, which ensures high signal quality for PQM. However, the PQM technique has a temporal limitation in application to a stationary fluid.

For exponential analysis, the phosphorescence signal must have low noise, which is provided by the selective collection of emitted phosphorescence and intense excitation pulse. But, the input of light causes phosphorescent consumption of oxygen, which is proportional to the excitation energy absorbed (Golub & Pittman, 2008). In stationary interstitial fluid, discrete \( O_2 \) depletions may be compensated by diffusion from capillaries and cells, but if the frequency of excitation pulses is too high, it could have a cumulative effect on measurements. The technical parameters of PQM setups are optimized so the method’s oxygen consumption is an insignificant fraction of total \( \text{PO}_2 \). It was previously estimated that in the muscle interstitium, the rate of excitation pulses should not exceed 5 per second (Golub & Pittman, 2008). While this 200 ms resolution is acceptable for tracking overall \( \text{PO}_2 \) dynamics between bouts of exercise and rest, it cannot report the rapid changes in interstitial \( \text{PO}_2 \) associated with muscle twitch.

It is known that after the onset of rhythmical contractions, the metabolic situation in the muscle comes to a new “active” steady-state with a lower microvascular \( \text{PO}_2 \) (Behnke et al., 2001; Golub et al., 2011; Nugent et al., 2016). At this new plateau, the stroboscopic principle with a phase rotation can be applied to \( \text{PO}_2 \) measurements and, through a series of 1 Hz excitation light flashes, compile a time course of \( \text{PO}_2 \) following contraction with a 20-ms temporal resolution. Three conditions are required: stationary \( \text{PO}_2 \) in the contracting muscle, synchronization between contraction and excitation light pulse, incrementing delay of the flash with respect to the onset of the electrical stimulation pulse. We have employed this approach to study the dynamics of interstitial \( \text{PO}_2 \) in response to muscle contraction with a high temporal resolution.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

SoBran Biosciences Inc. approved the following animal protocol and experimental procedures (IACUC Protocol no. SON-003-2018), which were executed by Song Biotechnologies LLC researchers. They are consistent with the National Institute 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. Six male Sprague Dawley rats (306 ± 10 g; Harlan, Indianapolis, IN, USA) were used in the experiments.

2.2 | Surgical preparation

Animals were inducted with 1–5% isoflurane in medical air for initial preoperative preparation and cannulations. The femoral vein was then accessed and cannulated with polyethylene tubing (PE-90) to enable the continuous infusion of anesthetic, alfaxalone acetate (Alfaxan; Schering-Plough Animal Health, Welwyn Garden City, UK), at rate ~0.1 mg/kg/min). Continuous infusion, with responsive adjustment to animal reflexes, heart rate, and oxygen saturation indicators, provided a steady plane of anesthesia through the conclusion of surgical preparation and measurement. A tracheal tube was inserted to maintain a patent airway. A femoral artery cannula was connected to a pressure transducer for monitoring systemic circulatory variables with a multichannel physiological monitoring system (BIOPAC MP-150; BIOPAC Systems, Goleta, CA, USA). The animal core and exteriorized spinotrapezius muscle temperatures were maintained.
by a custom animal platform (Golub & Pittman, 2003), and a rectal probe monitored the core temperature. The main parameters characterizing a physiological status in six rats were: mean arterial pressure 117 ± 4 mmHg; heart rate 433 ± 8 min⁻¹; body temperature 37 ± 0.2°C. Following the completion of experimental measurements, animals were euthanized with Euthasol (360 mg/ml of pentobarbital and 50 mg/ml of phenytoin sodium administered I.V. at 3 ml/kg; VetOne; Boise, ID).

2.3 | Spinotrapezius muscle preparation

The rat spinotrapezius muscle surgical preparation was similar to the original descriptions (Gray, 1973) with some modifications to measurements of interstitial PO₂ in isometrically contracting muscle. The exteriorized muscle was placed on a trans-illuminated pedestal of the 3-D printed animal platform, thermo-stabilized at 37°C. The edges of the muscle were fixed with seven sutures to a rigid frame to minimize muscle movement for isometric contractions. Two chlorinated silver wire electrodes were attached along the side edges of the muscle for electrical stimulation. A bout of electrical stimulation (1–5 s) was applied at the end of the preparation procedure to ensure proper muscle fixation and electrode connection.

The muscle was allowed to stabilize for 20 minutes, while the oxygen probe loaded into the interstitium (Golub et al., 2011). The phosphorescent oxygen probe employed in our measurements was a Pd(II) meso-Tetra(4-carboxyphenyl) porphine (PdT790; Frontier Scientific, Newark, DE) conjugated to bovine serum albumin as previously described (Golub & Pittman, 2016; Vanderkooi et al., 1987). The probe solution, at concentration 8–10 mg/ml of Pd-TCPP, was directly applied to surgically opened muscle for 20–30 min and then blotted with a filter paper. The muscle was covered with the gas barrier film Krehalon, CB-100 (Kureha Corporation, Tokyo, Japan).

An objective-mounted, transparent (Krehalon) airbag was inflated to provide organ compression at a pressure of 8 mmHg. Low-pressure surface contact allowed free blood circulation, while providing a tight seal of the gas barrier film to the muscle surface. The pressure was necessary to prevent the accumulation of a fluid layer between the film and tissue and to exclude a convective transfer of oxygen into the region of measurement due to muscle contraction.

2.4 | Intravital microscopy and PQM

Measurements of interstitial PO₂ were carried out using an Axioimager-A2m microscope with a 20X/0.8 Plan-Apochromat objective lens (Carl Zeiss, Germany). The measurement technique described in detail in our previous publications (Nugent et al., 2016) had several modifications to upgrade its performance. The excitation light source was a 520 nm green laser diode (NDG7475 1 W; Nichia Tokushima, Japan) powered by laser driver iC-HKB (www.ichaus.com). An optical cube in the path of the epi-illumination train contained a dichroic beam splitter (567 nm DMLP Longpass; Thorlabs, Newton, NJ, USA) and a wide-band, interference filter (Longpass Cut-on >650 nm; Thorlabs) for emitted phosphorescence light.

The circular (diameter 450 µm) epi-illuminated area covered more than ten fiber’s width (33.6 ± 0.7 µm; 22 measurements). The beam area was limited by a field diaphragm to employ the most homogeneous central part of the beam. The laser pulse duration was set to 1 μs, and the energy of a single pulse delivered to the muscle surface was 8 pJ/μm². With the combination of a low probe concentration and intensity of excitation illumination, oxygen consumption by phosphorescence quenching reduced PO₂ measurements by a minimal 0.4% per excitation flash, as previously described (Golub et al., 2018).

The phosphorescence decay curve was detected with a photomultiplier tube R9110 and a socket C12597-01 (Hamamatsu Photonics, Japan). The detector was assembled in a PXT-1 housing (Thorlabs) mounted on the microscope upper port. The current output of the detector was converted into a voltage signal with the precision operational amplifier LT1028. The initial segment of the phosphorescence decay signal containing the excitation flash and fast fluorescence transients was truncated by disabling the amplifier output for 5 μs with an analog switch ADG419. The phosphorescence decay analog signal was sampled at 200 kS/s and digitized with 16-bit accuracy using the software LabView and hardware NI PCIe-6361 board (National Instruments, Austin, TX, USA). The duration of the recorded phosphorescence decay curves was 2 ms with 400 data points collected per curve. The period starting from excitation flash and ending when a decay curve data is collected is a PO₂ measurement time interval.

The principle of exponential analysis of the phosphorescence decay curves and fitting model for recovery of a mean PO₂ from heterogeneous decay curves is as previously described (Golub & Pittman, 2016; Golub et al., 1997). The computer program for data acquisition, processing of curves, and the calculation of PO₂ during the measurement procedure were composed in-house as a Virtual Instrument in LabView.

2.5 | Muscle stimulation and location of sites

The built-in electrical stimulator produced a symmetrical 10 volt and 20 ms duration pulse delivered at 1 Hz to a pair of chlorinated silver wire electrodes placed alongside the
muscle. The stimulator circuit was optically isolated from other electronic units.

Measurement sites were clustered in the central part of the muscle, where contraction displacement was minimal. Excitation pulses were targeted to a 450 µm circular muscle region between transversal arterioles so that the vessels were represented mainly by capillaries and interstitial PO2 values belonged to the interface between the capillaries and muscle cells.

2.6 Experimental procedure

Event timing during the experiment was governed by a micro-controller board Arduino Uno R3 (https://www.arduino.cc/), according to a sketch illustrated by the top diagram in Figure 1. Interstitial PO2 was measured in a selected site of the muscle for 100 seconds and divided into one second time intervals, from #1 to #100. Each PO2 measurement interval (2 ms duration, shown as a black bar on the top diagram Figure 1) started with an excitation flash, so the total set of obtained 100 PO2 values was plotted versus the flash number, not versus time in seconds to avoid complications after measurement #60 when the intervals between measurements increment for 20 ms every second. With this in mind, the number of a flash is the same as for the corresponding one second time interval.

Measurements began after animal recovery and stabilization from surgery. Each measurement series (100 PO2 samples) was preceded by at least 4 minutes of rest after the previous bout of stimulated twitches. The first 10 PO2 values (#1-10 s) were recorded without electrical stimulation and averaged as a baseline PO2 (Figure 1). Starting from flash #11 to #60, the electrical stimulation pulse (20 ms duration, presented as a white bar on the top diagram Figure 1) occurred after the PO2 measuring flash (black bar), so the PO2 was sampled about a second after stimulation and twitch of the muscle. Stimulated muscle contractions resulted in a decrease in interstitial PO2, which stabilized after approximately 30 seconds/contractions. Ten values of PO2 for seconds #51-60 were averaged as an active steady-state PO2.

The pattern of flash preceding a stimulating pulse recorded data that is presented in real-time and lasted until measurement #60 (see the top diagram in Figure 1). Measurement #60 was taken as the zero value for the PO2 transient that followed the stimulation pulse. The next measurement of PO2 (#61) followed the stimulating pulse, that is, had a delay of 20 ms relative to the front of the stimulating pulse. This

![FIGURE 1 Protocol diagram and example of a recorded PO2 trace obtained in accordance with the experimental protocol. PO2 is plotted versus the number of the excitation flash, which corresponds to the time in seconds. However, data after measurement #60 no longer reflect continuity in time due to the incrementing delay of PO2 measurements. The top diagram shows the arrangement between the PO2 measurement time period (2 ms, black bars) and the pulse of electrical stimulation (20 ms, white bars). The first 10 seconds were taken to measure PO2 without stimulation, then starting from #11 and up to #60 inclusive, the PO2 measurement period immediately followed by the stimulator pulse. Starting from #61 the PO2 measurement period followed the stimulation pulse with an increment of 20 ms for every next pulse (stroboscopic mode). That permitted the reconstruction of the PO2 transient with time resolution 20 ms at a real flash rate of less than 1 per second. The bottom graph shows the result of PO2 measurements during the experimental procedure: (1) baseline PO2 as a mean of #1-10 values; (2) start of 1 Hz electric stimulations from #11 to #100; (3) averaged PO2 values #51-60 as a characteristic of an active steady-state PO2 in rhythmically contracting muscle; (4) start of the stroboscopic mode of PO2 measurements #61-100; (5) amplitude of the PO2 peak induced by muscle contraction; (6) duration of the PO2 peak considering 20 ms intervals between data points; (7) averaged PO2 values #81-90 to characterize the (8) trend to post-contractile steady-state PO2.](image)
delay increased an additional 20 ms with every subsequent flash reaching 800 ms by the end of the recording. Thus, respective to the front of the stimulation pulse, point #61 had a 20 ms delay, #62 had a 40 ms delay, and #63 a 60 ms … up to 800 ms at flash #100. Since an active steady-state of PO2 was established during measurements #50-60, it was assumed that muscle status was maintained until measurement #100. This “stroboscopic mode” with the incremental time shift was compiled to produce a 0.8 s profile of the changes in PO2 associated with single muscle contraction with a time resolution of 20 ms, without exceeding the actual flash rate of 1 per second (Figure 2).

The amplitude and duration of the PO2 transient were compiled by the set of incremental measurements #61-100 with the time intervals between adjacent values of 20 ms. Some of the compiled PO2 tracks had a slow positive trend in PO2 after reaching a stationary state, which exceeded the active stationary level (#51–60). We used the average value of the “post-contractile” steady-state PO2 (#81–90) to characterize this effect.

2.7 | Statistics

In each of the six muscles, 4–5 PO2 records were carried out in 3–4 tissue sites; a total of 21 sites were explored. The number of PO2 records made in each muscle was 15–19; a total of 101 records were obtained. A Descriptive Statistics and Paired t-test were used from the Data Analysis Tools in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). All data are presented as mean ± SEM (number of measurements). Diagrams were built with a graphical tool DataGraph 4.4 (http://www.visualdatatools.com/DataGraph/).

3 | RESULTS

The PO2 time courses showed a high degree of reproducibility between different tissue sites and animals (Figure 3). A 4–5 min rest between experimental recordings was sufficient to restore the baseline PO2, which, averaged over all experimental records, was 71 ± 1.4 mmHg (flash #1–10, Table 1). The onset of 1 Hz electrical stimulation after flash #11 caused a gradual decrease in interstitial PO2 until it reached an active steady-state of 43 ± 1.5 mmHg, in about 30 s. The average difference in PO2 between the baseline and active steady-state PO2’s (PO2 span) was 29 ± 1.2 mmHg (p < 0.001, Table 1).

Incremental PO2 data from the stroboscopic mode were used to reconstruct the 800 ms following the front of the electric stimulator pulse. This profile revealed that muscle contraction produced a substantial and rapid increase in interstitial PO2. The maximum value was reached at an average of 68.7 ± 3.3 ms, followed by a decline to a post-contractile steady-state. The total duration of the PO2 spike from the beginning of the stimulating pulse to the decrease of PO2 to the active steady-state level was 204.8 ± 6.3 ms. The amplitude of the PO2 peak was 57.6 ± 1.8 mmHg, 15.1 mmHg above the active steady-state PO2 level (#51-60, see Figure 1). That difference was significant (p < 0.001) and amounted to 58% of the PO2 span between baseline and active steady-state (Table 1, Figure 1).

An essential condition for this technique is to achieve stationarity, that is, the identity of all tracks of PO2 after each stimulating impulse. This condition was well satisfied for the spike, but a slow increase in PO2 was observed at the tail end. In many recordings, an active steady-state had a small positive trend (Figure 3), but on average, the difference between two stationary PO2 values, active and post-contractile, was 4.1 ± 0.4 mmHg and was not statistically significant.

FIGURE 2 Compilation of the PO2 profile following the 20 ms stimulation pulse. The dots represent the mean PO2 values for five records made in the same muscle site, and the vertical lines represent SE. The graph of 40 PO2 values from #60 through 100 is plotted against time (ms), corresponding to the fast interstitial PO2 spike following muscle stimulation. The duration of this spike is about 260 ms, and the total reconstruction is 800 ms.
DISCUSSION

The central finding of this experimental study is the spike of interstitial PO2 that follows muscle contraction. An explanation of this phenomenon’s mechanism is possibly found in the results of classical studies by G.V. Anrep (Anrep & Saalfeld, 1935). In this work, the inflow and outflow of blood from a contracting muscle were recorded using a fast-acting anemometer. During stimulated muscle contraction, interruption and “back thrust” of inflow occurred, while the venous outflow increased several times over a spike of 200 ms. Spike magnitude did not depend on muscle load but the intensity of the electric stimulus. This spike of venous blood flow during contraction was confirmed by more modern methods (Dobson & Gladden, 2003). Our PO2 measurements were made in the interstitial fluid exclusively surrounding capillary networks. If we assume that at the time of muscle contraction, a bolus of blood is squeezed from arterioles through the capillaries and into venules, then the resultant fast enrichment of the peri-capillary fluid with oxygen is responsible for the observed increase in PO2.

Blood flow is the primary indicator of the interstitial O2 supply and is often regulated by the arteriolar diameter. In exercising muscle, arterioles dilate to increase blood flow through the capillary beds, which are the major sites of gas exchange. The 50 second period of 1 Hz contractions preceding the switch to stroboscopic mode was designed to prevent changes in vascular resistance from confounding the 800 ms contraction profile, which is confirmed by reaching satisfactory equilibrium of the active steady-state. To our knowledge, there are no existing tracings of PO2 in response to contraction on the millisecond time scale. Thus, our finding is novel. Where our studies do overlap those of other laboratories is with the development of the active steady-state in response to rhythmic contractions, and our #1–60 profile is in agreement (Hirai et al., 2018; Poole et al., 2008; Poole & Ferreira, 2007).

One cannot conflate the circulatory phenomenon described by G.V. Anrep in a contracting muscle with the lately debated “muscle pump” theory (Folkow et al., 1970; Hamann et al., 2003). The mechanics of that hypothetical muscle pump are based on compression of the veins equipped with the anatomical venous valves. There are no valves in the microvasculature of the spinotrapezius muscle, and another explanation is needed for unidirectional blood flow during muscle compression. Possibly, the big difference between arterioles and venules in vascular resistance and blood pressure ensures the rectification of blood flow caused by a rapid increase of intramuscular pressure.

Following the muscle contraction-induced decrease in interstitial PO2 (29 mmHg), the increase in PO2 closes the gap between active steady-state and baseline by 58% for 200 ms,
which amounts to an absolute increase in oxygenation of less than 10%. The contribution is modest but noteworthy since it occurs at a moment of high oxygen demand for ATP regeneration. However, this contribution may be more significant in higher frequency and intensity rhythmic contractions. At five and more contractions per second, these 200 ms PO2 spikes may cumulatively provide a more substantial fraction of oxygen supply, working in concert with local oxygen capacitance in myoglobin, acting as a smoothing filter for pulsing PO2. Rhythmically contracting muscles, like myocardium, especially in small animals with high heart rates, may use this mechanism as a vital source of O2. Additionally, this effect may contribute to the oxygen supply in the tachycardic human heart.

The small positive trend in PO2 that appeared after 30 seconds of serialized contractions was probably due to the slow development of exercise hyperemia. This trend continued during the stroboscopic mode measurements, but it had a low magnitude, which was not significant and did not affect the detection of the PO2 spike.

5 | CONCLUSIONS

After the onset of stimulated, rhythmical contraction of the rat spinotrapezius muscle, the interstitial PO2, observed at a 1 Hz resolution, generally decreases to an active steady-state level. However, at higher temporal resolutions, it is observed that each muscle contraction is accompanied by a spike of interstitial PO2, which indicates the forced flush of arterial blood through a capillary network. The contribution of the additional oxygen delivered through that mechanism may become higher with increased frequency of contraction and intensity of stimulation. The impact of this phenomenon on oxygen supply in a heart muscle at high heart rates is of great interest.

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None.

DISCLOSURE

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.S.G. and B.K.S. conceived and designed the research; A.S.G. and B.K.S. performed the experiment. A.S.G., W.H.N., and B.K.S. analyzed the data; A.S.G., W.H.N., and B.K.S. interpreted the results of experiments; A.S.G. prepared the figures; A.S.G., W.H.N. drafted the manuscript; A.S.G., W.H.N., and B.K.S. edited and revised the manuscript; A.S.G., W.H.N., and B.K.S. approved the final version of the manuscript.

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