Oxygen regulation and limitation to cellular respiration in mouse skeletal muscle in vivo

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ABSTRACT

In skeletal muscle intracellular PO$_2$ can fall to as low as 2 – 3 mm Hg. This study tests whether oxygen regulates cellular respiration in this range of oxygen tensions through direct coupling between phosphorylation potential and intracellular PO$_2$. Oxygen may also behave as a simple substrate in cellular respiration that is near saturating levels over most of the physiological range. A novel optical spectroscopic method was used to measure tissue oxygen consumption ($\dot{M}$O$_2$) and intracellular PO$_2$ using the decline in hemoglobin and myoglobin saturation in ischemic hindlimb muscle of Swiss-Webster mice. $^{31}$P magnetic resonance spectroscopic determinations yielded [PCr] and pH in the same muscle volume. Intracellular PO$_2$ fell to <2 mm Hg during the ischemic period without change in the muscle [PCr] or pH. The constant phosphorylation state despite the decline in intracellular PO$_2$ rejects the hypothesis that direct coupling between these two variables results in a regulatory role for oxygen in cellular respiration. A second set of experiments tested the relationship between intracellular PO$_2$ and $\dot{M}$O$_2$. In vivo $\dot{M}$O$_2$ in mouse skeletal muscle was increased by systemic treatment with 2 and 4 mg/kg 2,4-dinitrophenol to partially uncouple mitochondria. $\dot{M}$O$_2$ was not dependent on intracellular PO$_2$ above 3 mm Hg in the three groups, despite a three-fold increase in $\dot{M}$O$_2$. These results indicate that $\dot{M}$O$_2$ and the phosphorylation state of the cell are independent of intracellular PO$_2$ throughout the physiological range of oxygen tensions. Therefore, we reject a regulatory role for oxygen in cellular respiration and conclude that oxygen acts as a simple substrate for respiration under physiological conditions.

Keywords: cellular respiration, oxygen limitation, oxygen regulation, critical PO$_2$
INTRODUCTION

Low oxygen tensions are characteristic of active muscle. During maximal aerobic exercise, intracellular PO₂ in skeletal muscle falls to as low as 2 – 3 mm Hg based on an average myoglobin saturation of about 50% (24, 27). The intracellular oxygen tension at the maximal rate of sustained exercise sets the lower end of the range of physiologically relevant intracellular PO₂ values. These values are just above the threshold where isolated mitochondria (12, 29), isolated cells (39, 40), and intact muscle (28) begin to become oxygen limited. Thus, the intracellular PO₂s reached during heavy muscle exercise may approach the threshold for limiting cellular respiration in vivo.

Low intracellular PO₂ may also affect the regulation of mitochondrial respiration. Wilson and colleagues have proposed that oxygen tension is coupled to the phosphorylation state of the cell ([ATP]/[ADP][Pi]) through oxidative phosphorylation (41). According to this hypothesis, a decline in intracellular PO₂ will reduce the rate of oxidative phosphorylation below that which is required to meet the ATP demand of the cell. This leads to an increase in the [ADP] and reduction in the phosphorylation potential. The increased [ADP] then stimulates oxidative phosphorylation to increase until it is sufficient to meet the ATP demand of the cell. Under this proposed mechanism, intracellular PO₂ contributes to the regulation of cellular respiration by increasing the [ADP] (a decrease in the phosphorylation potential) necessary to elicit a given rate of oxidative phosphorylation as oxygen tension decreases throughout the physiological range. Experiments in isolated mitochondria and cells have shown that respiration rate remains relatively constant over the physiological PO₂ range (12, 36), with a clear reduction occurring only at PO₂s below 2 – 3 mm Hg. However, the effect of intracellular PO₂ on the
phosphorylation state of the cell is not as well documented experimentally. Thus, it is not clear whether oxygen plays a regulatory role in modulating mitochondrial respiration or is simply a substrate for respiration that becomes limiting at low intracellular PO₂.

Combining optical and magnetic resonance spectroscopic (MRS) methods permits testing the role of oxygen in mitochondrial respiration in vivo. Near infrared optical spectroscopy (NIRS) has been used to estimate local muscle oxygen consumption (Ṁ O₂) in vivo in the same tissue volume as magnetic resonance measurements of phosphometabolite concentrations (8, 30). In these studies, muscle Ṁ O₂ is determined by occluding blood flow and monitoring the rate of decline of the oxygen-dependent heme signals (hemoglobin plus myoglobin) as muscle metabolism consumes oxygen. A new advance in in vivo optical spectroscopy is the ability to separately measure myoglobin and hemoglobin saturation in heart and skeletal muscle (4, 33). This separation permits the quantitative measure of muscle Ṁ O₂ in parallel with intracellular PO₂ based on myoglobin saturation in intact tissue. Thus, it is possible to determine intracellular PO₂ over the range of Mb saturations in muscle, simultaneous with measurement of Ṁ O₂ in the same volume of tissue. In combination with magnetic resonance spectroscopy of phosphometabolite levels (PCr, ATP, etc.), this new optical approach permits determining whether oxygen limits and/or regulates mitochondrial respiration in vivo.

The goal of this study is to evaluate the regulation and limitation roles of oxygen in respiration by combining ³¹P MRS and optical spectroscopy in mouse hindlimb muscle. MRS provides a measure of [PCr] and pH, which are the principal determinants of phosphorylation state. Optical spectroscopy is used to determine the intracellular PO₂ and oxygen consumption rate after oxygen delivery to the muscle is blocked with ischemia. We use a mitochondrial
uncoupler, 2, 4-dinitrophenol (DNP), to achieve a range of oxygen consumptions independently of muscle contraction and test for an effect of respiration rate on the relationship between mitochondrial respiration and PO2. The end result is the in vivo measurement of the key factors setting the oxygenation and phosphorylation states that permits testing the role of oxygen in mitochondrial respiration.

**METHODS**

*Animal preparation*

All experiments were approved by the Animal Care and Use Committee of the University of Washington. Female Swiss-Webster mice (31.8 ± 2.3g) were anaesthetised with an intraperitoneal injection of Avertin (0.55mg/g body wt) in saline. Supplemental anesthetic was given subcutaneously throughout the experiment. For optical experiments, the hair was removed from the right hindlimb using a commercial hair removal cream (Neet®) and a 3.2-mm wide cord was wrapped around the upper leg for use in inducing ischemia. The leg was secured between fiber optic bundles by fixing the ankle and foot in place with laboratory tape such that the light passed through the skin on either side of the leg from anterior to posterior just distal to the knee. The fiber optic bundles lightly contacted the skin on both the anterior and posterior side of the leg to avoid occlusion of blood flow in the resting condition. For the MRS experiments, the leg was secured so that the portion of the hindlimb just distal to the knee was sampled by a 3-turn solenoidal coil with an 8-mm internal diameter. The entire volume of this region of the musculature of the lower limb was sampled during both the optical and MRS experiments. The animal was warmed to maintain the temperature of the leg at 37 ± 0.5°C.
Optical spectroscopy

Optical transmission spectra were acquired using a 7-mm optical fiber bundle (Edmund scientific #K42-347) to carry illuminating light and a 2-mm fiber bundle for transmitted light. The fiber bundles were mounted with a fixed separation distance of 6 mm in a custom-made probe stand. Illumination from a constant intensity quartz-tungsten-halogen white light source (Oriel Instruments model #66184) was passed through a 1.0-inch water filter and electromechanical shutter (Oriel Instruments #76995) prior to transmission to the optical probe in order to decrease tissue heating. Constant light intensity was insured by a photo-feedback system (Oriel Instruments #68850). Spectra from 450 to 950 nm were acquired via a diffraction spectrograph (American Holographics #100S) with a 512-pixel photodiode array (Hamamatsu #C4350), using a 200-ms exposure time. Spectral acquisition was gated to acquire data at 1-s intervals and the data were converted into digital form using a 16 bit analog to digital converter (National Instruments #AT-MIO-16X).

$^{31}$P MRS

The magnetic resonance experiments were performed in a 4.7 tesla Bruker horizontal bore magnet. $B_0$ field homogeneity was optimized by off-resonance shimming of the proton peak from tissue water. Unfiltered PCR line widths were 20-30 Hz. Before the start of the experiment, a high signal-to-noise $^{31}$P MRS spectrum was taken under fully-relaxing conditions (128 acquisitions with a 16-s interpulse delay) at a spectral width of 3500 Hz consisting of 1024 data points. During the experimental procedure, free induction decays (FIDs) were acquired with a standard one-pulse sequence and a 1.2-s interpulse delay. The spectrum for each time
point consisted of 16 summed FIDs, increasing the signal to noise over individual FIDs and yielding 26-s time resolution. The free induction decays were fourier transformed, baseline corrected, line broadened with a 10 Hz exponential filter, and zero-filled. Fully relaxed peak areas were calculated by integration of the processed spectra using the Omega software on a General Electric console. Metabolite concentrations were determined from the partially saturated summed spectra collected throughout the experiment. The peak size was quantified using the Fit to Standard algorithm (18) and multiplied by the metabolite/gamma-ATP ratios from the fully relaxed spectra collected before the experiment. Metabolite concentrations were then absolutely quantified by measuring ATP concentrations with HPLC from extracts of frozen hindlimbs (42). pH was determined from the chemical shift of Pi relative to PCr in each spectrum (37).

Metabolite concentrations were used to determine the phosphorylation state of the muscle during ischemia. The [PCr] and pH are the main determinants of the phosphorylation state. These factors are representative of a change in [ATP]/([ADP][Pi]) because they are linked to both ADP and Pi levels. The [PCr] and pH changes determine [ADP] via the creatine kinase reaction:

$$\text{PCr + ADP + H}^+ \leftrightarrow \text{ATP + Cr.}$$  \hspace{1cm} (eq. 5)

[PCr] is also linked to [Pi], with the breakdown in [PCr] resulting in a stoichiometric increase in [Pi]. Thus, with both total creatine (Cr) and ATP concentration constant in muscle, any change in [ATP]/([ADP][Pi]) during ischemia will be reflected by changes in [PCr] and pH measured by $^{31}$P MRS.
Experimental design

In the first set of experiments, optical and MRS studies were performed on subsequent days on the same animals. During the optical experiments, the mice breathed 100% oxygen to maintain high Hb and Mb saturation in the resting state. Resting spectra were collected for 5 minutes followed by 5 minutes of ischemia and a 10-minute recovery period. This cycle was immediately repeated in each animal. There were no significant differences in the rate of oxygen consumption during the first and second ischemic periods (two-tailed t-test, p > 0.05). The MRS experiments were carried out as follows: off-resonance shimming followed by the acquisition of resting spectra for 6.5 minutes before inducing ischemia. Ischemia was maintained for 6.5 minutes followed by a 13-minute recovery period. After the last experiment the legs were removed under anesthesia and frozen between aluminum blocks in liquid nitrogen. The animals were then sacrificed with an overdose of anesthetic. These frozen tissues were used for the in vitro analysis of Hb, Mb, and ATP concentrations.

For the second set of experiments, only optical studies were performed. The control portions of the optical experiments were conducted as described above. After the first recovery period the animal was given an intraperitoneal injection of 2 (n=5) or 4 (n=6) mg DNP/kg body wt. Twenty minutes were allowed for the effect of DNP on oxygen consumption to stabilize before initiating the second 5-minute ischemic period. After the second ischemia, the leg was allowed to recover for 10 minutes before it was removed and frozen between aluminum blocks in liquid nitrogen. The animal continued to breathe 100% oxygen and the temperature of the leg was maintained at 37 ± 0.5 ºC while the leg was removed. After freezing the legs, the animals were sacrificed with an overdose of anesthetic.
Partial least-squares analysis (PLS) was used to extract the Hb and Mb saturations from the optical spectra of the mouse leg. PLS is an extension of linear regression that is useful for extracting information on specific spectral components of complex spectra (16). In this study, the PLS algorithm was applied to each spectrum twice, once to determine Mb saturation and once for Hb saturation. The analysis generates weighting factors for each wavelength that correspond to a known value for Hb or Mb saturation in a calibration set. The wavelengths used in this analysis were 560 – 850 nm. The weighting factors are then applied to each experimental spectrum to determine the unknown saturation of Hb or Mb. Before applying the PLS algorithm, spectra were pre-processed by taking the second derivative with respect to wavelength to remove the effect of baseline offsets. For a more complete description of the PLS analysis see refs. (3, 16, 33).

The calibration spectra used for PLS analysis must contain the same information as spectra acquired in the living tissue of interest. In this case, the absorbers Hb, Mb and cyt c were included in the calibration. Other absorbers, such as the other cytochromes, are present in lower concentrations in the tissue and can therefore be omitted from the calibration set (9). In addition to the absorbers, the calibration set must also include a scatterer to mimic the scattering properties of living tissue. The scattering media for this analysis was 20% Intralipid® (Baxter) diluted to one of four concentrations (1, 2, 3, or 4%). Another requirement of the calibration set is that the oxygenation state (or redox state in the case of cyt c) of the absorbers must vary independently of one another. This was accomplished by collecting spectra from oxy and deoxy Hb and Mb and reduced and oxidized cyt c in each concentration of scatterer. These spectra
within each scattering level were then mathematically added in different proportions to generate the full range of saturations or redox states for each absorber. Hereafter, these mathematically generated complex spectra will be referred to as composite spectra.

Concentrations of the absorbers were chosen to approximate the tissue concentrations. The calibration set for analysis of Mb saturation contained 30 µM Mb, 10 µM cyt c, and 20, 100, and 200 µM Hb. For the analysis of myoglobin saturation, multiple concentrations of Hb were used to bracket the range of concentrations expected in vivo from rest through hyperemic recovery. For the analysis of Hb saturation, only 100 µM Hb was used in the calibration set, because the PLS algorithm for determining saturation requires that the concentration of the absorber of interest does not vary. Therefore the analysis of Hb saturation is only valid for the resting and ischemic conditions, where Hb content of the tissue is relatively constant and approximately 100 µM. An adequate match between the combined calibration set and the experimental spectra from the hindlimb was insured by comparing the residuals from the calibration and experimental spectra after fitting with the PLS weighting factors (residual ratio test) (1).

Myoglobin from horse skeletal muscle and cytochrome c (cyt. c) purchased from Sigma were used for the calibration spectra. Hemoglobin was prepared from mouse blood by lysing red blood cells with deionized H$_2$O and centrifuging to separate the soluble Hb from cellular debris. Mb and Hb were completely reduced by the addition of excess sodium dithionite and passed over a Sephadex G-25 size exclusion column at pH 7.0. OxyMb and oxyHb were prepared by bubbling the solutions with 100% oxygen. Oxidized cyt. c was prepared by adding excess potassium ferricyanide. Deoxygenated and reduced forms of the proteins were prepared by
adding excess sodium dithionite to each solution. Fifty spectra were collected from each oxy and deoxy (or oxidized and reduced for cyt c) solution in a 6-mm pathlength cuvette.

Two calibration sets were used to test the sensitivity and predictability of the PLS method for determining Hb and Mb saturation (31). One calibration set was used to determine the PLS parameters for Mb and Hb saturation. These parameters were then used to predict the saturation of Mb and Hb from the second calibration set. The predicted vs. known saturations are plotted in Fig. 1, A and B.

Converting from Relative to Absolute Saturations

In order to convert relative saturation values from the PLS analysis of the in vivo experimental spectra to absolute saturation, two points of known saturation were necessary for both Mb and Hb. Figure 2 shows a typical pattern of Mb saturation over the course of an ischemic experiment cycle from which the resting Mb saturation level was established. The nadir in desaturation during ischemia was assumed to be 0% Mb saturation, while the peak saturation following release of ischemia represents 100% Mb saturation. This resulted in a Mb saturation of 86% in resting muscle. Hb was assumed to be 100% saturated at rest in muscle (Fig. 3). In order to ensure maximal saturation of the blood, the experiments were done while the animals were breathing 100% oxygen. Both Mb and Hb were assumed to be completely desaturated at the end of the 5-min ischemic period. This assumption is supported by the finding that both Hb and Mb saturation approached an asymptote toward the end of ischemia.
Calculation of Tissue Oxygen Content and Intracellular PO$_2$s

The rate of oxygen consumption was calculated from the change in Mb and Hb saturation over time throughout ischemia according to:

\[ \text{TotalO}_2 = \text{Hbsat} \times [\text{Hb}] + \text{dissO}_2\text{vasc} + \text{Mbsat} \times [\text{Mb}] + \text{dissO}_2\text{cell} \quad (\text{eq. 1}) \]

\[ \dot{M} \text{O}_2 = \Delta \text{Total O}_2/\Delta t \quad (\text{eq. 2}) \]

where \( \dot{M} \text{O}_2 \) is the oxygen consumption rate in \( \mu\text{mol}(\text{g s})^{-1} \) and is equal to the slope of a plot of total \( \text{O}_2 \) vs. time, [Hb] and [Mb] are expressed in \( \mu\text{mol g}^{-1} \) and dissO$_2$vasc and dissO$_2$cell are the amount of oxygen dissolved in the vascular and intracellular compartments, respectively.

Dissolved oxygen was calculated by first determining the vascular and intracellular PO$_2$s from the Hb and Mb saturations using the following equations.

\[ \text{intracellular PO}_2 = (\text{Mbsat} \times P_{50})/(1 - \text{Mbsat}) \quad (\text{eq. 3}) \]

\[ \text{intravascular PO}_2 = ((\text{Hbsat} \times P_{50}^n)/(1 - \text{Hbsat}))^{1/n} \quad (\text{eq. 4}) \]

where \( P_{50} \) for Mb and Hb are 2.39 (32) and 44 (34), respectively, and \( n \) is the Hill coefficient for Hb and equals 2.9 (2). A solubility coefficient of 0.0014 \( \mu\text{mol O}_2(\text{ml mm Hg})^{-1} \) was used to calculate the dissolved oxygen from the PO$_2$ determinations (22).
Measurement of Hemoglobin and Myoglobin Concentrations

The frozen muscles of the hindlimb distal to the knee were separated from skin and bone on an iced aluminum block and refrozen. Extracts for SDS-PAGE were made by pulverizing the muscle at liquid N\textsubscript{2} temperatures. Approximately 20 mg of pulverized muscle tissue was added to 250 µl of frozen SDS sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 5% β-mercaptoethanol). The frozen tissue was pulverized an additional 3 times for 20 seconds against the frozen buffer, then allowed to thaw, boiled for 6 minutes, and centrifuged at 9500g for 10 minutes. The supernatant was stored at -80°C until used. Hb and Mb were quantified by first separating on an 18% tris/glycine Biorad Criterion gel. The gels were stained with Coomasie Blue, imaged, and quantified using NIH image. Horse Mb and mouse Hb were used as standards and run on each gel. Quantification was repeated 3 times for each sample and the mean Hb and Mb concentrations were used for the calculation of the oxygen consumption rates. Hb and Mb concentrations were measured for each animal. Since the mice for both sets of experiments were females of approximately the same size, we assumed that the resting Hb concentration would be the same for both groups. Therefore, the mean Hb concentration from the 5 animals used in the first set of experiments (MRS/optical experiments) was used for the calculation of oxygen consumption rates for the control ischemic periods in the second set of experiments.

Determination of $\dot{M} O_2$

The decline in total oxygen content vs. time and intracellular PO\textsubscript{2} vs. time from each experiment were fit with fine-scale lowess curves using GraphPad Prism v.3.0a software for the Macintosh (GraphPad Software, Inc.). The lowess curve is model independent and fits the trend
in the data. An example of the lowess fit to the O2 content and intracellular PO2 are presented in Fig. 4. Oxygen consumption was determined from the least-squares slope of the decline in total oxygen content vs. time using five pairs of x-y coordinates generated by the lowess fits. This procedure was performed for the time points corresponding to the intracellular PO2 values from 10 to 1 mm Hg in steps of one mm Hg and also at a PO2 of 0.5 mm Hg for each experiment. The plots of \( \dot{M} O_2 \) vs. PO2 for each experiment were then divided into two segments – one representing the range from 10 – 3 mm Hg and the other from 3 – 0.5 mm Hg. The dependence of \( \dot{M} O_2 \) on intracellular PO2 from each experiment was determined over both ranges of intracellular PO2.

**Statistical Analyses**

All statistical analyses were done using GraphPad Prism v. 3.0a software for the Macintosh (GraphPad Software, Inc.). Repeated measures ANOVAs were used to test for changes in [PCr] and pH with time of ischemia. The dependence of \( \dot{M} O_2 \) on PO2 as a function of both oxygen tension and treatment was tested with a two-way ANOVA. One-sample t-tests were used to determine if the mean slopes of \( \dot{M} O_2 \) vs. PO2 between 10 – 3 mm Hg were significantly different from zero. The significance level used for all tests was P<0.05.

**RESULTS**

Our experiments consisted of two stages. The first stage involved optical calibrations that permitted measurement of muscle oxygen consumption and intracellular PO2 in vivo. The
second stage used these measurements to test for the regulatory and limiting role of oxygen in respiration.

**PLS analysis**

The partial least-squares algorithm predicts the saturation of Hb and Mb from the composite spectra by comparing the known and predicted saturations of a set of calibration spectra containing Hb, Mb, and cyt. c. Fig. 1 A and B demonstrate the high correlation between the known saturations of Hb and Mb in the composite spectra and those predicted by the PLS analysis. The slopes in each plot have residuals of 0.046 and 0.074 for Hb and Mb, respectively. These standard deviations indicate prediction errors for Hb and Mb saturations from complex spectra of 4.6% and 7.4%, respectively.

A typical pattern of Mb saturation over the course of an ischemic experiment cycle is presented in Fig. 2. This figure shows that Mb saturation is stable during the resting period prior to ischemia while the animals were breathing 100% oxygen. At the onset of ischemia (bold horizontal line), Mb saturation rapidly falls as resting oxygen consumption continues consuming the oxygen bound to Hb and Mb in the muscle. After 30 – 50 s. muscle oxygen stores have been reduced to low levels, indicated by the low saturations of Mb. The rate of decline of Mb saturation slows due to the inhibition of mitochondrial respiration by oxygen limitation as muscle oxygen stores are diminished. Restoration of blood flow results in the recovery of Mb saturation. We use these saturation values to scale the Mb levels: the peak during hyperemia is set to 100% Mb saturation and the lowest level at the end of ischemia reflective of intracellular anoxia is set to 0% Mb saturation. This procedure for scaling Mb saturation resulted in a resting Mb saturation of 86%.
A plot of Hb and Mb desaturation with time is shown in Fig. 3. Time zero in the plots indicates the onset of ischemia. The small dip in Hb saturation prior to ischemia is due to a motion artifact associated with the onset of ischemia. This figure demonstrates that both Hb and Mb begin to deoxygenate within seconds of the onset of ischemia and are near 10% saturation after 60 seconds of ischemia.

Intracellular PO$_2$, [PCr] and pH during ischemia

In the first set of experiments, we test the hypothesis that oxygen plays a regulatory role in mitochondrial respiration through coupling to the phosphorylation state. According to this hypothesis, the phosphorylation state, [ATP]/([ADP][Pi]), should change as intracellular PO$_2$ declines over the physiological PO$_2$ range with ischemia. To test this hypothesis, we used MRS to measure the two key determinants of phosphorylation state: [PCr] and pH.

Figure 5, A and B shows the [PCr] and pH (solid vertical bars) in relation to the intracellular PO$_2$ (open circles) at rest and for the first two data points collected after the onset of ischemia. Each post-ischemic data point represents a 26-s period. Neither the [PCr] (Fig. 5A) nor the pH (Fig. 5B) changed significantly from the resting levels during this period of ischemia, despite the fall in intracellular PO$_2$ (P=0.63 for PCr, P=0.54 for pH by repeated measure ANOVAs). The concentrations of ATP and Pi after 39 seconds of ischemia were also not significantly different from the resting values (8.23 ± 0.30 vs. 7.76 ± 0.62, P=0.37 for ATP; 3.83 ± 0.40 vs. 3.88 ± 0.67, P=0.95 for P$_i$). Constant values for [PCr], pH, [ATP] and [P$_i$] indicate that there was no change in the [ATP]/([ADP][P$_i$]) in the skeletal muscle of the mouse hindlimb. No change in the calculated phosphorylation potential was confirmed by a repeated measures ANOVA (P=0.35) (data not shown).
**$\dot{M} O_2$ and Intracellular $PO_2$**

In the second set of experiments, we test the extent to which oxygen limits cellular respiration. Mice were treated with two concentrations of DNP (2 and 4 mg DNP/[kg body mass]) to raise the rate of oxygen consumption in the skeletal muscle. Baseline oxygen consumption rates were determined for each group between intracellular $PO_2$ values of 10 and 3 mmHg (6.86 ± 0.14, 13.35 ± 0.21, and 21.77 ± 0.44 nmol O$_2$(g s)$^{-1}$ for control, DNP2, and DNP4 groups, respectively). DNP treatment significantly increased the baseline rate of oxygen consumption in a dose dependent manner (ANOVA, $P<0.001$). Plots of the average $\dot{M} O_2$s at each $PO_2$ in Fig. 6 A-C demonstrate a biphasic response of cellular respiration to intracellular oxygen tensions. Above 3 mm Hg there is little effect of decreasing oxygen tension on respiration rate, while below this value respiration rate decreases with decreasing intracellular PO$_2$. This relationship is independent of the baseline rate of oxygen consumption as demonstrated in the plots of $\dot{M} O_2$ normalized to the value at 10 mm Hg vs. $PO_2$ in Fig. 7. Table 1 compares the percent decrease in $\dot{M} O_2$ as a function of $PO_2$ calculated from each experiment over the two ranges of oxygen tension. There is a small but insignificant decrease in oxygen consumption with decreasing $PO_2$ between 10 – 3 mm Hg for each group (two-tailed, one sample $t$-tests, $P=0.25$, $P=0.54$, and $P=0.61$, for control DNP2, and DNP4, respectively). Consistent with the results presented in Fig. 7, there are no differences among treatments in the dependence of $\dot{M} O_2$ on $PO_2$ within each oxygen tension range (two-way ANOVA, $P=0.83$ for treatment and $P<0.001$ for $PO_2$ range).
DISCUSSION

We used a combination of optical and MR spectroscopy to determine the role of oxygen in regulating and/or limiting cellular respiration in intact skeletal muscle. The novel innovation that allows us to measure the oxygen sensitivity of respiration in vivo is the independent determination of Mb and Hb saturation from optical spectra. This is accomplished using a standard analytical method from chemometrics – partial least-squares analysis – which is used to extract quantitative Hb and Mb saturation from complex spectra (16). The success of PLS analysis in extracting Mb saturation data from spectra has been demonstrated in cardiac tissue ex vivo (31) and in vivo (33). This separation of Hb and Mb saturations expands on previous near-infrared spectroscopy studies that have used the sum of these saturations to estimate tissue oxygen consumption. The benefit of separation is that a more accurate measurement of oxygen consumption is possible using the saturation changes of known concentrations of Hb and Mb. The oxygen consumption derived from these measurements agrees with that estimated on the same muscle volume from ATP use by $^{31}$P MR (using the ATP/O$_2$ to convert values) (23). A second benefit of this separation is that a parallel measure of average intracellular PO$_2$ accompanies the measurement of tissue oxygen consumption. These parallel measurements make it possible to examine the relationship between oxygen consumption, phosphometabolites (e.g. PCr) and intracellular PO$_2$ in vivo.

$O_2$ regulation

A prediction of the oxygen regulation model is that the phosphorylation state ($[ATP]/([ADP][P_i])$) should decline with decreasing intracellular PO$_2$ as the cell adapts to the
lower oxygen tensions (41). Figure 5 shows no evidence for a change during ischemia in the two variables that determine the phosphorylation state, [PCr] and pH. Thus, the drop in intracellular PO$_2$ from 15 mm Hg to near anoxia occurs without change in the key factors determining phosphorylation state. This result is consistent with other in vivo studies that demonstrate no change in [PCr] concentration in human muscle until several minutes after the onset of ischemia, when the muscle is nearly anoxic (5, 30, 38). An interaction between inspired oxygen level and [PCr] has been reported for exercising human muscle during breathing of hypoxic air (17, 19). However, these studies did not measure intracellular PO$_2$. In the absence of a measure of intracellular oxygen tension, it is possible that breathing hypoxic air caused the intracellular PO$_2$ to fall low enough to limit cellular respiration (below 2 – 3 mm Hg) resulting in the consumption of PCr to maintain [ATP]. This would be consistent with the results presented here and support the simple oxygen limitation to respiration model. Our in vivo results from the mouse hindlimb indicate that until a threshold PO$_2$ is reached (2 – 3 mm Hg), there is no effect of oxygen tension on the phosphorylation state of the cell.

Earlier work with isolated cells reported a dependence of phosphorylation potential on intracellular oxygen tension at much higher PO$_2$ values than found in this study (40, 39). In the mouse hindlimb, the oxygen delivery system was intact and intracellular myoglobin was present to reduce intracellular oxygen gradients (43). In the experiments with isolated cells, oxygen tension was measured only in the extracellular medium and significant intracellular gradients likely existed between the intracellular and extracellular environments (29). The presence of larger oxygen gradients in isolated cell preparations would explain the dependence of phosphorylation potential at higher oxygen tensions than observed in this study.
**O₂ limitation**

A prediction of the model of oxygen as a simple substrate for respiration is that intracellular oxygen content will have little effect on respiration until a PO₂ is reached at which oxygen becomes limiting. We found that the cellular respiration is not limited by oxygen until intracellular PO₂ falls to low levels and Mb is near 50% saturation in vivo (Table 1). Values reported for limiting PO₂ and P₅₀s of isolated mitochondria support our conclusion that cellular respiration does not drop significantly until the PO₂ falls to very low levels. The P₅₀s for mitochondrial respiration from isolated mitochondria are generally within 0.5 – 1.0 mm Hg (7, 14, 29) of those for our in vivo measurements (1.04 – 1.42 mm Hg). This agreement is remarkable given that we are measuring a volume averaged Mb signal to determine intracellular PO₂ in vivo and indicates that intracellular PO₂ gradients in the presence of Mb are small (11, 29, 43). The presence of intracellular PO₂ gradients and the nonlinear oxygen equilibrium curve for Mb led Jürgens et al. (20) to question the validity of using a volume averaged Mb signal to determine the intracellular PO₂. However, the close agreement between the results from isolated mitochondria and those presented here indicates that the intracellular PO₂ gradients are small and supports the use of a volume averaged Mb signal in this study.

In mouse skeletal muscle in vivo, oxygen tension in the physiological range had no significant effect on cellular respiration over a three-fold range of baseline rates of oxygen consumption. This is the expected outcome if oxygen is acting as a simple substrate with no significant regulatory role under physiological conditions. A decrease in the P₅₀ for respiration has been reported between state 3 (ADP-saturated) and state 4 (ADP-free) conditions in isolated mitochondria (13) and with uncoupling in isolated cells and mitochondria (15, 29, 36). However, in neither case are the in vitro conditions and treatments reflective of our in vivo
experiment. First, mitochondria are not ADP-free in vivo. Therefore, the reduced mitochondrial oxygen affinity observed in vitro under state 4 (ADP-free) conditions are not relevant under physiological conditions. Second, the decrease in \( P_{50} \) reported with uncoupling in isolated mitochondria was attributed to the collapse of the protonmotive force in the mitochondria (15). The mice in this study were treated systemically with a dose of DNP that only partially uncouples mitochondria. Under these conditions the rate of oxygen consumption increases to meet the basal ATP demands of the cell and prevents the collapse of the protonmotive force. Therefore, our use of DNP to partially uncouple mitochondria would not be expected to influence the relationship between \( PO_2 \) and mitochondrial respiration. In support of this conclusion is a study using isolated endothelial cells, which found little change in \( P_{50} \) for cellular respiration with partial uncoupling (36). Thus, we found no evidence for a change in the relationship between respiration rate and intracellular \( PO_2 \) with a greater than 3-fold increase in the fully oxygenated respiration rate. This leads us to conclude that oxygen is not limiting to cellular oxygen consumption and therefore, does not play a significant role in regulating cellular respiration in vivo under these conditions.

Studies on exercising human muscle support the conclusion that oxygen tension in skeletal muscle does not fall to levels low enough to significantly inhibit cellular respiration, except under extreme physiological conditions (i.e. \( \dot{VO}_2_{\text{max}} \) in trained individuals). These studies indicate that Mb saturations at the aerobic capacity of human skeletal muscle are approximately 50% in vivo (2.4 mm Hg) (24, 27). Our results indicate that above this intracellular \( PO_2 \) there is little effect of oxygen tension on cellular respiration rate over the range tested in this study. Decreasing the capacity for oxygen delivery by breathing hypoxic air was found to drop Mb saturation below the 50% level and to reduce oxygen consumption during exercise (26). In
contrast, supplementing oxygen by breathing hyperoxic air during a maximum oxygen consumption test either did not effect or resulted in a small increase (~10%) in VO$_{2\text{max}}$ (6, 25).

Similarly, only small increases in VO$_{2\text{max}}$ were observed when the capacity for oxygen delivery was acutely increased above normal levels in endurance athletes and in high altitude natives (10, 26, 35). Together, these results indicate that at its aerobic capacity skeletal muscle may be working at the PO$_2$ threshold between oxygen-independent and oxygen-dependent respiration.

As intracellular oxygen tension decreases toward this threshold, Mb will release bound oxygen, thereby retarding the fall in intracellular PO$_2$. This buffering effect of Mb will be most effective around its P$_{50}$ for oxygen (2.4 mm Hg). The transition between oxygen-independent and oxygen-dependent respiration in vivo also occurs in this range of intracellular oxygen tensions (2 – 3 mm Hg). Therefore, an important role of Mb in skeletal muscle may be as an oxygen buffer to help maintain intracellular PO$_2$ above the point at which it becomes limiting to cellular respiration.

**Conclusion**

The findings of this study lead us to reject the hypothesis that oxygen plays a regulatory role in cellular respiration over physiological range of intracellular oxygen tensions. This conclusion is based on the absence of interaction between [PCr], pH (and therefore phosphorylation state), oxygen consumption and PO$_2$ above 3 mm Hg over a greater than 3-fold range in oxygen consumption rates. These results are consistent with the hypothesis that oxygen acts as a simple substrate for cellular respiration over the physiological range of oxygen tensions.
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FIGURE LEGENDS

Figure 1. Correlation between predicted and known Hb and Mb saturations. (A) Hb saturations of the in vitro test spectra predicted by the PLS analysis are highly correlated with the known saturations. (B) Mb predicted saturations are highly correlated with the known Mb saturations for the in vitro test spectra. The standard error for the residuals is 0.046 and 0.074 for the Hb and Mb plots, respectively.

Figure 2. Myoglobin saturation as a function of time during an ischemic bout. The period of ischemia begins at time zero and is indicated by the solid bar near the top of the plot. Mb saturation is set to 0% at the end of ischemia and to 100% at peak Mb saturation during hyperemia. This results in a resting saturation of 86%.

Figure 3. The decline in Hb (thick line) and Mb (thin line) saturations throughout an ischemic experiment. The onset of ischemia is time zero. Hb saturation was set to 100% saturation and Mb saturation is set to 86 % at the onset of ischemia as described in the text. The small dip in Hb saturation immediately prior to the onset of ischemia is a motion artifact caused by the mechanical induction of ischemia.

Figure 4. Model independent fits of the decline in total oxygen content and intracellular PO2 as a function of time during ischemia. The measured data is represented by the open symbols and the lowess fit to each data set by the solid lines. These data represent one ischemic period in one animal. Time zero is the onset of ischemia.
Figure 5. Intracellular PO$_2$, PCr, and pH during the ischemic protocol. Time zero represents the onset of ischemia in each plot. The bars represent the PCr (A) and pH (B) at rest (before time zero) and averaged over the first and second 26-s periods of ischemia. Neither [PCr] (A) or pH (B) fell significantly over this period of ischemia. Values represent the means (n=5) and error bars indicate standard error for PCr and the upper bound of the 95% confidence interval for the pH plot.

Figure 6. Mean oxygen consumption ($\dot{M}O_2$) plotted vs. intracellular PO$_2$ for control (A), DNP2 (B), and DNP4 (C) experiments. $\dot{M}O_2$ was calculated from the lowess fits as described in the methods for each intracellular PO$_2$ value in the plots.

Figure 7. Normalized plots of mean oxygen consumption vs. intracellular PO$_2$. $\dot{M}O_2$ was normalized to the value at 10 mm Hg for each experiment. The plot represents the mean normalized $\dot{M}O_2$ at each PO$_2$ for the different treatments. Error bars are omitted for clarity.
Table 1. *Percent decrease in $\dot{M} O_2$ as a function of intracellular $PO_2$ over two ranges of oxygen tension.*

<table>
<thead>
<tr>
<th>PO$_2$ range (mm Hg)</th>
<th>Control (%, n=10)</th>
<th>DNP2 (%, n=5)</th>
<th>DNP4 (%, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 - 3</td>
<td>1.5 ± 1.2</td>
<td>0.9 ± 1.4</td>
<td>0.7 ± 1.2</td>
</tr>
<tr>
<td>3 – 0.5</td>
<td>28.0 ± 3.7</td>
<td>25.8 ± 2.2</td>
<td>29.4 ± 2.1</td>
</tr>
</tbody>
</table>

The values represent the $d\dot{M} O_2/dPO_2$ for normalized $\dot{M} O_2$ data for each experiment. There was a significant effect of the range of intracellular PO$_2$, but no effect of treatment on the mean rate of change in respiration rate as a function of intracellular PO$_2$. The means of the slopes between 10 – 3 mm Hg for all three treatments were not significantly different from zero.
Figure 1

A

\[ y = 0.974x + 0.013 \]

\[ r^2 = 0.973 \]

Hb saturation (predicted) vs. Hb saturation (known)

B

\[ y = 0.934x + 0.028 \]

\[ r^2 = 0.937 \]

Mb saturation (predicted) vs. Mb saturation (known)
Figure 2
Figure 3

![Graph showing the effect of ischemia on Hb and Mb saturation over time.](image-url)
Figure 4.
Figure 5

A

Time (sec)

B

Time (sec)
Figure 6

A

B

C