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

David J. Marcinek, Wayne A. Ciesielski, Kevin E. Conley, and Kenneth A. Schenkman

Oxygen regulation and limitation to cellular respiration in mouse skeletal muscle in vivo. Am J Physiol Heart Circ Physiol 285: H1900–H1908, 2003. First published May 29, 2003; 10.1152/ajpheart.00192.2003.—In skeletal muscle, intracellular PO2 can fall to as low as 2–3 mmHg. This study tested whether oxygen regulates cellular respiration in this range of oxygen tensions through direct coupling between phosphorylation potential and intracellular PO2. 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 (M˙O2) and intracellular PO2 using the decline in hemoglobin and myoglobin saturation in the ischemic hindlimb muscle of Swiss-Webster mice. 31P magnetic resonance spectroscopic determinations yielded phosphocreatine concentration ([PCr]) and pH in the same muscle volume. Intracellular PO2 fell to <2 mmHg during the ischemic period without a change in the muscle [PCr] or pH. The constant phosphorylation state despite the decline in intracellular PO2 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 testing the relationship between intracellular PO2 and M˙O2 in vivo MO2 in mouse skeletal muscle was increased by systemic treatment with 2 and 4 mg/kg body wt 2,4-dinitrophenol to an average myoglobin (Mb) saturation of ∼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 PO2 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 (41) have proposed that oxygen tension is coupled to the phosphorylation state of the cell ([ATP]/[ADP][Pi]) through oxidative phosphorylation. 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 [ADP] and a 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 [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 the respiration rate remains relatively constant over the physiological PO2 range (12, 36), with a clear reduction occurring only at PO2 below 2–3 mmHg. 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 of the role of oxygen in mitochondrial respiration in vivo. Near-infrared optical spectroscopy has been used to estimate local muscle oxygen consumption (MO2) in vivo in the same tissue volume as magnetic resonance measure-
ments of phosphometabolite concentrations (8, 30). In these studies, muscle $\text{MO}_2$ is determined by occluding blood flow and monitoring the rate of decline of oxygen-dependent heme signals [hemoglobin (Hb) plus Mb] as muscle metabolism consumes oxygen. A new advance in vivo optical spectroscopy is the ability to separately measure Mb and Hb saturation in the heart and skeletal muscle (4, 33). This separation permits the quantitative measurement of muscle $\text{MO}_2$ in parallel with intracellular $\text{PO}_2$ based on Mb saturation in intact tissue. Thus it is possible to determine intracellular $\text{PO}_2$ over the range of Mb saturations in muscle simultaneously with measurement of $\text{MO}_2$ in the same volume of tissue. In combination with MRS of phosphometabolite levels [phosphocreatine (PCr), ATP, etc.], this new optical approach permits determination of whether oxygen limits and/or regulates mitochondrial respiration in vivo.

The goal of this study was to evaluate the regulation and limitation roles of oxygen in respiration by combining $^{31}\text{P}$ MRS and optical spectroscopy in mouse hindlimb muscle. MRS provided a measure of [PCr] and pH, which are the principal determinants of the phosphorylation state. Optical spectroscopy was used to determine the intracellular $\text{PO}_2$ and oxygen consumption rate after oxygen delivery to the muscle was blocked with ischemia. We used a mitochondrial uncoupler, 2,4-dinitrophenol (DNP), to achieve a range of blocked mitochondrial respiration states, which permits testing of the role of oxygen phosphorylation state. Optical spectroscopy was used and pH, which are the principal determinants of the hindlimb muscle. MRS provided a measure of [PCr] and pH, which are the principal determinants of the phosphorylation state. Optical spectroscopy was used to determine the intracellular $\text{PO}_2$ and oxygen consumption rate after oxygen delivery to the muscle was blocked with ischemia. We used a mitochondrial uncoupler, 2,4-dinitrophenol (DNP), to achieve a range of oxygen consumptions independently of muscle contraction and tested for an effect of respiration rate on the relationship between mitochondrial respiration and $\text{PO}_2$. The end result was the in vivo measurement of the key factors setting the oxygenation and phosphorylation states, which permits testing of 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 ± 2.3 g) were anesthetized with an intraperitoneal injection of Avertin (0.55 mg/g body wt) in saline. Supplemental anesthetic was given subcutaneously throughout the experiment. For optical experiments, the hair was removed from the right hindlimb given subcutaneously throughout the experiment. For optical experiments, the hair was removed from the right hindlimb with Avertin (0.55 mg/g body wt) in saline. Supplemental anesthetic was given subcutaneously throughout the experiment.

**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 sustain high Hb and Mb saturation in the resting state. Resting spectra were collected for 5 min, followed by 5 min of ischemia and a 10-min 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.

$\text{PO}_2$ over the range of Mb saturations in muscle simultaneously with measurement of $\text{MO}_2$ in the same volume of tissue. In combination with MRS of phosphometabolite levels [phosphocreatine (PCr), ATP, etc.], this new optical approach permits determination of whether oxygen limits and/or regulates mitochondrial respiration in vivo.

The goal of this study was to evaluate the regulation and limitation roles of oxygen in respiration by combining $^{31}\text{P}$ MRS and optical spectroscopy in mouse hindlimb muscle. MRS provided a measure of [PCr] and pH, which are the principal determinants of the phosphorylation state. Optical spectroscopy was used to determine the intracellular $\text{PO}_2$ and oxygen consumption rate after oxygen delivery to the muscle was blocked with ischemia. We used a mitochondrial uncoupler, 2,4-dinitrophenol (DNP), to achieve a range of oxygen consumptions independently of muscle contraction and tested for an effect of respiration rate on the relationship between mitochondrial respiration and $\text{PO}_2$. The end result was the in vivo measurement of the key factors setting the oxygenation and phosphorylation states, which permits testing of 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 ± 2.3 g) were anesthetized with an intraperitoneal injection of Avertin (0.55 mg/g body wt) in saline. Supplemental anesthetic was given subcutaneously throughout the experiment. For optical experiments, the hair was removed from the right hindlimb with Avertin (0.55 mg/g body wt) in saline. Supplemental anesthetic was given subcutaneously throughout the experiment.

**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 sustain high Hb and Mb saturation in the resting state. Resting spectra were collected for 5 min, followed by 5 min of ischemia and a 10-min 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.

$\text{PO}_2$ over the range of Mb saturations in muscle simultaneously with measurement of $\text{MO}_2$ in the same volume of tissue. In combination with MRS of phosphometabolite levels [phosphocreatine (PCr), ATP, etc.], this new optical approach permits determination of whether oxygen limits and/or regulates mitochondrial respiration in vivo.

The goal of this study was to evaluate the regulation and limitation roles of oxygen in respiration by combining $^{31}\text{P}$ MRS and optical spectroscopy in mouse hindlimb muscle. MRS provided a measure of [PCr] and pH, which are the principal determinants of the phosphorylation state. Optical spectroscopy was used to determine the intracellular $\text{PO}_2$ and oxygen consumption rate after oxygen delivery to the muscle was blocked with ischemia. We used a mitochondrial uncoupler, 2,4-dinitrophenol (DNP), to achieve a range of oxygen consumptions independently of muscle contraction and tested for an effect of respiration rate on the relationship between mitochondrial respiration and $\text{PO}_2$. The end result was the in vivo measurement of the key factors setting the oxygenation and phosphorylation states, which permits testing of the role of oxygen in mitochondrial respiration.
the case of cytochrome calibration set is that the oxygenation state (or redox state in analysis was 20% Intralipid (Baxter) diluted to one of four properties of living tissue. The scattering media for this calibration set must also include a scatterer to mimic the scattering calibrations in the tissue and can therefore be omitted from the such as other cytochromes, are present in lower concentrations. In this case, the absorbers Hb, Mb, and cytochrome 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 mg/kg body wt DNP (n = 6). Twenty minutes were allowed for the effect of DNP on oxygen consumption to stabilize before the second 5-min ischemic period was initiated. After the second ischemic period, the leg was allowed to recover for 10 min 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 °C while the leg was removed. After the legs were frozen, the animals were killed with an overdose of anesthetic.

Partial least-squares analysis and calibration sets. Partial least-squares (PLS) analysis was used to extract Hb and Mb saturations from 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 the present study, the PLS algorithm was applied to each spectrum twice: once to determine Mb saturation and once to determine 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 the PLS algorithm was applied, spectra were preprocessed 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, and 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 cytochrome c were included in the calibration. Other absorbers, such as 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 cytochrome 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 cytochrome 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 cytochrome c, and 20, 100, and 200 μM Mb. For the analysis of Hb 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 the Hb content of the tissue is relatively constant and ~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).

Mb from horse skeletal muscle and cytochrome c purchased from Sigma were used for the calibration spectra. Hb was prepared from mouse blood by lysing red blood cells with deionized H2O and centrifuging to separate the soluble Hb from cellular debris. Mb and Hb were completely reduced by the addition of excess sodium dithionite and passing 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 cytochrome 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 cytochrome 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 versus known saturations are shown in Fig. 1, A and B.

Converting from relative to absolute saturations. 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, whereas the peak saturation after the 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). To ensure maximal saturation of the blood, the experiments were done while the animals breathed 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 Mb and Hb saturation approached an asymptote toward the end of ischemia.

Calculation of tissue oxygen content and intracellular PO2. The rate of oxygen consumption was calculated from the change in Mb and Hb saturation over time throughout ischemia according to

\[
\text{Total O}_2 = \text{Hb}_{\text{sat}} \times [\text{Hb}] + \text{dissO}_2\text{vase} + \text{Mb}_{\text{sat}} \times [\text{Mb}] + \text{dissO}_2\text{cell}
\]

\[
\text{MO}_{2} = \Delta \text{Total O}_2/\Delta t
\]

where \(\text{MO}_{2}\) (in \(\text{μmol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}\)) is equal to the slope of a plot of total \(\text{O}_2\) versus time (t), [Hb] and [Mb] are expressed in \(\text{μmol/g}\), and dissO\(_2\)\(_{\text{vase}}\) and dissO\(_2\)\(_{\text{cell}}\) are the amount of oxygen dissolved in the vascular and intracellular compart-
Dissolved oxygen was calculated by first determining the vascular and intracellular $P_{O_2}$ from the Hb and Mb saturations using the following equations:

$$\text{Intracellular } P_{O_2} = \frac{(M_{\text{sat}} \times P_{50})}{(1 - M_{\text{sat}})}$$

$$\text{Intravascular } P_{O_2} = \frac{[(H_{\text{sat}} \times P_{50})]/(1 - H_{\text{sat}})]^{1/n_H}}$$

where $P_{50}$ for Mb and Hb are 2.39 (32) and 44 mmHg (34), respectively, and $n_H$ is the Hill coefficient for Hb and equals 2.9 (2). A solubility coefficient of $0.0014 \frac{\mu \text{mol } O_2}{\text{ml } 1\text{mmHg}}$ was used to calculate the dissolved oxygen from the $P_{O_2}$ determinations (22).

Measurement of Hb and Mb 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 N2 temperatures. Approximately 20 mg of pulverized muscle tissue were added to $250 \mu l$ of frozen SDS sample buffer ($62.5 \text{ mM Tris}, 2\% \text{ SDS}, 10\% \text{ glycerol}, 0.001\% \text{ bromophenol blue}, \text{ and } 5\% -\text{mercaptoethanol}$). The frozen tissue was pulverized an additional three times for 20 s against the frozen buffer, allowed to thaw, boiled for 6 min, and then centrifuged at 9,500 g for 10 min. The supernatant was stored at $-80^\circ \text{C}$ until use. Hb and Mb were quantified by separation on an 18% Tris-glycine Bio-Rad Criterion gel. The gels were stained with Coomassie blue, imaged, and quantified using NIH Image. Horse Mb and mouse Hb were used as standards and run on each gel. Quantification was repeated three 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. Because 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 five 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 versus time and intracellular $P_{O_2}$ versus time from each experiment were fit with fine-scale lowess curves using GraphPad Prism version 3.0a software for the Macintosh (GraphPad Software). The lowess curve is model independent and fits the trend in the data. Examples of the lowess fit to the $O_2$ content and intracellular $P_{O_2}$ are presented in Fig. 4. Oxygen consumption was determined from the least-squares slope of the decline in total oxygen content versus 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 $P_{O_2}$ values from 10 to 1 mmHg in steps of 1 mmHg and also at a $P_{O_2}$ of 0.5 mmHg for each experiment. The plots of $\dot{M}O_2$ versus $P_{O_2}$ for each experiment were then divided into two segments: one representing the range from 10 to 3 mmHg and the other representing the

---

Fig. 1. Correlation between predicted and known hemoglobin (Hb) and myoglobin (Mb) saturations. $A$: Hb saturations of the in vitro test spectra predicted by the partial least-squares (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 SDs for the residuals are 0.046 and 0.074 for the Hb and Mb plots, respectively.

Fig. 2. Mb saturation as a function of time during an ischemic bout. The period of ischemia begins at time 0 and is indicated by the solid bar (top). Mb saturation was set to 0% at the end of ischemia and to 100% at peak Mb saturation during hyperemia. This resulted in a resting saturation of 86%.

Fig. 3. Decline in Hb (thick solid line) and Mb (thin solid line) saturations throughout an ischemic experiment. The onset of ischemia is time 0. Hb saturation was set to 100% saturation, and Mb saturation was set to 86%, at the onset of ischemia, as described in the text. The small dip in Hb saturation immediately before the onset of ischemia is a motion artifact caused by the mechanical induction of ischemia.

---

AJP-Heart Circ Physiol • VOL 285 • NOVEMBER 2003 • www.ajpheart.org
range from 3 to 0.5 mmHg. The dependence of \( \dot{M}_O_2 \) on intracellular \( P_O_2 \) from each experiment was determined over both ranges of intracellular \( P_O_2 \).

**Statistical analyses.** All statistical analyses were done using GraphPad Prism version 3.0a software for the Macintosh (GraphPad Software). Repeated-measures ANOVA was used to test for changes in [PCr] and pH with time of ischemia. The dependence of \( M_O_2 \) on \( P_O_2 \) as a function of both range of oxygen tension and treatment was tested with a two-way ANOVA. One-sample \( t \)-tests were used to determine whether the mean slopes of \( \dot{M}_O_2 \) versus \( P_O_2 \) between 10 and 3 mmHg 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 \( M_O_2 \) and intracellular \( P_O_2 \) in vivo. The second stage used these measurements to test for the regulatory and limiting role of oxygen in respiration.

**PLS analysis.** The PLS 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 cytochrome c. Figure 1, A and B, demonstrates 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 SDs 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. Figure 2 shows that Mb saturation is stable during the resting period before ischemia, while the animals breathed 100% oxygen. At the onset of ischemia (horizontal line in Fig. 2), 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, as 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 used these saturation values to scale the Mb levels: the peak during hyperemia was set to 100% Mb saturation and the lowest level at the end of ischemia, reflective of intracellular anoxia, was 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 0* in the plots indicates the onset of ischemia. The small dip in Hb saturation before ischemia is due to a motion artifact associated with the onset of ischemia. Figure 3 demonstrates that both Hb and Mb begin to deoxygenate within seconds of the onset of ischemia and are near 10% saturation after 60 s of ischemia.

**Intracellular \( P_O_2 \), [PCr], and pH during ischemia.** In the first set of experiments, we tested 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][P_i]), should change as intracellular \( P_O_2 \) declines over the physiological \( P_O_2 \) range with ischemia. To test this hypothesis, we used MRS to measure the two key determinants of the phosphorylation state: [PCr] and pH.

Figure 5, A and B, shows [PCr] and pH (solid vertical bars) in relation to the intracellular \( P_O_2 \) (open circles).
at rest and for the first two data points collected after the onset of ischemia. Each postischemic data point represents a 26-s period. Neither [PCr] (Fig. 5A) nor pH (Fig. 5B) changed significantly from the resting levels during this period of ischemia despite the fall in intracellular \( \dot{\text{O}}_2 \) (\( P = 0.63 \) for PCr, \( P = 0.54 \) for pH by repeated-measures ANOVA). The concentrations of ATP and Pi after 39 s 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; 3.88 ± 0.40 vs. 3.88 ± 0.67, \( P = 0.95 \) for Pi). Constant values for [PCr], pH, [ATP], and [Pi] indicate that there was no change in [ATP]/[ADP][Pi] in the skeletal muscle of the mouse hindlimb. No change in the calculated phosphorylation potential was confirmed by repeated-measures ANOVA (\( P = 0.35 \)) (data not shown).

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

**DISCUSSION**

We used a combination of optical spectroscopy and MRS 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 was accomplished using a standard analytic method from chemometrics, PLS 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 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 with the use of \( ^{31} \text{P} \) MRS (using ATP/\( \dot{\text{O}}_2 \) to convert values) (23). A second benefit of this separation is that a parallel measure of average intracellular \( \dot{\text{O}}_2 \) accompanies the measurement of tissue oxygen con-
dependence of phosphorylation potential at higher oxygen tensions than observed in the present study.

O$_2$ 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 P$_{O2}$ is reached at which oxygen becomes limiting. We found that the cellular respiration is not limited by oxygen until intracellular P$_{O2}$ falls to low levels and Mb is near 50% saturation in vivo (Table 1). Values reported for limiting P$_{O2}$ and P$_{50}$ of isolated mitochondria support our conclusion that cellular respiration does not drop significantly until the P$_{O2}$ falls to very low levels. P$_{50}$ values for mitochondrial respiration from isolated mitochondria are generally within 0.5–1.0 mmHg (7, 14, 29) of those from our in vivo measurements (1.04–1.42 mmHg). This agreement is remarkable given that we measured a volume-averaged Mb signal to determine intracellular P$_{O2}$ in vivo and indicates that intracellular P$_{O2}$ gradients in the presence of Mb are small (11, 29, 43). The presence of intracellular P$_{O2}$ 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 P$_{O2}$. However, the close agreement between the results from isolated mitochondria and those presented here indicates that the intracellular P$_{O2}$ 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 threefold 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 P$_{50}$ 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 af-

**Table 1. Percent decrease in $\dot{M}$O$_2$ as a function of intracellular P$_{O2}$ over two ranges of oxygen tension**

<table>
<thead>
<tr>
<th>P$_{O2}$ Range, mmHg</th>
<th>Control</th>
<th>DNP 2 mg/kg body wt</th>
<th>DNP 4 mg/kg body wt</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>25.9 ± 3.7</td>
<td>25.8 ± 2.2</td>
<td>29.4 ± 2.1</td>
</tr>
</tbody>
</table>

Values (in %) are means ± SE and represent the change in tissue oxygen consumption ($\dot{M}$O$_2$) per change in P$_{O2}$ for normalized $\dot{M}$O$_2$ data for each experiment; n = 10 control mice, 5 mice treated with 2 mg/kg body wt 2,4-dinitrophenol (DNP), and 6 mice treated with 4 mg/kg body wt DNP. The values represent the d$\dot{M}$O$_2$/dP$_{O2}$ for normalized $\dot{M}$O$_2$ data for each experiment. There was a significant effect of the range of intracellular P$_{O2}$ but no effect of treatment on the mean rate of change in respiration rate as a function of intracellular P$_{O2}$. The means of the slopes between 10 and 3 mmHg for all three treatments were not significantly different from zero.
finity observed in vitro under state 4 (ADP free) conditions are not relevant under physiological conditions. Second, the decrease in P50 reported with uncoupling in isolated mitochondria was attributed to the collapse of the protons force in 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 protons force. Therefore, our use of DNP to partially uncouple mitochondria would not be expected to influence the relationship between PO2 and mitochondrial respiration. In support of this conclusion is a study (36) using isolated endothelial cells, which found little change in P50 for cellular respiration with partial uncoupling. Thus we found no evidence for a change in the relationship between respiration rate and intracellular PO2 with a greater than threefold 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., maximum oxygen consumption (VO2 max) in trained individuals]. These studies indicate that Mb saturations at the aerobic capacity of human skeletal muscle are ~50% in vivo (2.4 mmHg) (24, 27). Our results indicate that above this intracellular PO2, there is little effect of oxygen tension on the cellular respiration rate over the range tested in the present 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 of hyperoxic air during a maximum oxygen consumption test either did not effect or resulted in a small increase (~10%) in VO2 max (6, 25). Similarly, only small increases in VO2 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 PO2 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 PO2. This buffering effect of Mb will be most effective around its PO2 for oxygen (2.4 mmHg). The transition between oxygen-independent and oxygen-dependent respiration in vivo also occurs in this range of intracellular oxygen tensions (2–3 mmHg). Therefore, an important role of Mb in skeletal muscle may be as an oxygen buffer to help maintain intracellular PO2 above the point at which it becomes limiting to cellular respiration.

In conclusion, the findings of the present study lead us to reject the hypothesis that oxygen plays a regulatory role in cellular respiration over the 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 PO2 above 3 mmHg over a greater than threefold 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.

The authors thank Rudolph Stippard, Eric Shankland, and Rod Gronka for technical assistance. We thank Dr. Eric O. Feigl for critical reading of the manuscript and Martin J. Kushmerick for input throughout this work.

DISCLOSURES

This work was supported by National Institutes of Health Grants AR-45184, AR-36281, and AG-00057.

REFERENCES

14. Gnaiger E, Méndez G, and Hand SC. High phosphorylation efficiency and depression of uncoupled respiration in mitochondri-


