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Intramyocellular Oxygenation During Ischemic Muscle Contractions In Vivo

Michael A. Tevald, P.T., Ph.D.¹, Ian R. Lanza, Ph.D.¹, Douglas E. Befroy, D.Phil², and Jane A. Kent-Braun, Ph.D.¹

¹Kinesiology Department, University of Massachusetts, Amherst MA, USA

²Departments of Radiology and Internal Medicine, Yale University School of Medicine, New Haven CT, USA

Abstract

There is some evidence that the fall in intramyocellular oxygen content during ischemic contractions is less than during ischemia alone. We used proton magnetic resonance spectroscopy to determine whether peak deoxy-myoglobin (dMb) obtained during ischemic ankle dorsiflexion contractions attained the maximal dMb level observed during a separate trial of ischemia alone (resting max). In 6 healthy young men, the rate of myoglobin desaturation was rapid at the onset of ischemic contractions and then slowed as contractions continued, attaining only $75 \pm 3.3\%$ (mean $\pm$ SE) of resting max dMb by the end of contractions ($p=0.03$). Myoglobin continued to desaturate while ischemia was maintained following contractions, reaching $98 \pm 1.8\%$ of resting max within 10 minutes ($p = 0.03$ vs. end of contractions). Notably, contractions performed after 10 min of ischemia did not affect dMb (dMb = $100 \pm 1.5\%$ of resting max; $p > 0.99$), suggesting that full desaturation had already been achieved. The blunting of desaturation during ischemic contractions is likely a result of slowed mitochondrial oxygen consumption due to limited oxygen availability.

Keywords

deoxymyoglobin; ischemia; critical PO_2 ; mitochondria; bioenergetics

INTRODUCTION

An adequate supply of oxygen is critical to maintaining energy homeostasis in skeletal muscle. Studies in isolated mitochondria (Wilson et al., 1977; Wilson et al., 1979), cells (Rumsey et al., 1990), and whole skeletal muscle in situ (Hogan et al., 1992b; Hogan et al., 1992a) have shown that, over a range of oxygen tensions (PO_2), the rate of oxygen consumption can be maintained as PO_2 falls through changes in mitochondrial redox and/or phosphorylation state. If the PO_2 in the cell falls below “critical PO_2 ”, respiration will become limited, thereby slowing the rate of mitochondrial ATP production (Connett et al., 1990). The cell must then either decrease energy demand or increase ATP production by non-oxidative processes (e.g., anaerobic glycolysis or net breakdown of phosphocreatine) in order to avoid depleting cellular ATP.

Due to the crucial role of oxygen in oxidative energy metabolism, much effort has been devoted to the development of techniques to measure intracellular oxygenation in working muscle.

Address correspondence to: J.A. Kent-Braun, Ph.D. Kinesiology Department, Totman 108 University of Massachusetts Amherst MA 01003 USA 413.545.9477; 413.545.2906 (fax) E-mail: janekb@kin.umass.edu.

Proton magnetic resonance spectroscopy (^1H -MRS) has been used since 1990 (Wang et al., 1990) to observe the deoxygenated form of myoglobin (dMb) via the N- δ proton of F8 proximal histidine. Myoglobin (Mb) is an oxygen-binding protein found exclusively in muscle, and the appearance of dMb reflects intramyocellular deoxygenation. The area of the dMb peak in a ^1H spectrum can be used to estimate intramyocellular PO_2 from the oxymyoglobin dissociation curve (Richardson et al., 1995). The complex role that oxygen plays in human skeletal muscle energetics during contractions has been studied in healthy (Brillault-Salvat et al., 1997; Duteil et al., 2004; Mole et al., 1999; Richardson et al., 1995; Richardson et al., 1999; Richardson et al., 2001) and clinical populations (Kreis et al., 2001; Mancini et al., 1994) using this noninvasive technique.

During tourniquet-induced ischemia, Mb gradually desaturates in the cell at an approximately linear rate before reaching a plateau. This plateau of dMb is thought to reflect full desaturation of Mb (Wang et al., 1990), and is typically used to calibrate the dMb signal (Mole et al., 1999; Richardson et al., 1995; Wang et al., 1990). A similar calibration approach is used in studies of muscle oxygenation using near-infrared spectroscopy (Amara et al., 2007; Hamaoka et al., 1996). Performing muscle contractions during cuff occlusion rapidly increases the rate of muscle deoxygenation compared to ischemia alone (De Blasi et al., 1992). This is due to the increased metabolic demand imposed by the contractions, which leads to an increase in the rate of mitochondrial oxygen consumption. Under these conditions, one would expect that mitochondria would continue to consume oxygen at an elevated rate until critical PO_2 was reached and respiration became limited by oxygen availability. At this point, the rate of oxidative ATP production would fall, and any further force production would then rely largely on ATP generation by anaerobic pathways. However, to date this has not been demonstrated in vivo.

We recently observed that peak dMb during repeated ischemic contractions was less than that achieved during resting ischemia alone (Lanza et al., 2006). The estimated PO_2 during the ischemic contractions was 1 mmHg (Lanza et al., 2006), which is well above the critical PO_2 value of ≈ 0.5 mmHg reported in isolated cell (Rumsey et al., 1990) and animal models (Gayeski et al., 1987). Similarly, DeBlasi and colleagues (1992) reported a submaximal plateau in muscle deoxygenation, measured by near-infrared spectroscopy, during ischemic contractions. Under ischemic conditions, a plateau in muscle deoxygenation suggests a cessation of mitochondrial oxygen consumption. A submaximal plateau in dMb suggests that oxygen consumption is inhibited by some factor associated with muscle contractions, rather than oxygen availability per se. Since there is some evidence that by-products of energy metabolism, most likely H^+ and inorganic phosphate (P_i ; (Harkema and Meyer, 1997; Jubrias et al., 2003; Suleymanlar et al., 1992; Walsh et al., 2002) can inhibit mitochondrial function, it is possible that, under these conditions, the metabolic state of the cell may inhibit mitochondrial oxygen consumption.

The first aim of this study was to investigate the effects of ischemic contractions on muscle deoxygenation in vivo using ^1H -MRS to measure dMb. We hypothesized (hypothesis 1) that dMb would reach a submaximal plateau during ischemic contractions of the tibialis anterior muscle. To determine whether this submaximal plateau resulted from metabolic inhibition of mitochondrial oxygen consumption, we continued to monitor dMb while maintaining occlusion after the contractions were stopped, thereby preventing any metabolic recovery and “clamping” the metabolic state of the muscle (Harris et al., 1976). We hypothesized (hypothesis 2) that no further Mb desaturation would occur during this period of ischemia, suggesting that mitochondrial oxygen consumption was inhibited by the metabolic state of the cell despite available oxygen.

To address the first aim of this study, it is necessary to define “maximal” Mb desaturation. Typically, Mb is considered to be completely desaturated once a stable plateau of dMb is achieved during ~ 10 minutes of cuff ischemia (Mole et al., 1999; Richardson et al., 1995; Wang et al., 1990). However, there is some evidence to suggest that the mere existence of a plateau may not indicate full Mb desaturation (Tran et al., 1999). Our second aim, therefore, was to determine if 10 min of resting ischemia is sufficient to fully desaturate Mb in the ankle dorsiflexors. In a separate experiment, we imposed a period of increased metabolic demand following the establishment of a plateau in dMb induced by cuff ischemia. We hypothesized (hypothesis 3) that performing ischemic muscle contractions after 10 min of resting ischemia would further increase dMb in the tibialis anterior muscle, thereby suggesting that 10 min of resting ischemia is insufficient to achieve full desaturation. The aims of this study therefore address important physiological and methodological issues related to bioenergetics in human skeletal muscle in vivo.

METHODS

Ethical Approval and Subjects

Six healthy young men between the ages of 22 and 35 years participated in this study. All were non-smokers and free from cardiovascular, respiratory, neuromuscular, or metabolic disease. Physical activity levels of the subjects ranged from sedentary to endurance-trained. All subjects gave written informed consent, as approved by the appropriate review boards at the University of Massachusetts, Amherst and Yale University School of Medicine. All procedures conformed to the standards set by the Declaration of Helsinki.

Experimental Arrangement and Acquisition Parameters

Subjects lay supine in a 4.0 Tesla whole-body superconducting magnet (Bruker Biospin, Rheinstetten, Germany). A coplanar 7 cm ^1H and 3×5 cm ^{31}P elliptical surface coil was taped over the belly of the tibialis anterior muscle, and the right foot was secured in a custom-built, non-magnetic apparatus to measure ankle dorsiflexor muscle force production. The ankle was fixed at 30° of plantarflexion, and the lower leg was immobilized with Velcro straps placed just below the knee joint and midway between the knee and the ankle. A pneumatic cuff was placed around the thigh and connected to a rapid cuff inflator (Hokanson, Inc., Bellevue, WA) to occlude blood flow to the lower leg. Transverse gradient-echo scout images were obtained to ensure correct positioning of the limb in the isocenter of the magnet and to select a region of interest within the tibialis anterior muscle for localized shimming on the muscle water peak using the FASTMAP method (Shen et al., 1997).

Proton MR spectra were acquired before, during, and after each contraction protocol using a 500 μs , frequency-selective Gaussian pulse (repetition time = 25 ms, sweep width = 30 kHz, number of acquisitions = 80, temporal resolution = 2 s) centered approximately 88 ppm downfield from the water peak, at the resonance of the dMb signal. To minimize baseline artifacts due to the water signal, a water suppression pulse (500 μs Gaussian pulse centered on water, 5 ms crusher gradient) was also implemented.

To evaluate the metabolic state of the muscle during the two contraction protocols we acquired interleaved ^1H and ^{31}P spectra from a single subject using the Bruker Multiscan Tool. Phosphorous scans were acquired using a 125 μs hard pulse with a nominal 60 degree flip angle (4 s repetition time, 2048 data points, 8 kHz spectral width). Parameters for the ^1H scan were identical to those listed above with the exception of temporal resolution, which was 4 s, and the lack of water suppression.

Procedures

To address the two aims of this project, each subject performed 2 isometric dorsiflexion protocols with the same leg. The protocols were separated by at least 30 min of rest with the cuff deflated. The order of the protocols was alternated for each subject to minimize any procedural effects. Prior to the contraction protocols, baseline muscle strength was assessed with 2 maximum voluntary isometric contractions (MVIC), each lasting approximately 4 s and separated by 2 min of rest. After MRS data acquisition was initiated, one minute of baseline data was acquired in the resting muscle before inflating the thigh cuff to 240 mmHg (pressure achieved within 30 s), thereby inducing ischemia. During the contraction protocols, subjects were given visual feedback via an LED display and strong verbal encouragement in order to ensure maximal effort, and peak force (N) was recorded for each contraction. Contractions were cued by verbal command (early contractions; EC) or audible tone (late contraction; LC). Cues were timed by the spectrometer and each subject was familiarized with the contraction protocols to ensure that timing of the contractions was accurate.

Protocol 1 - Early Contractions (EC)—To address our first aim, each subject performed 6 MVIC's (12 s on, 12 s off) 1 minute after the cuff was inflated. The contractions were followed by 6 min and 48 s of “ischemic recovery”. Therefore, the total duration of ischemia was 10 min, after which the cuff was deflated, and recovery data were collected for 5 min under free-flow conditions.

Protocol 2 - Late Contractions (LC)—To address our second aim, and to establish maximal resting dMb, subjects performed 3 min of ischemic MVIC contractions (1.2 s on, 1.8 s off) after 10 min of ischemia alone. Contractions were followed by 2 min of ischemic rest. This rapid contraction protocol was chosen to impose a high metabolic demand, since the metabolic cost of force development is higher than that of force maintenance (Bergstrom and Hultman, 1988; Chasiotis et al., 1987; Russ et al., 2002). The total duration of ischemia was 15 minutes. Recovery data were collected under free-flow conditions for 5 min following cuff deflation.

Spectral Analysis

Spectral analysis was performed using NUTS software (Acorn NMR, Livermore, CA). Free induction decays (FIDs) were averaged to yield a temporal resolution of 12 s. The FIDs were then apodized with a mixed Lorentzian/Gaussian function (-300 Hz, 0.1 fraction Gaussian), followed by zero-filling and Fourier transformation. The resulting spectra were phased manually and baseline-corrected using a 5th order polynomial fit of the baseline region (-60 to -120 ppm). The area of the dMb peak was quantified using an iterative, least-squares curve-fitting routine to optimize peak width, chemical shift, amplitude, and lineshape. For each subject, dMb is expressed relative to the average of the 3 highest consecutive values (i.e., a 36-s average) achieved during 10 minutes of resting ischemia (from the first 10 minutes of LC protocol). This approach is commonly used in studies employing ¹H-MRS of dMb (Mole et al., 1999; Richardson et al., 1995; Wang et al., 1990).

Phosphorus FIDs from interleaved acquisitions in a single subject were averaged to yield a time resolution of 12 (EC) or 24 (LC) s, apodized using an exponential function corresponding to 10 Hz, zero-filled and Fourier transformed. After manual phasing, the underlying broad peak due to the phosphorus in bone was removed with a polynomial fit to obtain a flat baseline. Peaks corresponding to phosphocreatine (PCr) and inorganic phosphate (P_i) were then fit with Lorentzian-shaped curves to quantify their respective areas. Phosphorus metabolite levels are expressed relative to resting values. Intracellular pH was calculated from the chemical shift (σ), in ppm, of P_i relative to PCr (Taylor et al., 1986):

$$\text{pH}=6.75+\log\frac{\sigma-3.27}{5.69-\sigma} \quad (1)$$

Curve-Fitting and Calculations

In an ischemic limb, the rate of Mb desaturation reflects the rate of oxygen consumption, although it is an underestimate since it fails to account for the diffusion of oxygen into the cell from the stagnant blood and the interstitium. To estimate the rates of Mb desaturation for both protocols, the change in dMb, beginning with the initial detection of a dMb peak, was fit for each subject using a bilinear, segmented regression model (PROC NLIN, SAS Institute, Cary, NC), as follows (Wigmore et al., 2004):

$$Y=a_0-[ip \cdot id \cdot (a_1-b_1)]+[(a_1-b_1) \cdot (ip+time \cdot id)]+(b_1 \cdot time) \quad (2)$$

where Y is the predicted dMb (% resting max), a_0 is the intercept of the first line, a_1 is the slope of the first line, b_1 is the slope of the second line, and ip is the inflection point of the 2 lines. For all cases where $time < ip$, id was assigned a value of 1, indicating that the data point belonged to the first line. When $time \geq ip$, id was assigned a value of 0, indicating that the data point belonged to the second line. The NLIN procedure was used to simultaneously estimate a_0 , a_1 , b_1 , and ip based on a two-stage, iterative fitting procedure to minimize the error sums of squares for the above model.

Since the appearance of dMb during resting ischemia has been described previously as sigmoidal (Wang et al., 1990), the time-course of Mb desaturation for each protocol was also fit with a sigmoid function using curve-fitting software (SigmaPlot, SPSS Inc., Chicago, IL):

$$y=y_0+\frac{a}{1+e^{-\left(\frac{t-t_0}{b}\right)}} \quad (3)$$

where y is dMb (% resting max), y_0 is the asymptote value at the start of the function, t is time, t_0 is time at the starting point of the function, and a and b are coefficients describing the shape of the curve. However, because the objective of this study was to identify the point at which the rate of dMb desaturation slowed, presumably reflecting a slowing of mitochondrial oxygen consumption, the bilinear fit parameters were the primary outcome variables of interest.

The intracellular PO_2 at which the Mb desaturation rate slowed was estimated for each subject from the oxygen dissociation curve for myoglobin:

$$\text{PO}_2=P_{50} \cdot \frac{1-f}{f} \quad (4)$$

where f is the fractional desaturation of myoglobin at the dMb ip , and the P_{50} is 2.39 mmHg (Schenkman et al., 1997).

Statistical Analyses

To determine if dMb reached a submaximal level during ischemic contractions (hypothesis 1), dMb peak area during the last contraction ($t = 192$ s in EC) was compared to maximal resting dMb (maximum 3-point average during first 10 min of LC protocol). To determine if dMb remained submaximal during the ischemic “recovery” period (hypothesis 2), dMb peak area

at the end of the EC protocol (3-point average) was compared to maximal resting dMb. To address our secondary aim (hypothesis 3), maximal resting dMb was compared to dMb peak area observed during contractions in the LC protocol (maximum 3-point average between $t = 600$ to 840 s).

Bilinear fit parameters, including the slopes and the PO_2 at the inflection point for each protocol were compared in order to evaluate the time-course of Mb desaturation in each of the protocols. Fatigue (end MVIC/baseline MVIC) during the protocols was also compared, and the r^2 values for the sigmoid and bilinear models were compared. Due to the small sample size, non-parametric statistics were employed to avoid making assumptions about the distribution of the data; all comparisons were made using the Wilcoxon signed-rank test, and α was set at 0.05. All statistics were performed using JMP v.4 (SAS Institute, Cary, NC). Data are expressed as mean $\pm$ SE.

RESULTS

Representative stackplots of the 1H -MRS spectra from a single subject are shown in Fig. 1; panel A shows the changes in the dMb peak that occur during the EC protocol, while panel B is from the LC protocol. In both protocols, the dMb peak increased progressively during ischemia and recovered rapidly upon cuff deflation (Figures 2 and 3 for EC and LC, respectively).

Submaximal Desaturation During Ischemic Contractions (Protocol 1 - EC)

In the EC protocol, dMb increased sharply at the onset of ischemic contractions (Fig. 2). In support of hypothesis 1, Mb desaturation at the end of ischemic contractions ($t = 192$ s) was less than maximal resting dMb observed during the LC protocol ($75 \pm 3.3\%$ of resting max, $p = 0.03$); the corresponding PO_2 was 0.82 ± 0.13 mmHg. In contrast to hypothesis 2, however, Mb desaturation gradually increased during ischemic “recovery” ($98 \pm 1.8\%$ of resting max, $p = 0.031$ vs. end of contractions); this value was similar to resting max dMb ($p = 0.69$).

Full Desaturation Achieved during Resting Ischemia (Protocol 2 - LC)

In 5 of 6 cases, a dMb peak became visible in the 1H spectrum between 90 and 120 s after the start of cuff inflation (Fig. 3). In the one subject whose data were recorded using the interleaved 1H - ^{31}P -MRS without water suppression, a dMb peak became visible soon after cuff inflation (Fig. 4). Notably, the overall pattern of desaturation was similar in all subjects, despite the differences in acquisition parameters. In all subjects, dMb reached a plateau within 10 min (Fig. 3). In contrast to hypothesis 3, the addition of maximal isometric contractions after 10 min of ischemia did not affect dMb ($100 \pm 1.5\%$ of max resting; $p > 0.99$).

Comparison of Mb Desaturation - EC vs. LC

Overall, Mb desaturation during resting ischemia (first 10 min of LC protocol) follows a sigmoidal pattern, although portions of the data were well described by the bilinear model (mean $r^2 = 0.973 \pm 0.004$ and 0.944 ± 0.014 for sigmoid and bilinear models, respectively; Fig. 1B, Table 1). Similarly, Mb desaturation during the EC protocol was well fit by both sigmoid and bilinear functions (Fig. 1A; mean $r^2 = 0.899 \pm 0.017$ and 0.885 ± 0.016 for sigmoid and bilinear models, respectively). The r^2 values for both models were similar ($p = 0.17$ and 0.50 for LC and EC, respectively). An advantage of the bilinear model over the sigmoid function is the ease of comparison of Mb desaturation rates and inflection points between the two protocols. The initial rate of desaturation was more than 4 times faster in EC than LC ($p = 0.005$; Table 1). Consistent with the faster rate of desaturation, the ip occurred earlier in EC than in LC ($p = 0.005$). In the EC protocol, dMb reached the ip during contractions (102 s into the protocol, corresponding to 42 s after the start of contractions), which were not completed until 192 s into

the protocol. The predicted dMb at the *ip* was lower in the EC protocol than in the LC protocol (Table 1; $p = 0.02$). Accordingly, the calculated PO_2 at the *ip* was higher in the EC protocol (Table 1; $p = 0.02$). The rate of Mb desaturation after the *ip* was similar in the two protocols (Table 1; $p = 0.13$), and was significantly greater than zero in both cases ($p = 0.031$ and 0.034 for EC and LC, respectively), indicating that Mb continued to desaturate during this period.

Exploratory Interleaved ^{31}P and 1H Studies

To further explore the effects of ischemic contractions on intracellular metabolism, a single subject performed the EC and LC protocols while both dMb and phosphorus metabolites were monitored by interleaved 1H and ^{31}P magnetic resonance spectroscopy. As seen in Fig. 4A, Mb (top panel) desaturated rapidly during the first several minutes of the EC protocol, while PCr fell and P_i increased after contractions were initiated. Intracellular pH (bottom panel) increased initially, a result of the release of protons by the breakdown of PCr, and then began to fall. For this subject, the dMb *ip* occurred 108 s into the protocol (48 s after the start of contractions). At this point, PCr had fallen to 53% of resting values and $pH \approx 7.04$. Following the *ip*, Mb continued to desaturate at a diminished rate, while PCr and pH continued to fall precipitously until the end of contractions (end contraction PCr = 18% of resting and $pH = 6.80$).

During the LC protocol (Fig. 4B), Mb began to desaturate shortly after the start of ischemia and reached an inflection point 343 s after the start of ischemia. PCr fell slightly at the onset of ischemia, and reached 91% of resting at the dMb *ip*, while $pH \approx 7.05$. Near the time of the dMb *ip* ($t = 343$ s), the rate of PCr breakdown increased. At the end of 10 min of resting ischemia, PCr had fallen to 72% of resting and pH was 7.09.

Force During Ischemic Contractions

Baseline force, obtained prior to cuff inflation, was 449 ± 42.2 N. During EC, peak force was $91 \pm 2.8\%$ of baseline and ultimately fell to $64 \pm 6.3\%$ of baseline MVIC during the final contraction (Fig. 2). During LC, peak force was $85 \pm 0.04\%$ of baseline MVIC and fell to $12.2 \pm 4.6\%$ of baseline by the final contraction (Fig. 3). Thus, fatigue was significantly greater during the LC protocol ($p = 0.03$).

DISCUSSION

In support of our first hypothesis, Mb desaturated to a submaximal level during repeated ischemic contractions. After contractions, Mb continued to desaturate at a diminished rate during ischemic recovery, such that maximal desaturation was achieved within 10 min. The fact that full desaturation was achieved while cuff occlusion was maintained indicates that oxygen consumption continued after the contractions were completed. These data are inconsistent with our second hypothesis that mitochondrial oxygen consumption would be inhibited during contractions by the metabolic state of the cell. In contrast to our third hypothesis, contractions performed after 10 min of ischemia did not produce additional Mb desaturation, suggesting that full desaturation of Mb had already been achieved. This demonstrates that 10 min of ischemia, induced by cuff inflation to 240 mmHg, is sufficient to fully deoxygenate the ankle dorsiflexor muscles *in vivo*.

Muscle Deoxygenation During Ischemic Rest and Contractions

The first part of this study confirms and extends the results of previous studies demonstrating that Mb desaturation during ischemic contractions is less than ischemia alone (De Blasi et al., 1992; Lanza et al., 2006). Since there is some evidence that high levels of H^+ and P_i , such as those which are present during ischemic contractions (Lanza et al., 2006), may directly or indirectly inhibit mitochondrial oxygen consumption (Hall and DeLuca, 1984; Harkema and

Meyer, 1997; Jubrias et al., 2003; Suleymanlar et al., 1992; Walsh et al., 2002), we hypothesized that submaximal dMb may occur during contractions as a result of metabolic inhibition of respiration. To gain further insight into this phenomenon, we maintained ischemia following cessation of the contractions and hypothesized that Mb desaturation would remain submaximal. We reasoned that maintaining cuff occlusion after ischemic contractions would prevent recovery by “clamping” the metabolic state of the muscle (Harris et al., 1976), thereby preserving the inhibitory influence during the ischemic post-contraction period. The progressive desaturation of Mb (Fig. 2) following the cessation of contractions is not consistent with the hypothesis that oxygen consumption was inhibited by the metabolic state of the muscle. Why, then, does the muscle not fully desaturate during ischemic contractions?

Another explanation for the current results is suggested by the model of cellular energetics proposed by Connett and colleagues (Connett et al., 1990), who proposed that critical PO_2 , the level of oxygenation at which the rate of mitochondrial oxygen consumption becomes limited by oxygen availability, is directly related to the rate of mitochondrial respiration. The mechanism for this effect of respiratory rate on critical PO_2 may be related to the effect of metabolic activity on the K_m of oxygen for cell respiration (Oshino et al., 1972; Sugano et al., 1974), or the effect of the respiratory rate on the intracellular oxygen gradient (Rumsey et al., 1990). According to the model of Connett et al. (Connett et al., 1990), critical PO_2 in resting muscle is low, so Mb can desaturate to very low levels before the rate of desaturation, which reflects the rate of oxygen consumption, becomes oxygen-limited. In a contracting muscle, however, the critical PO_2 rises in proportion to the respiratory rate. Therefore, the rate of Mb desaturation must slow before Mb is desaturated to the same level as observed in the resting muscle. Once critical PO_2 is reached, oxygen consumption, and therefore Mb desaturation, continues at a diminished rate until the oxygen in the muscle is depleted.

The pattern of Mb desaturation in both the LC and EC protocols in this study is consistent with the model proposed by Connett and others (Connett et al., 1990). During resting ischemia, Mb desaturated gradually and eventually reached a plateau, as shown in the first 10 min of the LC protocol (Fig. 3). The onset of Mb desaturation was much earlier when no water suppression pulse was used (compare Figure 4, with no water suppression, to Figure 1), suggesting that off-resonance effects of the water suppression pulse decreased the intensity of the dMb peak. However, the overall pattern of myoglobin desaturation was similar in all subjects, and the difference in the onset time does not affect the interpretation of these results. Since performing maximal contractions after the plateau was achieved did not produce further Mb desaturation, we conclude that this plateau represents full desaturation. While the time-course of desaturation under these conditions has been described as sigmoidal (Wang et al., 1990), portions of the current data were well-approximated by a bilinear fit. The initial phase of desaturation was rapid, followed by a much slower phase (Fig. 1B). These two phases are separated by the *ip*, which estimates the point at which mitochondrial oxygen consumption slowed; the *ip* occurred, on average, 280 s into the protocol. The PO_2 at this point may therefore reflect the critical PO_2 , and the slow rate of Mb desaturation after the *ip* indicates oxygen-limited mitochondrial respiration.

Assuming that the energy demand of the resting muscle remains constant during 10 minutes of ischemia, one would expect that other pathways of ATP production would be called upon to compensate for the decline in mitochondrial respiration. Indeed, other investigators have shown that net PCr breakdown begins after an average of 250 s of ischemia in resting muscle (Amara et al., 2007; Blei et al., 1993), and that the onset of net PCr breakdown corresponds to a slowing of the rate of muscle deoxygenation assessed by optical spectroscopy (Amara et al., 2007). P_i and pH values were similar resting values when PCr started to fall (Blei et al., 1993), excluding the possibility that mitochondrial respiration was inhibited by the accumulation of H^+ and P_i under these conditions. Our results and our interpretation are wholly

consistent with these prior reports. The small discrepancy between the onset of net PCr breakdown in these studies and (250 s) the dMb *ip* in the current study (282 s) is likely due to the slower onset of ischemia in the current study (full ischemia achieved within 30 s). Furthermore, the results from our exploratory interleaved ^1H - ^{31}P -MRS study are also strikingly similar to these results from the literature; the dMb *ip* corresponds to an acceleration of net PCr hydrolysis (Fig. 4), and we observed no change in pH and only a small increase in P_i at the dMb *ip*. Although the data from a single subject must be interpreted with caution, the consistency of these data with other studies in the literature (Amara et al., 2007; Blei et al., 1993) increases our confidence in these results.

During ischemic contractions, Mb desaturation exhibited a similar pattern that was well approximated by a bilinear model (Fig. 1A): an initial phase of rapid desaturation, followed by a slower phase (Fig. 2). As expected, the initial rate of desaturation was significantly higher in the EC protocol than during ischemic rest (Table 1), reflecting higher initial oxygen consumption as a result of the metabolic demand imposed by the contractions. In the EC protocol, the *ip* occurred during the contractions (Table 1, Fig. 1A), suggesting that critical PO_2 was reached before the contractions ceased. The calculated PO_2 at the *ip*, 1.1 mmHg, was significantly higher than that observed during ischemia alone. As in the LC protocol, the *ip* is unlikely to indicate the onset of metabolic inhibition of oxygen consumption, since 1) in a previous study from our lab using the identical contraction protocol (Lanza et al., 2006), intramuscular pH at the time of the dMb *ip* in this study (40 s after the start of contractions) was 6.95, well above that required to inhibit respiration in skinned muscle fibers (pH = 6.6; Walsh et al., 2002) and similar to the value of 7.0 observed in the single subject for whom ^{31}P -MRS data are available in this study; and 2) the rate of Mb desaturation after the *ip* was similar in both the EC protocol and during the ischemic portion of the LC protocol, when the accumulation of P_i was small and unlikely to inhibit mitochondrial respiration. In the EC protocol, full desaturation was not achieved until nearly 5 minutes into “ischemic recovery”. Immediately following contractions, therefore, Mb was not yet fully desaturated.

While we believe that the PO_2 at the *ip* is a reasonable estimate of critical PO_2 in vivo, we must urge caution with this interpretation. First, the rate of Mb desaturation is an indirect measure of oxygen consumption. Secondly, the *ip* is a value predicted from the bilinear fit of portions of the dMb curve. A bilinear function was used in the current study in order to identify the point at which the rate of Mb desaturation slowed. It could be argued that Mb desaturation follows a nonlinear pattern, and therefore estimates derived from the bilinear fit are questionable. However, the bilinear fit closely approximates the data from both protocols (Fig. 1), and the r^2 values for the sigmoid and bilinear fits were not significantly different from each other in the EC or LC protocols. Therefore, we believe that the parameters derived from the bilinear fit accurately reflect the underlying physiology, particularly during the EC protocol.

There are few estimates of critical PO_2 from in vivo studies of human muscle with which to compare these results. The calculated PO_2 at the *ip* of the LC protocol (0.37 mmHg) is similar to the critical PO_2 estimate of ≈ 0.5 mmHg from studies of isolated cell preparations (Rumsey et al., 1990) and animal models (Gayeski et al., 1987). However, species, model and methodological differences make direct comparisons difficult. Using ^1H -MRS, Richardson and colleagues (1999) estimated that critical PO_2 was ≈ 4 mmHg in human muscle exercising at $\text{VO}_{2\text{max}}$. Re-calculating the PO_2 at the *ip* from the EC protocol using the same P_{50} (3.2 mmHg, rather than the 2.39 mmHg used in this study) yields a PO_2 of 1.5 mmHg in the current study. Since muscle oxygen consumption requires 2 - 3 min to reach $\text{VO}_{2\text{max}}$ (assuming a time constant of 30 to 40 s (Bangsbo et al., 2000)), oxygen consumption at the *ip*, which occurred 42 s after the start of contractions, was well below the muscle's $\text{VO}_{2\text{max}}$. Since we expect critical PO_2 at submaximal VO_2 to be lower than that at $\text{VO}_{2\text{max}}$, these results are in general agreement with those of Richardson and colleagues (1999).

Full Desaturation with Resting Ischemia

A second aim of this study was to address a calibration issue with the ^1H -MRS measurement of dMb. It has been shown that achieving a steady-state level of dMb during ischemia does not necessarily indicate that the muscle is fully deoxygenated (Tran et al., 1999). Our approach was to increase metabolic demand after a period of ischemia to determine if Mb would exhibit additional desaturation. As ATP is hydrolyzed to power muscle contraction, the pathways responsible for producing ATP, namely oxidative phosphorylation and anaerobic glycolysis, are up-regulated. During the LC protocol, dMb did not significantly increase during the 3 minutes of contractions, suggesting that there was no increase in mitochondrial oxygen consumption despite the increase in metabolic demand. The simplest and most likely explanation for this result is an oxygen limitation; mitochondrial oxygen consumption, and therefore Mb desaturation, was limited by oxygen availability. As discussed above, the modest changes in P_i and pH over the 10 min period of resting ischemia make it highly unlikely that mitochondrial respiration was inhibited by the accumulation of inhibitory metabolites such as P_i and H^+ .

As shown in Figure 3, mean dMb decreased briefly at the end of contractions in the LC protocol, then recovered during the 2 minutes before the cuff was deflated and the muscle reperfused. A fall in dMb suggests reoxygenation of the muscle, which seems unlikely after 13 minutes of ischemia. Inspection of the individual data revealed some variability in dMb around the maximal plateau in all subjects. While ischemic contractions did not increase dMb in any subject, dMb fell during the last minute of contractions in 2 subjects. In one of these subjects, dMb fell briefly to 61% of max, then recovered to 88% before reperfusion. The ^{31}P data for this subject showed no evidence of recovery of PCr (Figure 4), which would be expected if reperfusion had occurred. Although we cannot rule out the possibility of partial reperfusion in the other subject who exhibited a fall in dMb, we believe that this is unlikely because cuff pressure was maintained at 240 mmHg by an automated cuff inflator during the entire protocol. Since the oxygen affinity of Mb in vitro is independent of the salt composition of the medium (Antonini and Brunori, 1971), and we are not aware of any evidence that the ionic state of the muscle affects the detection of dMb by ^1H -MRS, it is unlikely that the fall in dMb in these two subjects reflects changes in oxygen affinity or dMb detection due to the ionic state of the muscle (i.e., elevated Ca^{2+} or Mg^{2+}). While the oxygen affinity of dMb is sensitive to temperature, one would not expect temperature fluctuations to occur as rapidly as the changes in dMb observed in these two subjects. One likely explanation for this observation in these 2 subjects is that the dMb signal intensity fell due to limb movement towards the end of the protocol. The broadening of the PCr peak at the end of the ischemic contractions in one of the two subjects who exhibited a fall in dMb is consistent with this interpretation, although these data are not available for the other subject.

The results of this study have implications for the calibration procedures used for both ^1H -MRS and NIRS studies of muscle oxygenation in humans. Some studies have employed a only brief period (i.e., 2 minutes) of ischemic contractions in order to “fully desaturate” Mb and hemoglobin (Amara et al., 2007). Our results suggest that this approach will underestimate myoglobin desaturation and lead to inaccurate calibration of the dMb signal. The results of the current study suggest that a full 10 min of resting ischemia, induced by cuff inflation at 240 mmHg, is sufficient to reach full desaturation in the ankle dorsiflexor muscles.

Muscle Force and Fatigue During Ischemic Contractions

Fatigue was significantly greater in the LC protocol than in the EC protocol. Since the muscle was fully desaturated before contractions were initiated in the LC protocol, it is likely that differences in muscle oxygenation at the start of the contractions may have contributed to the differences in fatigue. The differences in the contraction protocol may also have played a role;

the brief, rapid contractions performed in the LC protocol are more metabolically demanding than the sustained contractions with prolonged rest used in the EC protocol (Bergstrom and Hultman, 1988; Chasiotis et al., 1987; Russ et al., 2002).

Conclusions

We have investigated the phenomenon of submaximal muscle deoxygenation during ischemic contractions, which does not appear to be a result of the inhibition of mitochondrial oxygen consumption. Rather, the marked slowing of Mb desaturation during the ischemic contractions, evidenced by the *ip* of the bilinear fit, suggests that mitochondrial oxygen consumption becomes limited under these conditions due to low PO₂. Finally, the inability to further desaturate dMb with contractions after 10 min of resting ischemia supports the use of 10 min of resting ischemia as a calibration method for studies of intracellular oxygenation in human skeletal muscle in vivo.

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Figure 1. Myoglobin desaturation in a single subject

Representative stackplots of ^1H -MRS spectra during A) EC and B) LC in a single subject are shown in the left panels. The dashed line indicates ischemia alone and the solid line denotes contractions + ischemia. For each panel, temporal resolution is 60 s for the first spectrum and 12 s in all subsequent spectra. In the right panels, dMb is expressed relative to maximal dMb obtained during 10 min of resting ischemia, in the same subject. Contractions are indicated by the hatched bar. Sigmoid and bilinear fits of the dMb data are shown. A) $r^2 = 0.91$ and 0.90 , respectively. B) $r^2 = 0.98$ and 0.96 , respectively.

Figure 2. Changes in dMb and force during EC protocol

Deoxy-myoglobin (dMb), expressed relative to maximal dMb obtained during 10 min of resting cuff ischemia. Contractions are indicated by the hatched bar. Closed symbols indicate ischemia, open symbols indicate free-flow recovery period. In the inset, force is expressed relative to baseline maximum. Each contraction lasted 12 s, followed by 12 s of rest.

Figure 3. Changes in dMb and force during LC Protocol

Deoxy-myoglobin (dMb), expressed relative to maximal dMb, as in Fig. 2. In the inset, force is expressed relative to baseline maximum. Each contraction lasted 1.2 s, followed by 1.8 s of rest.

Figure 4. Deoxymyoglobin and PCr a single subject

Data were acquired in an interleaved manner during A) the EC protocol, and (B) 10-min resting ischemia during LC protocol. Observed dMb, along with the bilinear fit, are presented in the top panel. Phosphocreatine (PCr) is shown in the second panel, with inorganic phosphate (Pi) and intracellular pH below. PCr and Pi are expressed relative to resting levels. Contractions during the EC protocol are indicated by the hatched bar.

Table 1**Bilinear fit parameters**

Slope 1 corresponds to the rate of Mb desaturation from the start of desaturation (min 1 for EC; min 2 for LC) to the inflection point (*ip*), while Slope 2 corresponds to the time from the *ip* to min 10 in each protocol. dMb and PO₂ at *ip* refer to the predicted dMb at the inflection point of the bilinear fit and the corresponding PO₂.

	Early Contraction	Late Contraction
r^2	0.885 ± 0.016	0.944 ± 0.014
Slope 1 (% of peak·s ⁻¹)	1.68 ± 0.38	$0.38 \pm 0.034^*$
Slope 2 (% of peak·s ⁻¹)	0.054 ± 0.008	0.035 ± 0.006
<i>ip</i> (s)	103.6 ± 7.4	$282.1 \pm 12.7^*$
dMb at <i>ip</i> (% max)	69.7 ± 4.1	$86.6 \pm 2.4^*$
PO ₂ at <i>ip</i> (mmHg)	1.1 ± 0.2	$0.38 \pm 0.08^*$

*
(p<0.05)