Comparative analysis of NMR and NIRS measurements of intracellular P_{O_2} in human skeletal muscle

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[1]*Department of Biological Chemistry, School of Medicine, and* [3]*Department of Exercise Science, University of California, Davis, 5616;* [2]*General Electric Medical Systems, Fremont, California 94539; and* [4]*University of Tsukuba, Tsukuba-shi, Ibiraki-ken, 305-8573 Japan*

Tran, Tuan-Khanh, Napapon Sailasuta, Ulrike Kreutzer, Ralph Hurd, Youngran Chung, Paul Mole, Shinya Kuno, and Thomas Jue. Comparative analysis of NMR and NIRS measurements of intracellular P_{O_2} in human skeletal muscle. *Am. J. Physiol.* 276 (*Regulatory Integrative Comp. Physiol.* 45): R1682–R1690, 1999.—^{1}H NMR has detected both the deoxygenated proximal histidyl $N_\delta H$ signals of myoglobin (deoxyMb) and deoxygenated Hb (deoxyHb) from human gastrocnemius muscle. Exercising the muscle or pressure cuffing the leg to reduce blood flow elicits the appearance of the deoxyMb signal, which increases in intensity as cellular P_{O_2} decreases. The deoxyMb signal is detected with a 45-s time resolution and reaches a steady-state level within 5 min of pressure cuffing. Its desaturation kinetics match those observed in the near-infrared spectroscopy (NIRS) experiments, implying that the NIRS signals are actually monitoring Mb desaturation. That interpretation is consistent with the signal intensity and desaturation of the deoxyHb proximal histidyl $N_\delta H$ signal from the β-subunit at 73 parts per million. The experimental results establish the feasibility and methodology to observe the deoxyMb and Hb signals in skeletal muscle, help clarify the origin of the NIRS signal, and set a stage for continuing study of O_2 regulation in skeletal muscle.

myoglobin; hemoglobin; exercise; bioenergetics; oxygen; near-infrared spectroscopy

THE REGULATION of O_2 transport to the mitochondria is a key issue in biology, especially in exercising muscle, where O_2 consumption ($\dot{V}_{O_2}$) can vary over a wide range. The dramatic increase in $\dot{V}_{O_2}$ requires a coordinate response in the O_2 cascade from the lung to the cell. Whether convection, diffusion, or metabolic mechanisms are limiting maximum O_2 consumption ($\dot{V}_{O_{2\,max}}$) is still under debate (37). These mechanisms present a recurring O_2 gradient motif, which governs O_2 transport to the mitochondria (12, 36, 41). Of particular importance is the O_2 gradient from the capillary to the mitochondria (11, 30).

Assessing the vascular-to-cellular P_{O_2} gradient during exercise, however, poses many technical obstacles, which some investigators have attempted to hurdle with near-infrared spectroscopy (NIRS) methods. In the oxygenated and deoxygenated states of myoglobin (Mb) and Hb, the NIRS spectra exhibit distinct absorption bands. When Mb or Hb is oxygenated, an absorption band appears at 850 nm. When Mb or Hb is deoxygenated (deoxyMb or deoxyHb), an absorption band appears at 760 nm. The 760- and 850-nm absorption bands form, then, a basis to assess the overall tissue P_{O_2} (15). Recent experiments are now suggesting that the NIRS signals originate predominantly from Hb and therefore reflect the vascular P_{O_2} (34, 46).

In contrast, ^{1}H NMR methodology can follow the intracellular P_{O_2} with the Val E11 signal of oxyMb and the proximal histidyl $N_\delta H$ signal of deoxyMb. In buffer-perfused hearts, these signals appear at distinct chemical shift positions and unquestionably reflect the intracellular P_{O_2} (17, 18). In blood-perfused tissue, however, the corresponding signals from Hb should appear in the same spectral region and can potentially overlap with the Mb signal (17). Yet so far, no studies have reported any Hb signal contamination (25, 29, 43). Some researchers have now asserted that the erythrocyte Hb signals are therefore not NMR visible (44). Such a view is at odds with results from numerous erythrocyte Hb studies (8, 10, 23, 42).

Nevertheless, if the NIRS and NMR techniques do measure distinctly the vascular and intracellular P_{O_2}, then a unique research tool will emerge to help investigate $\dot{V}_{O_2}$ regulation in exercising skeletal muscle. A previous combined NIRS-NMR study has demonstrated the importance of the approach. NIRS is assumed to map the vascular P_{O_2}, and NMR monitors deoxyMb as a reflection of the intracellular P_{O_2}. The results indicate that O_2 limitation during exercise in normal vs. heart failure patients arises from an inadequate O_2 utilization in the mitochondria and not from any deficiency in O_2 supply (25). However, the critical tenets remain somewhat moot. Does the NMR signal assigned to the deoxyMb proximal histidyl $N_\delta H$ reflect only the intracellular P_{O_2}, and do the NIRS signals reflect predominantly Hb P_{O_2}?

We have undertaken a study to examine the NMR deoxyMb signal in human skeletal muscle and to form a relationship between the NMR and the NIRS data. When blood flow to the human gastrocnemius muscle is reduced, the NMR spectra exhibit clearly the deoxyMb signal at 78 parts per million (ppm), which gradually rises with O_2 desaturation. Upfield at 73 ppm is a signal corresponding to the deoxyHb β-subunit histidyl F8 $N_\delta H$, which rapidly reaches a steady-state level (8, 10, 42). The kinetics of Mb desaturation match the NIRS observed decline in the composite MbO_2 and HbO_2 signal. Even after Mb has attained a steady-state level, the high-energy phosphate levels, as reflected in the ^{31}P spectra, still remain unperturbed. The experi-

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mental results indicate that NMR can distinguish the proximal histidyl $N_\delta H$ signals of deoxyMb and deoxyHb and that the NIRS signals in these experiments are monitoring Mb, instead of Hb, desaturation. The study sets, then, a framework for the combined NMR and NIRS approach to study $\dot{V}O_2$ regulation in skeletal muscle.

MATERIALS AND METHODS

NMR. NMR measurements were performed on a 1-m bore diameter GE Signa scanner at 1.5 T. ^{1}H (63.86 MHz) NMR signal acquisition used a body coil transmit-surface coil (5-in. diameter) receive configuration. Magnetic field shimming used a three-point Dixon method to improve the field homogeneity, yielding a water line width of ~40 Hz (9, 33). A selective excitation pulse sequence was optimized to excite the deoxyMb and deoxyHb histidyl F8 $N_\delta H$ signals, ~4.6 kHz from the water resonance (27; Tran and Jue, unpublished data). Numerical simulation and experimental data verified that the experimental pulse length of 800 μs had a full width at half-maximum excitation of 2 kHz. At an offset of 800 Hz or 13 ppm from the excitation maximum, the pulse power dropped by 25%. Each data block was comprised of 200 transients, or 45-s signal-averaging times. The repetition time was 160 ms. The spectral width was 16 kHz, and the data block size was 512. All spectra were referenced to the water signal as 4.65 ppm at 35°C, which in turn was calibrated against sodium-d_4-(trimethylsilyl)propionate as 0 ppm.

^{31}P (25.85 MHz) signal acquisition used a conforming flexible coil, which wrapped around the subject's leg. A 50-mm slice was selected and then excited with a self-refocused 45° radio-frequency pulse. The effective echo time was set at 2.5 ms (22). The other acquisition parameters were as follows: spectral width, 2.5 kHz; data points, 2,048; acquisition time, 820 ms; recycle time, 2 s. Each ^{31}P NMR spectrum consisted of 50 transients and required a total acquisition time of 140 s. All spectra were apodized with a 15-Hz exponential function and referenced to phosphocreatine (PCr) as 0 ppm.

Intracellular pH was calculated from the P_i signal using the equation

$$\mathrm{pH} = \mathrm{p}K + \log\left(\frac{\delta_A - \delta_0}{\delta_0 - \delta_B}\right)$$

where $\mathrm{p}K = 6.9$, δ_A = change in ppm (δ_{ppm}) of $H_2PO_4^-$ at 3.290 ppm, $\delta_B = \delta_{ppm}$ of HPO_4^{2-} at 5.805, and $\delta_0 = \delta_{ppm}$ of P_i referenced to PCr δ_{ppm} as 0 ppm.

A soft cast was made for the leg of each subject, from the knee down to the ankle, and was used to calibrate the area intensity of the observed Mb signal. The cast was prepared with Scotchast Plus (3M, Minneapolis, MN) and filled with 0.2 mM metHb solution, which approximates the physiological Mb concentration in tissue (38). ^{1}H metHb spectra were then acquired with acquisition parameters, which were identical to the ones used in the leg exercise experiment. The peak intensity at 85.5 ppm was then used as a basis to quantitate the observed Mb intensity in exercising gastrocnemius muscle (21, 38). No longitudinal relaxation time (T_1)-based saturation factor correction was necessary, because the T_1 values of both the Mb and Hb signals are sufficiently rapid to permit full recovery within the recycle time.

Data were imported from the Signa system to a SunSparc2 workstation and processed with the GE Omega 6.0 software package. All spectra were zero-filled to 2,000 and apodized with a 50-Hz Gaussian-exponential function. All spectra were baseline corrected and referenced to water at 4.65 ppm at 35°C.

NIRS. NIRS measurements were made with a continuous-light source dual-wavelength spectrometer with a pair of colored light-emitting diodes as light sources and a photodiode detector (HEO100; Omron, Japan) (46). This system used two wavelengths on either side of the oxyHb-deoxyHb (and oxyMb-deoxyMb) isosbestic point. All wavelengths other than 760 and 850 nm were filtered out. The probe was wrapped around the leg muscle with a Velcro strap and engineered for a photon depth penetration of 2–3 cm. Because O_2-ligated heme groups have a greater absorbance at 850 nm than at 760 nm, whereas the corresponding deoxy heme groups have greater absorbance at 760 nm than at 850, the difference signal between 760 and 850 nm can reflect the changes in HbO_2-MbO_2 saturation on a relative scale (15, 34, 46). The relative scale was calibrated against the difference signal observed when the muscle was at rest and at steady state after 15 min thigh occlusion at 250 mmHg, which corresponds to 0% and 100% Mb-Hb deoxygenation, respectively.

Exercise and cuffing protocol. Healthy male volunteers (n = 4; weight 120–150 lbs; age 20–27 yr) were placed supine inside the magnet of a GE Signa 1.5-T scanner. The subject's calf muscle was positioned on top of a 5-in.-diameter ^{1}H receiving coil and strapped down with Velcro. A ^{31}P conforming flexible coil was placed around the calf and the ^{1}H receiving coil. A pressure cuff connected to a manual air pump was wrapped around the subject's thigh just above the knee. Within a few strokes the cuff inflation reached the final pressure of 180–265 mmHg.

The ergometer used for plantar flexion was constructed of wood. It consisted of a three-sided box with dimensions of 25.4 (width) × 25.4 (height) × 91.4 cm (length) with a foot pedal on an axle at one end and a movable backplate at the other end of the box. Rubber tubing (1.3 cm diameter × 34.3 cm length) with a Hooke's constant of 31.12 N/cm was attached to the back plate and the axle of the foot pedal. Resistance to plantar flexion was adjusted by varying the number of tubes and/or by stretching the tubes to increase the distance between the axle and back plate. Mechanical work of plantar flexion involved moving the pedal against a specified resistance through an arc of 3.8 cm. The power output can be incremented by varying the contraction frequency with resistance held constant. In the exercise protocol, the contraction frequency was 1 Hz, and the power output was ~6 W.

In one protocol, the pressure cuff was inflated to 180 mmHg and maintained at that pressure for 10 min. In the second protocol, the pressure cuff was first inflated to 180 mmHg for 5 min and then increased to 265 mmHg for an additional 5 min. In the final protocol each subject performed light plantar flexion exercise for 4 min. Immediately after the cessation of exercise, the pressure cuff was inflated to 180 mmHg for an additional 3 min. During the course of each protocol, the spectrometer recorded ^{1}H or ^{31}P signals. At all times, the subject was monitored for any sign of discomfort, which would signal an end to the experiment.

All data are reported as means ± SE. Statistical significance in the Student's t-test is ascribed for $P < 0.05$. The NMR and NIRS data were analyzed with a nonlinear regression fit to an exponential recovery function, $y = y_0 + a(1 - e^{-bt})$ (SigmaPlot for Windows 4.0).

RESULTS

Figure 1 shows the time course of the ^{1}H NMR spectra on inflating a pressure cuff above the knee to reduce blood flow to the gastrocnemius muscle. Before

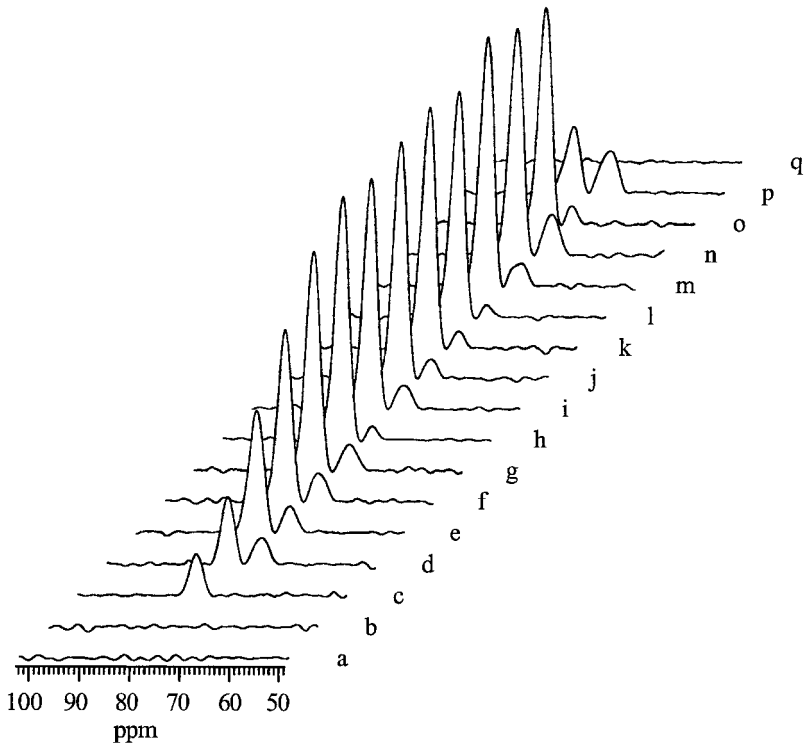

Fig. 1. ^{1}H NMR spectra from human gastrocnemius muscle during cuff occlusion at 180 mmHg. Peaks at 78 and 73 parts per million (ppm) correspond to proximal histidyl $N_\delta H$ of deoxyMb and β subunit of deoxyHb, respectively. Traces correspond to following experimental conditions: control (*a* and *b*), pressure cuffing at 180 mmHg (*c*–*o*), and reperfusion (*p* and *q*). Each spectrum required 45 s of signal averaging.

pressure cuffing, the reference spectrum shows no signal in the region between 50 and 100 ppm (Fig. 1, *a* and *b*). Pressure cuffing to 180 mmHg produces two signals from the gastrocnemius muscle, one at 78 ppm and the other at 73 ppm in the ^{1}H spectra. The 78-ppm signal is assigned to the paramagnetic shifted proximal histidyl $N_\delta H$ signal of deoxyMb, whereas the 73-ppm peak is assigned to the deoxyHb β-subunit histidyl F8 $N_\delta H$ (10, 16, 18, 20, 39). The deoxyHb β-subunit histidyl F8 $N_\delta H$ signal at 61 ppm is not visible under these experimental conditions.

The Mb signal emerges once the leg is cuffed (Fig. 1*c*). Within 45 s, the Hb signal also becomes apparent (Fig. 1*d*). Although the Hb signal intensity quickly reaches a plateau, the deoxyMb signal continues to increase during the cuffing period, rapidly at first and then more slowly after 270 s. During the cuffing period the deoxyHb β-subunit signal remains at a relatively constant intensity (Fig. 1, *d*–*o*). With reperfusion of blood, both the Mb and Hb signals disappear rapidly (Fig. 1, *p* and *q*). The corresponding ^{31}P spectra are shown in Fig. 2. No significant changes in the high-energy phosphate signals are observable. Figure 2*a* is the control spectrum. Figure 2, *b*–*f* corresponds to the cuffing period, whereas Fig. 2*g* corresponds to the postcuffing period.

During plantar flexion exercise of the gastrocnemius muscle at ~1 Hz, Mb desaturation is observed. The reference spectrum before exercise shows no signal between 50 and 100 ppm (Fig. 3*a*). With exercise the Mb signal emerges at 78 ppm (Fig. 3, *b*–*h*). Its signal intensity rises rapidly to a steady-state level with exercise but increases even further at the onset of pressure cuff inflation (Fig. 3, *i*–*m*). The maximal Mb signal intensity (Fig. 4*m*) is fourfold greater than the initial signal observed in Fig. 3*b*. The deoxyHb β-subunit signal is also detectable after pressure cuffing the leg. On reperfusion both signals disappear within 90 s (Fig. 3, *n* and *o*).

The corresponding ^{31}P NMR data are shown in Fig. 4. The control spectra are shown in Fig. 4, *a* and *b*. During leg exercise the PCr level decreases, whereas the P_i intensity increases (Fig. 4, *c* and *d*). On cuffing the leg, the PCr and P_i levels show a slight recovery to the control levels (Fig. 4, *e* and *f*). During postexercise reperfusion, the PCr and P_i signals recover fully to their control levels (Fig. 4, *g* and *h*). During the exercise phase the pH drops from 7.11 to 7.00, whereas PCr level drops ~25%. With cuffing the pH drops further to 6.96. After reperfusion, the pH recovers to the control value of 7.11 (data not shown).

Figure 5 graphs the deoxyMb signal and the NIRS signal for tissue deoxygenation as a function of cuffing time. Pressure cuffing the leg at 180 mmHg produces a gradual increase in deoxyMb signal, which reaches a steady-state level within 5 min. No further signal intensity change follows for the next 4 min. The NIRS data reveal a similar time course. Even though the NMR deoxyHb β-subunit signal is detectable, it reaches a steady state much more rapidly than the deoxyMb signal and contributes <10% to the overall measurement. Fitting the NMR and NIRS data to an exponential recovery equation yields rate constants of 0.30 ± 0.05 and 0.24 ± 0.02 min^{-1} (Table 1).

During exercise the deoxyMb signal is detectable, but the corresponding deoxyHb signal is not. Figure 6 shows the relationship between the Mb and Hb signals during an exercise and cuff protocol (n = 4). Within 45 s of exercise at 1 Hz, the signal of deoxyMb is visible and

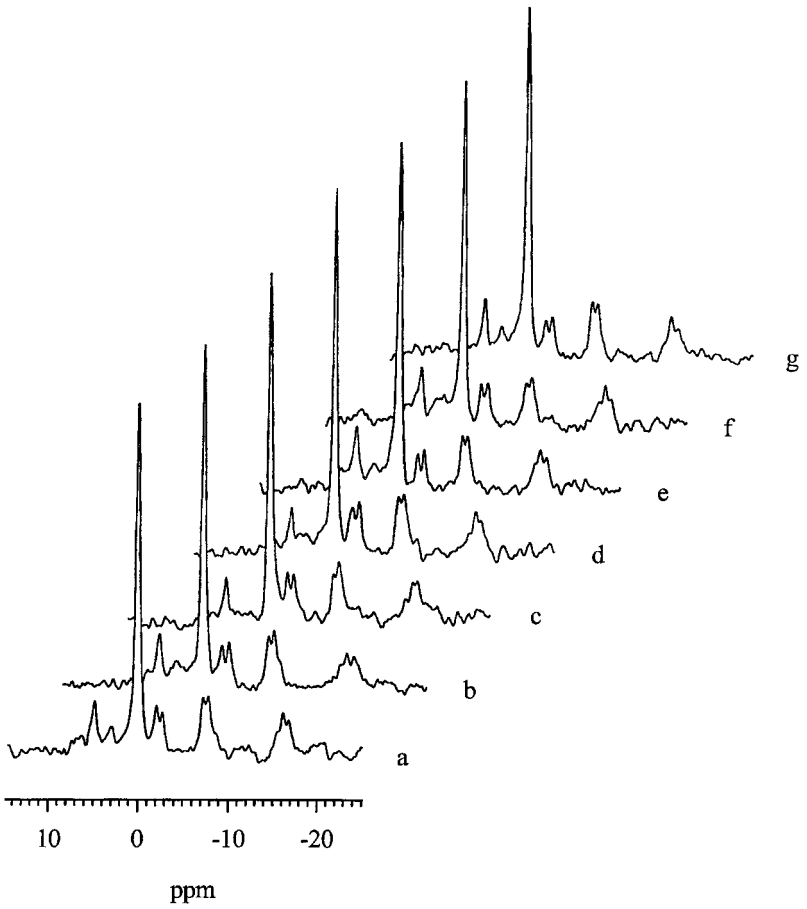

Fig. 2. ^{31}P NMR spectra from human gastrocnemius muscle during cuff occlusion at 180 mmHg. Spectra show signals of phosphocreatine [PCr (0 ppm)], P_i (−4.9 ppm), and ATP (2.4, −7.5, −16 ppm). Traces correspond to following experimental conditions: control (*a* and *b*), pressure cuffing at 180 mmHg (*c*–*e*), and reperfusion (*f* and *g*). No significant changes in signal intensity of PCr, P_i, and ATP appear during entire protocol.

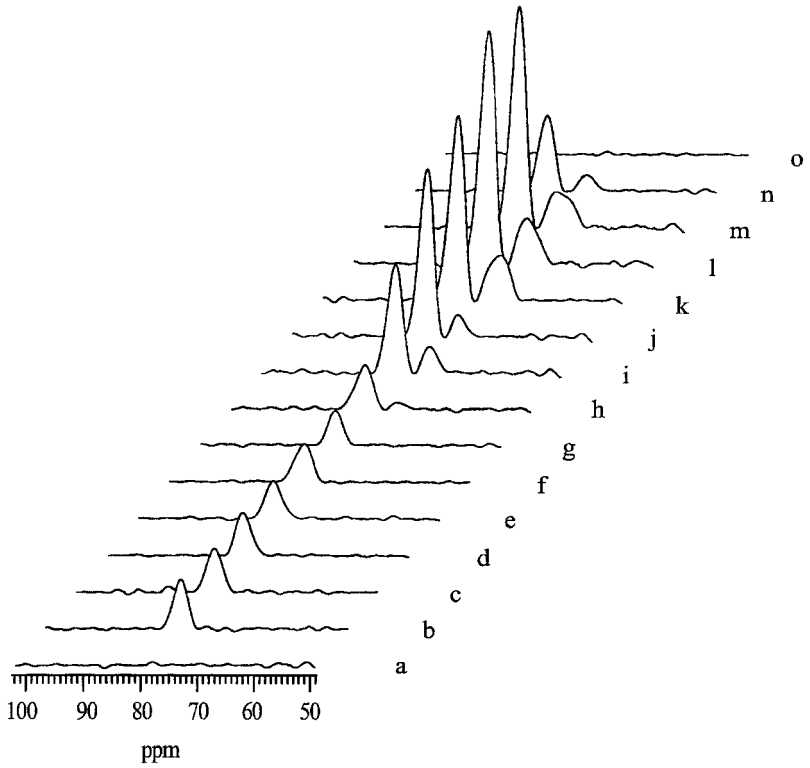

Fig. 3. ^{1}H NMR spectra from human gastrocnemius muscle during exercise and subsequent cuff occlusion at 180 mmHg. Traces correspond to control (*a*), plantar flexion exercise at 1 Hz (*b*–*h*), pressure cuffing at 180 mmHg (*i*–*m*), and postexercise (*n* and *o*). During exercise phase, histidyl F8 $N_{\delta}H$ signals of both deoxyMb and deoxyHb become visible and rapidly reach steady state. After pressure cuffing leg at 180 mmHg after exercise, deoxyMb and deoxyHb signal intensities rise sharply at 78 and 73 ppm, respectively. These signals vanish shortly after pressure cuff release.

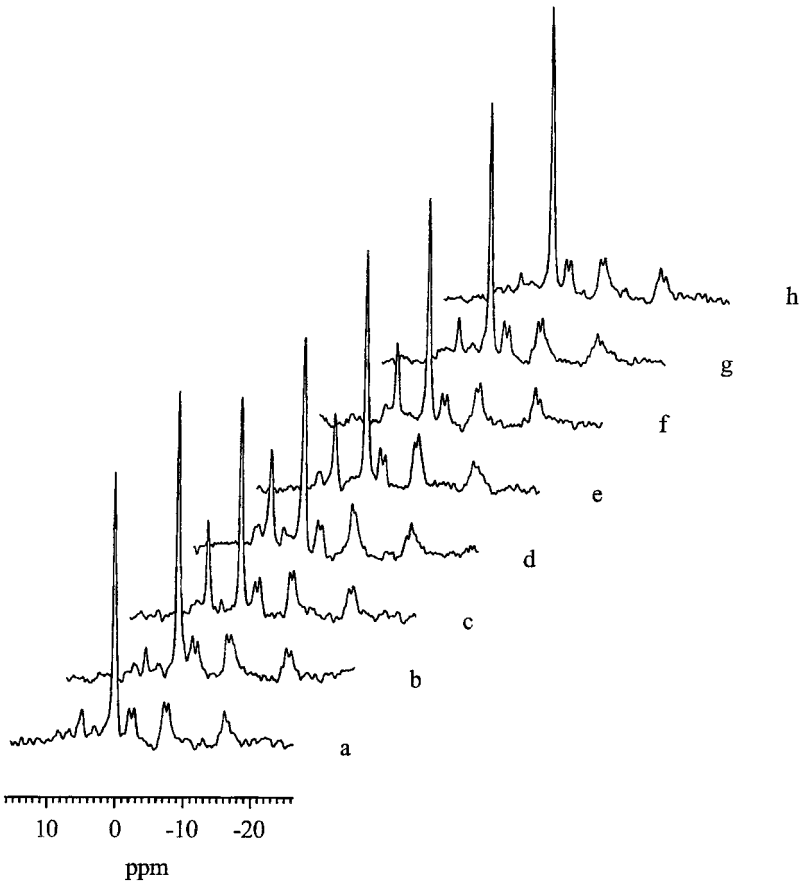

Fig. 4. ^{31}P NMR spectra from human gastrocnemius muscle during exercise and cuff occlusion at 180 mmHg. Traces correspond to following experimental conditions: control (*a* and *b*), plantar flexion exercise at 1 Hz (*c* and *d*), pressure cuffing at 180 mmHg (*e* and *f*), and postexercise (*g* and *h*). During exercise, PCr signal (0 ppm) drops, whereas P_i signal (−4.9 ppm) increases. These signals begin to recover during pressure cuffing and fully recover during postcuffing.

rapidly reaches a steady-state level. During the exercise period, the deoxyHb signal intensity is not distinguishable. Immediately after the exercise, the pressure cuff is inflated, and the deoxyMb signal intensity increases dramatically. The Hb signal now becomes evident and is calibrated against the maximum deoxyMb + deoxyHb signal as 100%. However, Hb desaturates and reaches a steady state more rapidly than Mb. The corresponding NIRS data are also shown in Fig. 6. The respective NIRS and Mb rate constants are 0.86 ± 0.12 and 0.73 ± 0.07 min^{-1}.

Pressure cuffing the leg at 180 mmHg produces a deoxyMb signal, which shows that Mb desaturates to a steady-state level within 5 min (Fig. 7). No further signal intensity change follows for the next 5 min. When the leg is cuffed first at 180 mmHg for 5 min and then at a higher pressure of 265 mmHg for the remaining 5 min, the deoxyMb signal increases further by 10% ($n = 4$; $P < 0.05$). At the steady-state condition during the 180-mmHg cuffing, a relaxation measurement yields the apparent transverse relaxation time (T_2) of 2.6 ms for the deoxyMb proximal histidyl $N_\delta H$ signal.

DISCUSSION

Visibility of Mb in human muscle. Once the pressure cuff is applied to the leg, the ^1H NMR spectra of human gastrocnemius muscle show clearly a signal at 78 ppm (Fig. 1). No signal is detected during the control period. On the basis of the unique chemical shift of the proximal histidyl $N_\delta H$ signal of human deoxyMb at 80.3 ppm at 25°C, the peak at 78 ppm is assigned to Mb at a cellular temperature of ~33°C (5, 16). The Mb signal rises linearly with time during ischemia and within 5 min reaches a steady-state level. With reperfusion the Mb signal rapidly disappears.

Even though the Mb signal reflects a gradual O_2 desaturation, the ^{31}P spectra are unperturbed and still indicate no apparent O_2 limitation (Fig. 2). The observation would imply that the resting cellular O_2 level does not partially saturate Mb and is well above the critical P_{O_2} point. During the cuffing protocol, ischemia induces a downregulation of energetic demands as well as the mobilization of glycolysis to buffer the O_2 loss.

Such is not the case during exercise. Even under light exercise, the rapid rise in the deoxyMb signal indicates a rapid drop of the cellular P_{O_2} (Fig. 3). Once exercise has stopped and the leg is cuffed, the deoxyMb signal rises sharply, indicating a further decrease in cellular P_{O_2}. The ^{31}P PCr and P_i signal intensity changes during exercise but is unaffected during pressure cuffing (Fig. 4).

Visibility of Hb in human muscle. Along with the Mb signal is an upfield peak at 73 ppm. The peak does not arise from Mb undergoing chemical exchange, because the chemical shift would imply that the subpopulation is at an unphysiological temperature of 46°C (16). Moreover, no solution or perfused myocardium studies have ever shown any dynamic exchange of Mb that

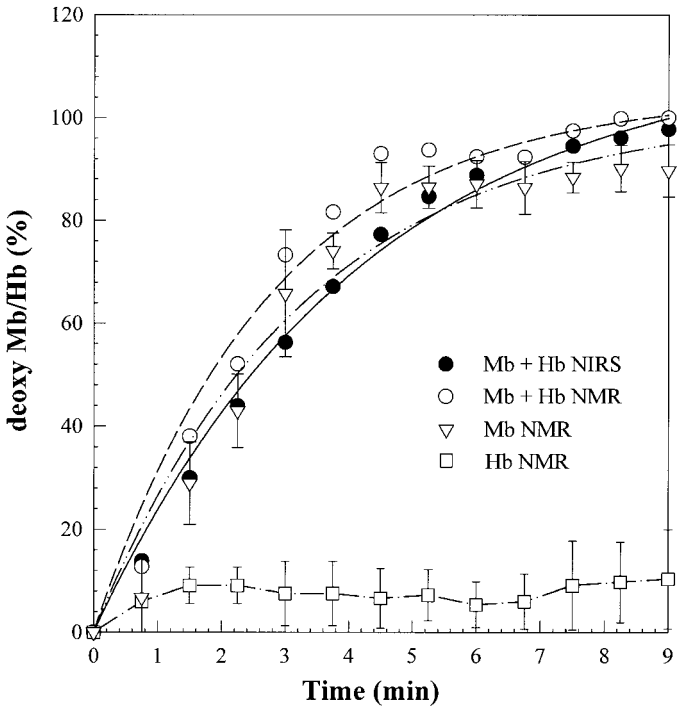

Fig. 5. Graph of NMR and NIRS signals during pressure cuffing of leg at 180 mmHg. ^{1}H NMR Hb signal (□) quickly reaches steady-state level and remains constant throughout entire ischemic cuffing protocol. Deoxy Mb signal (▽) exhibits slower kinetics and reaches steady state within 5 min. Because deoxyHb signal constitutes <10% of total deoxyMb-deoxyHb signal, composite deoxyMb and deoxyHb signal (○) yields kinetics that are dominated by Mb signal. NIRS difference signal (●), reflecting Mb-Hb desaturation, also shows a time response that reaches steady-state intensity within 5 min. Nonlinear fit of curves with an exponential recovery function confirms that Mb and NIRS kinetics are quite similar.

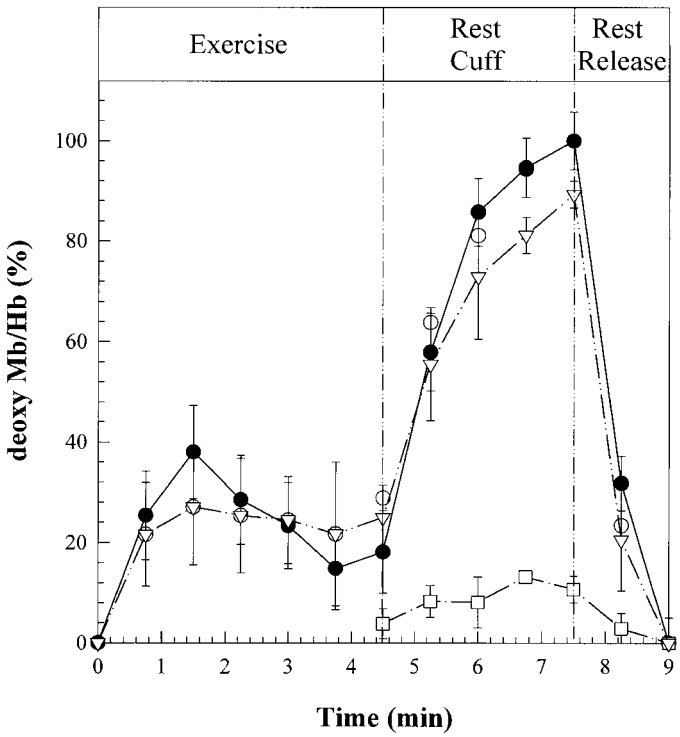

Fig. 6. Relative NMR and NIRS signal intensity changes as a function of exercise and subsequent cuffing. With exercise deoxyMb signal (▽) rises to a steady state within 90 s. Hb signal (□) is below detectable limits. Immediately after exercise, pressure cuffing produces a signal of deoxyHb and an increase in deoxyMb signal intensity. Hb signal reaches steady-state level much faster than Mb and represents a small contribution to overall Mb-Hb signal intensity (○). Corresponding NIRS data (●) closely match observed Mb kinetics. Once cuff pressure is released, both deoxyMb and deoxyHb signals disappear rapidly.

could lead to the rapid appearance of a low-intensity upfield Mb signal. So far no one has reported the hyperfine shifted signals of deoxyHb in tissue, which appear in the spectral window from 50 to 100 ppm.

The peak assignment to the β-subunit proximal histidyl $N_\delta H$ of deoxy-Hb is based on solution and erythrocyte studies, which show the resonance at 76 ppm at 25°C (8, 10, 42). Given the chemical shift, the Hb environment is ~37°C, consistent with previous reports of tissue temperature (31, 35, 45). However, the α-subunit proximal histidyl $N_\delta H$ signal of deoxy-Hb at 61 ppm is not detected, even though the pulse simulation and in vitro experimental data demonstrate that the selective pulse has sufficient bandwidth to detect the signal. Such an observation would imply that the α- and β-subunits of Hb have different O_2 affinity. Indeed, Hb studies have indicated that the α-subunit has a higher affinity for O_2 than the β-subunit, controlled largely by the "off" rate constant (1, 8, 40). As a result, Hb deoxygenation should begin with O_2 loss in the β-subunit, consistent with the observation in Figs. 1 and 3.

The observation of the proximal histidyl $N_\delta H$ signal of Hb in human muscle stands in contrast to previously reported skeletal muscle studies in which no Hb signal appears (29, 43, 44). Our results do not support the hypothesis that the erythrocyte environment has a

Table 1. *NMR and NIRS kinetic parameters*

	Exercise Cuff			Cuff		
	y_0	a	b, min^{-1}	y_0	a	b, min^{-1}
NIRS	17.59 ± 2.7	90.28 ± 4.9	0.86 ± 0.120	0	113.4 ± 3.5	0.24 ± 0.02
NMR Mb	25.14 ± 1.2	71.23 ± 2.7	0.73 ± 0.068	0	101.4 ± 7.1	0.30 ± 0.05
NMR Mb + Hb	29.03 ± 1.3	79.73 ± 2.7	0.74 ± 0.061	0	104.7 ± 4.1	0.36 ± 0.04

All values means ± SE. Near-infrared spectroscopy (NIRS) and NMR data are analyzed with an exponential recovery function, $y(t) = y_0 + a[1 - \exp(-bt)]$, where $y(t)$ is the normalized signal intensity of the NIRS or NMR signals as a function of time, t is time (in min), a is a constant, and b is a rate constant. Normalization value was obtained from observed signal after 5 min of pressure cuffing at 180 mmHg. Exercise-cuff data set is derived from experimental results during exercise followed by cuffing; cuff data set is derived from results during pressure cuffing only. Mb, myoglobin.

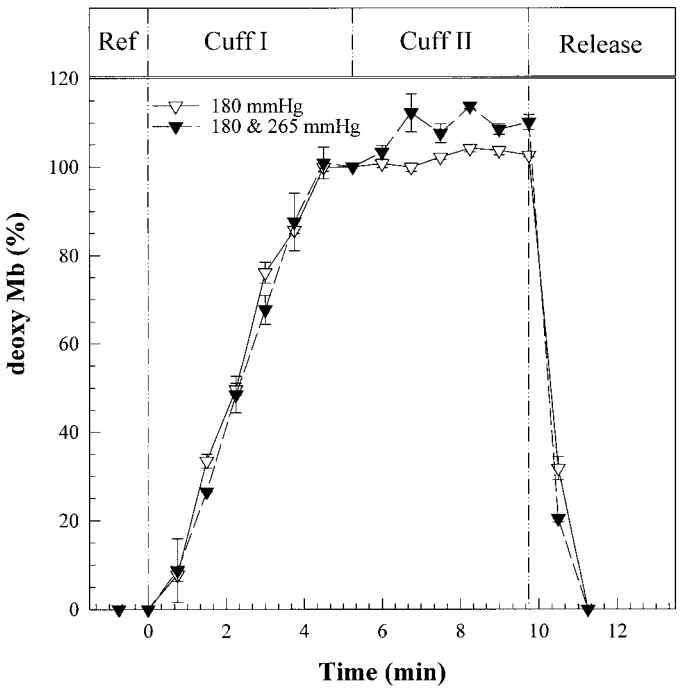

Fig. 7. DeoxyMb signal intensity during pressure cuffing. Within 5 min after pressure cuffing at 180 mmHg (▽), deoxyMb proximal histidyl $N_\delta H$ reaches steady-state level. Under an identical experimental protocol, if cuff pressure is increased to 265 mmHg after 5 min (▼), deoxyMb proximal histidyl $N_\delta H$ signal intensity increases by 10% and reaches a significantly higher steady-state level.

microviscosity that restricts Hb diffusion, which then broadens the proximal histidyl $N_\delta H$ signal in vivo beyond the NMR detection limit. The observation of the erythrocyte Hb signal is consistent with recent field-dependent relaxation and other erythrocyte Hb studies (3, 6, 7, 10, 19, 24, 42, 43). In fact the relatively sharp line width of the erythrocyte Hb observed in the muscle experiment indicates only a minor perturbation in the cellular microviscosity.

Relationship between the NMR and NIRS data. Because both the NMR Mb and Hb signals are well resolved, spectral contamination is no longer an issue. Studies can use these signals to explore the interaction between the vascular and intracellular P_{O_2}. In cuffed leg muscle, the deoxygenated Hb signal emerges with the Mb signal. However, the Hb signal reaches a steady-state level much more rapidly than the Mb signal. The progression is consistent with a rapid Hb desaturation followed by Mb desaturation. The exponential rate constant for Mb is 0.30 ± 0.05 min^{-1}. For Hb, the signal has reached a steady-state level within 40 s.

With the NIRS measurement, the kinetics of O_2 desaturation in tissue have a rate constant of 0.24 ± 0.016 min^{-1}, closely approximating the Mb kinetics. Such an observation is in contrast to a previous optical/NMR study, which indicated that Mb desaturates to a steady state while Hb desaturation continues, such that the optical changes reflect predominantly the time course of Hb deoxygenation (25). The ^{1}H NMR spectra clearly show that Hb desaturates first and reaches a steady state much more rapidly than Mb. Even though at present, the deoxyHb signal does not yield a P_{O_2} measurement because it cannot distinguish between volume vs. P_{O_2} changes, the rapid rise of the β-subunit proximal histidyl $N_\delta H$ signal of deoxyHb to a steady-state level would preclude any significant Hb contribution to the NIRS signal under these experimental conditions. Moreover the integrated signal areas indicate that the fractional contribution of Hb/Mb is <20%.

The detection of both the signals of deoxyMb and Hb leads to the potential of studying the relationship between O_2 availability and demand under different physiological conditions. Quite clearly during blood flow reduction, both MbO_2 and HbO_2 desaturate, but the high-energy phosphate levels are still unperturbed, even when Mb continues to desaturate to ~50% of the control level. Even at the P_{O_2} of half-maximal saturation ($[P_{O_2}]_{50}$) of Mb, ~2.39 mmHg (37°C), O_2 is still sufficient to maintain the basal energy demand of the muscle tissue, as reflected in the unperturbed ^{31}P spectra (32). Even though the experimental data at present will not yield the precise P_{O_2}, they do give an estimate of the vascular P_{O_2} when intracellular P_{O_2} becomes limiting. Because fully deoxygenated Hb will exhibit both an α-subunit as well as a β-subunit proximal histidyl $N_\delta H$, the appearance of only the β-subunit proximal histidyl $N_\delta H$ signal would suggest that the critical vascular P_{O_2}, as reflected in the HbO_2 saturation, is above the $[P_{O_2}]_{50}$.

Normalization of the deoxyMb signal. Even though the Mb peak is visible in gastrocnemius muscle, the physiological relevance depends on an accurate quantitation of the percent Mb desaturation. Previous studies have occluded blood flow for 6–8 min to produce a steady-state deoxyMb signal, which is then assumed to be the totally desaturated Mb state. Under the presumed totally desaturated state, the deoxyMb signal intensity represents the 100% deoxygenation point and is the basis for normalizing all the other Mb signals during different exercise/ischemia protocols (2, 4, 25, 29).

Reaching a steady-state level is a functional definition of the deoxygenated state but should be assessed carefully. If the cuff pressure is set at 180 mmHg, within 5 min the deoxyMb signal will reach a steady state. That steady-state value is higher if the cuff pressure is first set at 180 mmHg for 5 min and then stepped up to 265 mmHg for the remaining 5 min ($P < 0.05$), suggesting that the steady state is a necessary but not a sufficient condition to establish a fully desaturated state. The ^{31}P spectra also do not indicate a critical P_{O_2}.

Even at cuffing pressure of 265 mmHg, Mb may still not be completely desaturated. In totally ischemic muscle the deoxyMb signal should exhibit a temperature decrease. In fact, blood flow occlusion in human thenar muscle produces a tissue temperature drop to 27°C, which is directly reflected in the deoxyMb chemical shift (16). Such a temperature drop is also consistent with the observation in ischemic blood-perfused heart (14).

A comparative analysis using a leg cast phantom containing 0.2 mM metHb as the calibration standard indicates that the Mb signal at the end of the exercise-cuff experiment is only 50% desaturated (13, 26, 28, 38, 45). Such a phantom will overestimate the normalization intensity for the deoxyMb signal and relies on an approximate Mb tissue concentration. Clearly the P_{O_2} derived from the steady-state intensity of the deoxyMb signal vs. the P_{O_2} derived from a leg phantom can differ by a factor of two. However, any interpretation based on the normalized steady-state value of the deoxyMb signal intensity must assess the quantitation judiciously (25, 29, 43, 44).

Cellular temperature. The hyperfine shifted signals of deoxyMb and deoxyHb present an opportunity to investigate the link between released heat and chemical reactions during muscle contraction. In general, paramagnetic signals exhibit a large temperature-dependent chemical shift, which, if calibrated, yields the cellular temperature. Studies of the solution-state Mb and Hb have established that the human deoxyMb proximal histidyl $N_\delta H$ signal resonates at 80.3 ppm, and the corresponding human deoxyHbA α- and β-subunit signals resonate at 76.4 and 64.4 ppm, respectively, at 25°C. The deoxyMb and deoxyHb β-subunit $N_\delta H$ signals maintain a 4.1-ppm difference over the physiological temperature range (5, 16). In the experiment, the deoxyMb signal at 78 ppm reflects a tissue temperature of 33°C, whereas the deoxyHb signal at 73 ppm reflects a blood temperature of 37°C. Although the core body temperature is 37°C, the skin temperature can be as low as 27°C, depending on the ambient condition (31). Given the surface coil dimension and placement, most of the detected Mb signal originates most likely from the superficial layer and reflects a tissue temperature that is consistent with other reported measurements (16, 31, 35, 45). In contrast to previous measurements, however, the deoxyMb signal monitors specifically the intracellular temperature. Additional studies with comparative temperature determination are under way to define carefully the Mb approach to measure cellular temperature.

In conclusion, ^{1}H NMR can discriminate the deoxyMb proximal histidyl $N_\delta H$ signals from the corresponding signal of deoxyHb from human gastrocnemius muscle. Both the Mb and Hb signals are detected with 45-s time resolution. The deoxyMb signal intensity reaches a steady-state level within 5 min of pressure cuffing the leg, and its kinetics match the ones observed in the NIRS experiments, implying that the NIRS signals are actually monitoring Mb desaturation in these experiments. Since only the deoxyHb β-subunit proximal histidyl $N_\delta H$ exhibits a signal, the extracellular P_{O_2} is most likely above the $[P_{O_2}]_{50}$ when the intracellular P_{O_2} becomes limiting.

Perspectives

During exercise both blood flow and metabolism respond to meet the increased O_2 demand. Despite numerous experiments, the specific mechanism that limits respiration is still in question. Measuring cellular O_2 itself in exercising muscle has posed one of the most daunting hurdles. Although NIRS has followed the composite change in Mb and Hb desaturation, it cannot discriminate Mb desaturation from Hb desaturation and has often assumed that the NIRS signal arises predominantly from Hb. Our study demonstrates that the proximal histidyl $N_\delta H$ ^{1}H NMR signals of Mb and Hb from exercising human gastrocnemius muscle are detectable and can reflect the tissue P_{O_2}. In fact, the Mb desaturation kinetics match the NIRS observed kinetics, which suggests that the NIRS signal under these particular experimental conditions originates from Mb. The experimental results set the stage for definitive study of O_2 regulation in exercising skeletal muscle.

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