Observing the deoxy myoglobin and hemogobin signals from rat myocardium in situ

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Abstract ^{1}H NMR proximal histidyl $N_\delta H$ signals of deoxy hemoglobin (Hb) and myoglobin (Mb) are distinguishable in the rat myocardium in situ. In the normoxic resting state, the blood and tissue pO_2 is sufficient to saturate both Mb and Hb. No deoxy Mb or Hb signals are detected. Under 12% inspired O_2, the erythrocyte Hb is partially desaturated and yields the α and β proximal histidyl $N_\delta H$ signals of deoxy Hb. The detection of the Hb signals clarifies the debate about the NMR visibility of erythrocyte Hb in vivo and augments the strategy to observe tissue pO_2.

Key words: Myoglobin; Hemoglobin; Nuclear magnetic resonance; Myocardium

1. Introduction

In aerobic metabolism the oxygen supply must match demand to maintain cellular viability. Although researchers have investigated the O_2 cascade from lung to mitochondria, they have encountered formidable challenges in determining the regulatory steps between the vasculature and the mitochondria. Perhaps the most difficult hurdle is the measurement of pO_2 in vivo [1].

Both optical and NMR techniques have relied on the myoglobin (Mb) and hemoglobin (Hb) signals as indicators of the intracellular and vascular pO_2. With optical techniques, however, the myoglobin (Mb) and hemoglobin (Hb) signals overlap and are difficult to separate [2–4]. With magnetic resonance methods, the ^{1}H NMR signals of the proximal histidyl $N_\delta H$ and the Val E11 signals myoglobin (Mb) can also reflect the cellular pO_2 in tissue [5–7]. Indeed the ^{1}H NMR Mb signals in the buffer perfused myocardium have yielded insight into the critical oxygen level under different physiological conditions [8,9].

Whether the NMR technique can distinguish the Mb from Hb signals in myocardium or skeletal muscle in situ remains an open question. At issue is the visibility of the Hb signals as well as the potential spectral overlap with the Mb signals. Even though Hb in erythrocyte is approximately 9 mM, whereas Mb in the myocyte is only 0.1 mM, no Hb signal, however, has ever been reported in skeletal muscle and open chest myocardium studies, despite the visibility of the proximal histidyl $N_\delta H$ signals of solution Mb and Hb at 79 and 76 ppm as well as 64 ppm at 25°C, respectively [6,10–12].

Even though the Hb concentration is high, its vascular volume and therefore signal sensitivity is still very low and difficult to detect. Some researchers, however, have asserted that the cellular environment increases the Hb rotational correlation time and broadens the signal beyond detection. Erythrocyte Hb should not exhibit any detectable signal, implying that previously reported observation of erythrocyte Hb signals may arise from in vitro experimental artifacts [13–16]. Such a conclusion opposes the findings from numerous studies, indicating that the erythrocyte Hb signal should indeed be NMR visible and that the cellular microviscosity does not impede the Hb rotational diffusion [10,17–22].

The discrimination of the Hb signal is a crucial point, since it can be mistaken for Mb, given the broad line widths in some in vivo NMR spectra. On the other hand, the detection of the Hb signal, along with the Mb signal, would provide unique insights into the oxygen gradient between the vasculature and cell in vivo.

We have investigated the visibility of the deoxy Mb and Hb signals in the intact rat myocardium and report that both the proximal histidyl $N_\delta H$ signals of deoxy Mb and Hb from the myocardium are distinguishable. Since the experiments are conducted with the rat myocardium in situ, no in vitro experimental artifacts can contribute to the detection of the Hb signal. The results form a basis to map now both the intracellular and extracellular pO_2 in blood perfused tissue.

2. Materials and methods

2.1. Animal preparation

Male Sprague-Dawley rats (400–450 g) were anesthetized by an intraperitoneal injection of 60 mg/kg pentobarbital. Additional pentobarbital was administered in 15 mg doses every 45–60 min via an intraperitoneal catheter. The animal was positioned prostrate, and the heart was centered on the surface coil. For the hypoxia experiments, a breathing tube was inserted into the trachea and was connected to a ventilator. Two separate flow meters regulated the N_2 and O_2 gas flow to produce the different O_2 mixtures. Stroke volume was maintained between 2.5 and 3 ml; ventilation rate was 60–65 strokes per min. Heart rate and mean blood oxygen saturation was measured by placing the oximeter sensor (Nellcor) probe on the tail. During the control period, the animal was ventilated with 100% air (21% O_2). During the hypoxia phase, the percentage of oxygen was decreased with N_2. The animals were subsequently killed with a pentobarbital injection of 150 mg/kg.

2.2. NMR

All ^{1}H NMR signals were collected on a General Electric Omega 7T spectrometer. A home-built 30 mm diameter single-loop surface coil was used to detect the signals. At coil center, the 90° pulse length was 38 μs. Multi-slice spin-echo images determined the heart location and guided the animal positioning, relative to the coil center and plane. Imaging parameters were as follows: field of view 100 mm, slice thickness 2 mm, slice separation 3 mm, repetition time 50 ms, echo time 18 ms, and data block size 128. A microsphere provided a calibration check of the image dimensions. From the coil, the surface

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Abbreviations: DSS, dimethylsilapentane sulfonate; Mb, myoglobin; Hb, hemoglobin; pO_2, partial pressure of oxygen

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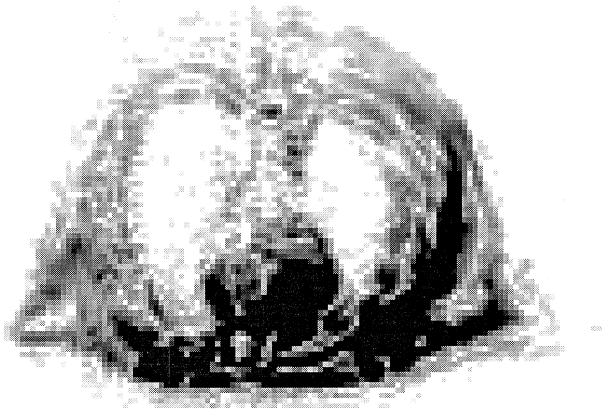

Fig. 1. A transverse gradient-echo image obtained from an intact rat. The animal is positioned such that the heart is centered on top of a 3 cm surface coil. The imaging parameters are as follows: slice thickness, 2 mm; field of view, 100 mm; TR, 50 ms; TE, 18 ms; and number of averages, 4. The image shows that the subcutaneous fat layer comprises the first 3 mm and that the heart volume is about 1 cm^3. The lungs appear as light intensity regions, and the center of the heart is 8 mm from the coil.

tissue layer comprised the first 3 mm, and the center of the heart was at approximately 8 mm depth. Total heart volume was about 1 cm^3. Magnet shimming resulted in a water line width of 100 Hz. A selective pulse then excited the deoxy Mb His-F8 $N_\delta H$ [7]. A typical ^1H spectrum consisted of 10 000 transients and required a total acquisition time of 7 min. The spectral width was 60 kHz; data block size 4000. All FIDs were apodized with Gaussian-exponential multiplication before Fourier transformation. Baseline correction was applied as previously described [23]. Spectra were referenced to water at 4.76 ppm at 25°C, which was in turn referenced to DSS.

3. Results

In all experiments, the whole, intact rat is the animal model. Except for anesthesia, no other surgical intervention is used. A typical experimental setup is shown in Fig. 1. The ^1H transverse image shows that the whole rat is positioned on top of a ^1H surface coil, such that the heart is centered. The lungs correspond to the two regions with minimal signal intensity, and the rat heart is positioned directly above the coil center.

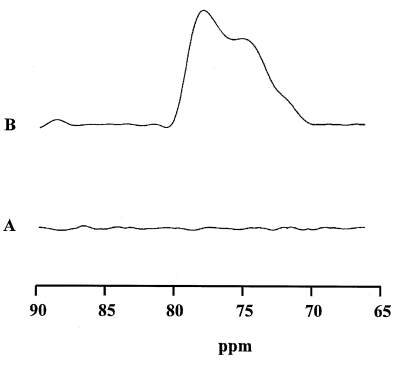

Fig. 2. ^1H NMR spectra obtained from rat myocardium in situ (A) during control and (B) after intraperitoneal injection of pentobarbital. During the control period, no signal appears in the spectral region between 60 and 100 ppm. With pentobarbital injection both the deoxy Mb and Hb proximal histidyl $N_\delta H$ signals appear at 77.0 ppm and 75.2 ppm respectively. The Mb/Hb ratio is approximately 1:1. A 180° excitation pulse reduces any signal contamination from muscle.

During the control period, the ^1H NMR spectrometer does not record any proximal histidyl $N_\delta H$ signals of Mb or Hb (Fig. 2A). Both blood and tissue are then well oxygenated. However when the animal is quickly killed, the sudden loss of tissue oxygen produces a signal corresponding to the deoxy Mb proximal histidyl $N_\delta H$ at 77 ppm (Fig. 2B). On the shoulder of the peak, about 2 ppm upfield of the Mb signal in approximately 1:1 area ratio, is a signal assigned to the proximal histidyl $N_\delta H$ signal from the β subunit of Hb. The assignment is consistent with previous ^1H NMR studies of solution and erythrocyte Hb [10,12,17,24]. Under these experimental conditions, the α proximal histidyl $N_\delta H$ signal approximately 12 ppm upfield is not observed. Although skeletal muscle also contains Mb, the observed signal originates predominantly from the myocardium, since the 180° excitation pulse reduces by 80% the surface muscle signal contamination, as confirmed by 1-dimensional image profile analysis.

The visibility of the Hb signal is more evident in hypoxia experiments, where the inspired oxygen will partially saturate Hb. Under normoxic conditions (21% O_2), the blood and tissue pO_2 is sufficient to saturate both Hb and Mb. No signal of the proximal histidyl $N_\delta H$ from deoxy Mb or Hb is observed (not shown). As the inspired pO_2 decreases to about 12% O_2, the proximal histidyl $N_\delta H$ signals of Hb appear prominently, both at 65.1 and 77.8 ppm (Fig. 3A). The chemical shift positions of the signals are in excellent agreement with those reported in solution Hb studies [10,24]. However, no deoxy Mb signal is detected. The cellular pO_2 is still sufficient to saturate Mb. Decreasing the initial pulse length by 0.5 and 0.25 accentuates the surface signals. However the Hb signal intensity (Fig. 3B,C) decreases, as expected for a signal originating from the heart rather than muscle tissue.

Detecting the distinct signals of deoxy Mb and Hb is further substantiated in experiments where the rat is first subjected to hypoxia and is then killed. Under control conditions, the spectra show no signal in the region from 60–100 ppm (Fig. 4A). With the described experimental protocol, the signal of deoxy Mb appears at 77.9 ppm, while the β $N_\delta H$ of Hb signal now appears at 73.9 ppm (Fig. 4B). An additional peak corresponding to the α subunit $N_\delta H$ of Hb is detectable at 61.7 ppm. The intensity and linewidth distortion observed in the α proximal histidyl $N_\delta H$ originates from the pulse and phase characteristics of the selective excitation [17,25]. The ratio of Hb/Mb is approximately 0.28/1.

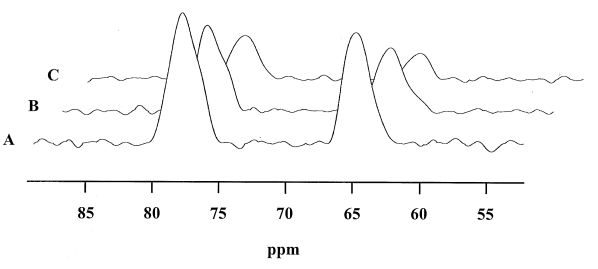

Fig. 3. ^1H spectra from the in situ rat myocardium during hypoxia observed at different tissue depths. Under control condition when the animal is breathing 21% O_2, no signal is detected in the 60–100 ppm region. With 12% O_2, the α and β proximal histidyl $N_\delta H$ signals of Hb appear. The signals attenuate as the surface pulse lengths decrease from (A) the π surface pulse to (B) the π/2 pulse and finally to (C) the π/4 pulse. No deoxy Mb signal is visible.

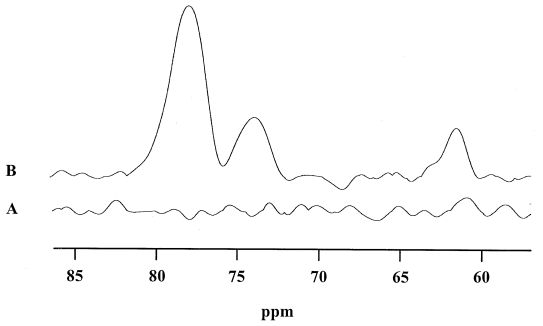

Fig. 4. ^{1}H spectra from the in situ rat myocardium during hypoxia followed by pentobarbital injection (A) during control (21% O_2) and (B) after hypoxia (12% O_2) and pentobarbital injection. During control the His F8 $N_\delta H$ signals of deoxy Mb and deoxy Hb are not observed. With O_2 concentration at 12% and pentobarbital injection, three peaks appear, corresponding to the proximal histidyl $N_\delta H$ of deoxy Mb (77.9 ppm) and the α and β deoxy Hb (61.7 and 73.9 ppm). The amplitude and linewidth distortion observed in the α proximal histidyl $N_\delta H$ originates from the property of the selective pulse.

4. Discussion

4.1. Detecting the Hb signal in vivo

The deoxy Mb proximal histidyl $N_\delta H$ signal at 78 ppm appears in a unique spectral window of the ^{1}H spectrum and has yielded insight into oxygen regulation in perfused myocardium and skeletal muscle [13,17,26]. Because deoxy Hb also resonates in the same spectral region, the measurement of any erythrocyte Hb interference is critical in determining accurately the intracellular pO_2, reflected in the Mb signal. So far the reported skeletal muscle studies have not detected any Hb signal and have presumed that the cellular microviscosity increases the rotational correlation time to broaden the linewidth beyond NMR detection [16]. But clearly the in situ myocardium does exhibit a detectable deoxy Hb signal, which cannot be attributed to any in vitro experimental artifact.

The deoxy Hb signals appear prominent as the inspired pO_2 decreases. Although the cell preserves its intracellular O_2 level and shows no Mb desaturation, the proximal histidyl $N_\delta H$ signals from the α and β subunits of erythrocyte deoxy Hb are clearly visible at 65.1 and 77.8 ppm, in precise agreement with the expected chemical shift positions observed in protein solution studies [10,12,17,24]. These signals arise predominantly from the myocardium as confirmed by pulse length dependent variation in the signal intensity. As the excitation pulse length decreases, any surface tissue signals should increase. Instead the deoxy Hb signal intensity decreases correspondingly, consistent with a predominant myocardial localization. Such an observation is inconsistent with any major signal contribution from superficial muscle. A visual inspection of the open chest animal after the NMR experiments also confirms a noticeable presence of deoxygenated Hb in the myocardial tissue but not in skeletal muscle.

The extent of Mb/Hb deoxygenation, however, is dependent upon the physiological conditions. When the rat is killed, the myocardium exhibits a deoxy Mb signal along with an upfield shoulder peak, assigned to the Hb β proximal histidyl $N_\delta H$. The upfield peak is not associated with Mb from another microenvironment, since the temperature dependent chemical shift of the Mb signal would then predict a temperature gradient from 29 to 41°C, which is quite unlikely within a cell [17]. Moreover no other hyperfine shifted signal in tissue has been reported in this spectral region. Such an observation of only the β proximal histidyl $N_\delta H$ of Hb is consistent with the preliminary data from regional ischemic rat myocardium and human gastrocnemius muscle (Tran et al. and Kreutzer et al., unpublished observations). The observation is consistent the α subunit binding O_2 more tightly than the β subunit [27,28].

Because the erythrocyte Hb signals are detectable, the analysis of the deoxy Mb signal in vivo must take the potential interference into account, since under different physiological conditions, either the Hb β or the α and β proximal histidyl $N_\delta H$ signals can appear in the ^{1}H spectra. Spectral deconvolution cannot rely solely on the presence of the α deoxy Hb signal at 64.4 ppm to distinguish the presence of any contaminating Hb peak next to the Mb signal.

4.2. Detecting the Mb signal in vivo

In the normoxic myocardium in situ, the deoxy Mb signal is not visible. The observation is consistent with open chest canine or rat myocardium results [15] (Kreutzer et al., unpublished observation). Even under hypoxic condition, 12% inspired O_2, when the deoxy Hb signal is detectable, no deoxy Mb signal appears. Unlike the perfused heart model, the in vivo myocardium exhibits compensating physiological mechanisms that maintain a sufficient cellular pO_2 as the inspired pO_2 declines [8,9].

In all cases the resting cellular pO_2 is sufficient to maintain MbO_2 in a saturated state. Such an observation appears inconsistent with the postulate of a partially saturated MbO_2 state in the resting myocardium. If the deoxy Mb signal in Fig. 2B represents the total Mb desaturation, then under normoxic conditions MbO_2 saturation must exceed 75% to avoid signal detection during the control period. Since the Mb signal in Fig. 2B is probably not fully desaturated, the MbO_2 saturation in a contracting myocardium must be well above 75% under resting conditions.

4.3. Temperature difference between the Mb and Hb microenvironment

Both the Mb and Hb proximal histidyl $N_\delta H$ signals can reflect their respective cellular environment. Solution studies show a temperature dependent shift of 0.3 ppm/°C over the physiological temperature range, and the deoxy Mb and the deoxy Hb β subunit signals maintain a 2.5 ppm separation [17]. At 25°C, the deoxy Mb proximal histidyl $N_\delta H$ resonates at 79 ppm, whereas the corresponding Hb signals appear at 76 and 64 ppm. The spectra in Figs. 2–4 demonstrate that the cellular temperature of Mb and Hb can vary over a substantial range depending upon the physiological condition. In the current study, the temperature range, reflected in the deoxy Mb signal, varies from 29 to 33°C, whereas for Hb it varies from 20 to 33°C.

5. Conclusion

The study has demonstrated that both the ^{1}H NMR deoxy Mb and Hb signals are distinguishable in the intact rat myocardium in situ. The percentage of Mb vs. Hb contribution depends upon the specific physiological conditions. The absence of either signal during the control period implies that MbO_2 is well saturated in the resting physiological state.

Under certain physiological conditions, only the β subunit deoxy Hb proximal histidyl $N_\delta H$ signal is detected. The capacity to measure both the Mb and Hb signals in vivo opens a new opportunity to investigate the relationship between vascular and intracellular pO_2 in myocardium and skeletal muscle.

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