^{1}H Nuclear Magnetic Resonance Studies of Sarcoplasmic Oxygenation in the Red Cell-Perfused Rat Heart

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ABSTRACT The proximal histidine $N_\delta H$ proton of deoxymyoglobin experiences a large hyperfine shift resulting in its ^{1}H nuclear magnetic resonance (NMR) signal appearing at ~76 ppm (at 35°C), downfield of the diamagnetic spectral region. ^{1}H NMR of this proton is used to monitor sarcoplasmic oxygen pressure in isolated perfused rat heart. This method monitors intracellular oxygenation in the whole heart and does not reflect oxygenation in a limited region. The deoxymyoglobin resonance intensity is reduced upon conversion of myoglobin to the ferric form by sodium nitrite. ^{1}H resonances of the $N_\delta H$ protons of the α and β subunits of bovine deoxyhemoglobin do not interfere with the measurement of myoglobin deoxygenation in blood-perfused rat heart. We find that steady-state myoglobin deoxygenation is increased progressively (and reversibly) as oxygenation of the perfusing medium is decreased in both saline and red blood cell-perfused hearts at constant work output. An eightfold increase in the heart rate of the blood-perfused heart resulted in no change in the deoxymyoglobin signal intensity. Intracellular P_{O2} of myoglobin-containing cells is maintained remarkably constant in changing work states.

INTRODUCTION

Myoglobin, a monomeric, oxygen-binding protein present at high concentration (200 μmol/kg wet weight) (Schuder et al., 1979) in the sarcoplasm of heart cells, delivers oxygen to mitochondria to form an integrated system supplying oxidatively generated ATP to the working heart (Wittenberg and Wittenberg, 1989). Expression of the myoglobin gene is subject to physiological control in response to hypoxia and other factors (Williams, 1990). Short-term controls regulate the instantaneous rate of aerobic ATP synthesis requiring increased oxygen uptake in the face of changing demand. Several recent reports have illuminated the role of myoglobin in maintaining intracellular oxygen supply and maximum work output in the heart. It has been shown that in the absence of functional myoglobin the phosphocreatine level in the heart falls faster (Taylor et al., 1986) and that cardiac function is diminished during hypoxia (Braunlin et al., 1986). Gayeski and Honig (1991) have observed that in working heart, intracellular myoglobin is close to half saturation with oxygen in several species (including dogs and rats), over a wide range of ventricular work and arterial oxygen supply. Because cardiac intracellular myoglobin is near equilibrium with oxygen (Tamura et al., 1978; Wittenberg and Wittenberg, 1985), these findings show that cardiac intracellular P_{O2} is between 1 and 5 torr, depending on the value taken for the P_{O2} at which intracellular myoglobin is half saturated with oxygen. Within each cell, the oxygen pressure is kept everywhere the same (without detectable oxygen pressure gradients) by "buffering" of free O_2 by reversible binding to myoglobin, and by myoglobin-facilitated diffusion of oxygen from regions of high oxygen pressure (e.g., capillary wall) to regions of low oxygen pressure (e.g., mitochondria), (Cole et al., 1982). Nuclear magnetic resonance (NMR) evidence shows that myoglobin is mobile and undergoes unhindered rotational diffusion in the heart (Livingston et al., 1983). Because myoglobin is maintained, partially deoxygenated in situ myocytes are protected from short-term fluctuations in oxygen supply. The respiration of cardiac myocytes does not become oxygen limited until myoglobin is completely deoxygenated (Wittenberg and Wittenberg, 1985) and myoglobin deoxygenation in the saline-perfused heart precedes the loss of intracellular energy reserves (Kreutzer and Jue, 1991).

NMR spectroscopy provides a noninvasive and specific technique to measure metabolites in cells and tissues. Because NMR is a nondestructive technique, time courses of different events can be followed in the same preparation, enabling one to study the same heart under a variety of perfusion and work states. The method permits real-time continuous monitoring, and evaluation of reversibility. ^{31}P NMR has been applied extensively to study intracellular high-energy phosphate levels, intracellular free Mg^{2+}, and intracellular pH of heart (Radda, 1986). High-energy phosphate signals reflect cellular bioenergetics and serve as an indirect measurement of intracellular oxygenation. However, under certain conditions, such as during moderate hypoxia, the ^{31}P NMR signals may not be sufficiently sensitive to accurately report on changes in oxygen delivery/utilization. Recently Jue and co-workers (Kreutzer and Jue, 1991; Kreutzer et al., 1992) have demonstrated that ^{1}H NMR is a very sensitive direct marker of myoglobin oxygenation in perfused heart. The ^{1}H NMR method monitors oxygenation in the entire volume of the whole heart rather than reporting oxygenation at the surface of the heart or in a limited region of the left ventricle as do optical techniques. The ^{1}H NMR method monitors the exchangeable proximal histidine imidazole proton of deoxymyoglobin. This is a uniquely resolvable proton

Received for publication 19 July 1994 and in final form 7 February 1995.
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0006-3495/95/05/2129/08 $2.00

in the NMR spectrum, which experiences a large hyperfine shift (to ~79 ppm at 25°C) and rapid relaxation (Livingston et al., 1983). This resonance disappears upon oxygenation of myoglobin in solution, consistent with a shift of the heme iron from the paramagnetic electronic state in deoxymyoglobin to the diamagnetic electronic state in oxymyoglobin (Livingston et al., 1983). The resonance has been shown to be sensitive to temperature but does not exhibit any pH-dependent shift between pH 6 and 7 (La Mar et al., 1978).

Cellular P_{O2} reflects the balance between O_2 uptake and delivery, and delivery depends on capacity, oxygenation, and flow rate of the perfusion medium. To validate the method we have designed our study to examine myoglobin deoxygenation under a variety of oxygenation levels and flow rates as well as during perfusion with blood, which has a higher O_2 capacity than saline-based media. Kreutzer and Jue (1991) have previously observed the deoxymyoglobin resonance in isolated saline-perfused well-oxygenated hearts at flow rates below 2 ml/min. In our study, using the ^{1}H NMR method we have found that myoglobin is partially deoxygenated in red blood cell-perfused rat heart studied at flow rates of 0.3–4 ml/min even at low heart rates. The effects of sodium nitrite (which inactivates myoglobin), decreased oxygen pressure, and no-flow ischemia on the oxygenation of cardiac cells perfused with minimal essential medium (MEM) or red blood cell-perfused hearts were investigated. Finally we determined the effect of an eightfold-increased electrical pacing on the maintenance of intracellular oxygen pressure of the heart.

MATERIALS AND METHODS

Heart preparation and perfusion

Adult male Wistar rats (400–500 g, obtained from Charles River Laboratories, Wilmington, MA) were heparinized and decapitated in accordance with institutional guidelines. The hearts were isolated and perfused in the Langendorff mode at 35°C at constant pressure (90 cm H_2O) with a modified HEPES-buffered MEM containing 117 mM NaCl, 5.7 mM KCl, 9.0 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 21.1 mM HEPES, 10.8 mM glucose, 10 mM taurine, 2 mM glutamine, 2 mM $CaCl_2$, and 4 mM beta-hydroxybutyrate; pH was adjusted to 7.2 with NaOH. The solution was 285 milliosmolar and was gassed with 95% O_2 and 5% CO_2. The perfusion apparatus consisted of two water-jacketed Langendorff vessels connected via a Y joint to a water-jacketed "umbilical cord" delivering perfusion medium to the heart. In some experiments, after initial perfusion with MEM, we began red-cell perfusion. Bovine blood (containing citrate) was obtained from Max Insel Cohen (Livingston, NJ). One liter of the blood was centrifuged for 5 min at 3500 rpm, after which the buffy coat was removed. The blood was then rinsed twice with 500 ml MEM (centrifuged for 10 min at 1300 rpm) and was then stored at 4°C until used for the experiment. Bovine serum albumin (1%), heparin (40 units/ml), and 20 μM adenosine (in all experiments except one heart-perfused at a flow rate of 0.3 ml/min) were added at the time of the NMR experiments. The perfusing blood in the second Langendorff reservoir was passed through an inline filter to remove particulates and then through a hollow fiber cellulose acetate dialysis unit (Baxter Healthcare Corporation, Deerfield, IL). The gas-equilibrated MEM solution from the first reservoir was then used as a dialyzing fluid to exchange gas with the blood in the dialyzer. The temperature of the bath supplying circulating water to the water-jacketed perfusion apparatus was adjusted such that the temperature of perfusate at the cannula was 35°C. Humidified gas was delivered directly to the MEM. The content of oxygen, nitrogen, and CO_2 delivered to the medium was regulated by a mass flow controller (Tylan, Torrence, CA).

A small latex balloon (size 4, obtained from Hugo Sachs Elektronik, March-Hugstetten, Germany) attached to a fluid-filled line (saline) and connected to a Gould P23ID pressure transducer and Gould 2200S recorder (Gould Electronics, Valley View, OH) was introduced into the left ventricle for isovolumic perfusion. Left ventricular pressure was increased by incrementing the balloon volume using a calibrated Gilmont syringe (Gilmont Instruments, Barrington, IL). The balloon volume was adjusted to give an end diastolic pressure of ~10 mm Hg. Constant flow experiments with MEM were performed using a peristaltic pump in the inflow line. Ischemia was induced by stopping the medium flow. Cardiac pacing in the NMR magnet was accomplished with agar electrodes as described by Burstein and Fossel (1987). Silver pacing leads were attached to 6-inch-long agar electrodes connected to shielded wires leading to a Grass S44 physiological stimulator. Heart rate and flow rate were monitored during the experiments.

NMR spectroscopy

^{1}H spectra were acquired using a GE Omega/Bruker 400WB spectrometer equipped with a 20-mm ^{1}H/^{19}probe with a Helmholtz-type NMR observation coil. Hearts, after attachment to the cannula and umbilical cord, were placed in 20-mm (outer diameter) air-filled sample tubes (perfusate was removed from the bottom of the NMR tube by an outflow line attached to a peristaltic pump). The spectrometer was not field-frequency locked, and the magnetic field was homogenized using the ^{1}H signal of H_2O (typical linewidth was 50 Hz). Typically each spectrum resulted from signal averaging 15,000 transients acquired using the 1331 solvent suppression pulse sequence (Hore, 1983) to suppress the H_2O resonance. In some experiments 50,000 transients acquired with shorter recycle time were signal averaged to achieve better signal-to-noise ratio. The pulse sequence was tailored to give maximum excitation at 76 ppm. The sequence recycle time was ~0.04 s (each spectrum required ~10 min of data acquisition). The 90° pulse width was typically 40 μs. The spectral width was 100,000 Hz, and 2048 data points were used. The ^{1}H resonances were referenced to the water peak. Data were processed with the standard Omega software (G. E., Fremont, CA) using a 200-Hz line broadening function and polynomial baseline correction. The baseline points were selected using the ischemic spectrum of each heart; the same points were then used for correction of all earlier spectra from that heart. The % oxygenation of myoglobin was determined by comparing the areas of the ^{1}H resonance under various conditions with that acquired under no-flow ischemia as described by Kreutzer and Jue (1991).

^{31}P spectra were acquired with a 20 mm broad-band probe. Typically each ^{31}P NMR spectrum resulted from signal averaging 200 transients acquired with a 45° radio frequency pulse (16 μs) and a 2.0 s sequence recycle time (each spectrum required ~6 min of data acquisition). The spectral width was set at 10,000 Hz. Data were processed using the standard Omega software. Time domain data were multiplied with a 50-Hz linebroadening function to improve the signal-to-noise ratio.

In some experiments we used a perfusion medium containing no inorganic phosphate (P_i) to accurately measure intracellular pH under a variety of conditions. The chemical shift of the ^{31}P resonance of P_i was then used to measure intracellular pH (pH_i) from the following equation (Gupta and Wittenberg, 1991):

$$pH = 6.73 + \log(\partial_{obs} - 2.90)/(5.70 - \partial_{obs}) \quad (1)$$

where ∂_{obs} is the chemical shift difference between the P_i and phosphocreatine resonances in ppm.

Statistical analysis was performed where possible. Data are reported as mean ± SE. The null hypothesis was tested using a paired Student's t-test for analysis of data obtained with one heart, and unpaired Student's t-test for comparison of data from different hearts. Data are considered significantly different when $p \leq 0.05$.

RESULTS AND DISCUSSION

The spectrum of a solution of purified myoglobin, deoxygenated in the NMR tube at 35°C, exhibited a peak at 76 ppm assigned as the proximal histidine exchangeable $N_\delta H$ (data not shown). This resonance was not observed when oxymyoglobin or nitrite-treated metmyoglobin solutions were used. Subsequent to their ^1H NMR studies on deoxymyoglobin in perfused hearts (Kreutzer and Jue, 1991), Kreutzer et al. (1992) reported the observation of the ^1H NMR signal of a valine methyl group of oxymyoglobin as a marker of myoglobin oxygenation in myocardium. Under our experimental conditions we have been unable to use this resonance to monitor myoglobin oxygenation accurately. We did observe this resonance in a sample containing 2.7 mM oxymyoglobin; however, the oxymyoglobin concentration in the heart is ~200 μM, and the amount of time required for signal averaging was prohibitively long. Because of the sensitivity and reasonable time for acquisition of data obtained monitoring the deoxymyoglobin histidine resonance we have performed all of our experiments monitoring the deoxymyoglobin signal.

Fig. 1 shows that, as expected (Kreutzer and Jue, 1991), the magnitude of the deoxymyoglobin resonance increased progressively as the oxygen content of the gas mixture was decreased from 95% to 20% and was finally switched to zero oxygen, 95% nitrogen. The % deoxymoglobin was indistinguishable from 0 during perfusion with 95% 0_2,5% CO_2 equilibrated MEM ($n = 5$) (representative spectra are shown in Figs. 1 and 3) and increased significantly to 33.5 ± 5.3% ($p < 0.003$, $n = 4$ hearts) when the oxygen pressure of the perfusion medium was decreased by equilibration with 15–20% 0_2, 5% CO_2. Stopping the flow entirely gave rise to a downfield-shifted ischemic signal. The magnitude of this shift is consistent with a drop in temperature of up to 10°C, which is within the expectations with this perfusion system, given that the heart was not bathed in warmed medium. The signal shifted upfield when the flow of warmed medium was restarted with 95% nitrogen and sodium nitrite. We have previously shown by absorption spectroscopy that 1–2 mM sodium nitrite converts intracellular myoglobin to the inactive high-spin ferric form (Doeller and Wittenberg, 1991) and that the effects can be reversed by removal of nitrite (Gupta and Wittenberg, 1991). During perfusion in the presence of nitrite, the deoxymyoglobin signal, which is normally large in the presence of zero oxygen, 95% nitrogen, began to disappear because of slow anaerobic conversion of intracellular myoglobin to the ferric and ferric nitrite forms. There was very little evidence of a signal in the final spectrum (Fig. 1, *upper trace*), indicating that the conversion of deoxymyoglobin was virtually complete after 90 min. Fig. 2 shows the spectra of a sample of high-spin ferric myoglobin (1 mM) before (Fig 2, *top trace*) and after (Fig. 2, *bottom trace*) an addition of excess nitrite. The middle trace shows a wider region of the spectrum (shown in Fig. 1 *e*) of the perfused heart obtained after 30 min of nitrite perfusion. The low intensity of the hyperfine shifted resonances between 65 and

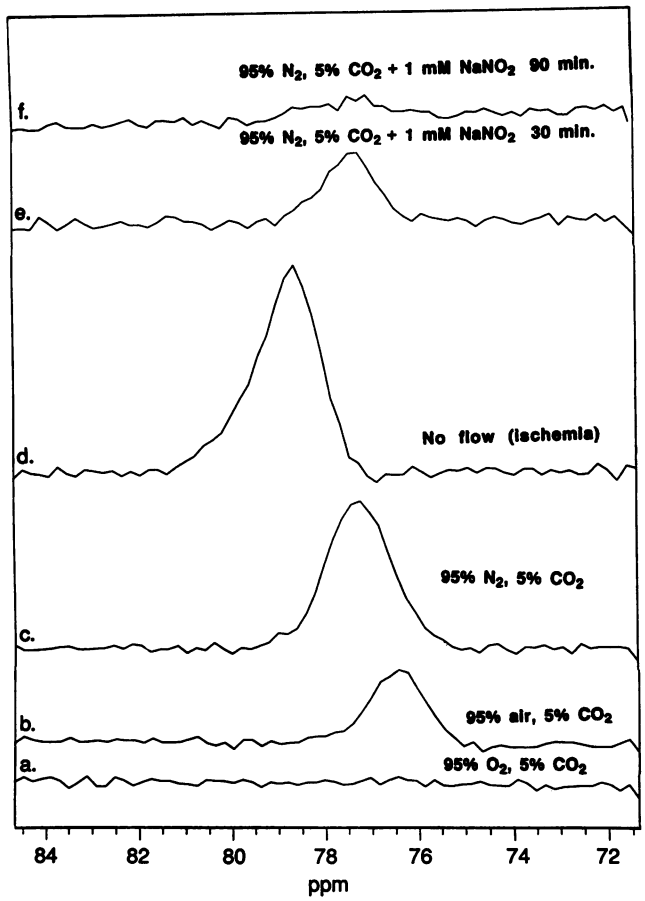

FIGURE 1 ^1H NMR spectra of an MEM-perfused rat heart showing the effect of oxygenation, ischemia, and conversion of myoglobin to ferric myoglobin and ferric myoglobin nitrite (using sodium nitrite) on intracellular deoxymyoglobin. The magnitude of the single spectral peak in each record is proportional to the concentration of deoxymyoglobin in the heart. The % intracellular myoglobin in the deoxy form is vanishingly small during perfusion with medium equilibrated with 95% oxygen (*trace a*) and increases to 30% (*trace b*) and 70% (*trace c*) when the oxygen content of perfusion medium is progressively reduced to 20% and 0%. The ischemia spectrum (*trace d*) shows 100% deoxymyoglobin. After reinitiation of perfusion for 30 min with medium equilibrated with 95% nitrogen and containing 1 mM sodium nitrite, the magnitude of the deoxymyoglobin peak drops markedly (*trace e*; only 30% of intracellular myoglobin is in the deoxy form) and there is an increase in ferric myoglobin and/or ferric myoglobin nitrite resonances as shown in Fig. 2. After 90 min of anaerobic nitrite perfusion, the deoxymyoglobin peak had become vanishingly small (≤ 10%, *trace f*) indicating complete conversion of intracellular deoxymyoglobin to the ferric and ferric nitrite forms. Intracellular oxygen can no longer be monitored, because the endogenous probe has been inactivated. In this experiment and those shown in Figs. 2 and 3, pyruvate replaced β-OH butyrate as substrate, and adenosine and heparin were omitted from the medium.

95 ppm in the lower trace strongly suggests that ferric myoglobin is not the sole product. This suggests that ferric nitrite myoglobin may also be present (Bondoc and Timkovich, 1989). Presumably deoxymyoglobin is not converted to the oxy form, because no oxygen is present. The deoxymyoglobin $N_\delta H$ resonance appears in a region of the spectrum where there are no resonances observed for either ferric or ferric nitrite myoglobin (see Fig. 2, *dashed line*). The spectral features of high-spin ferric myoglobin shown in this figure

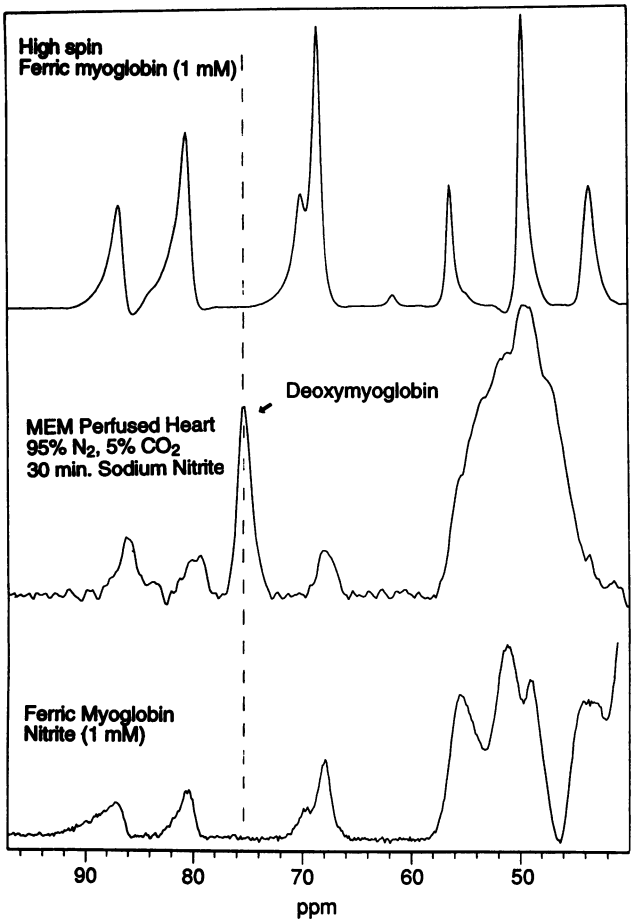

FIGURE 2 Comparison of ^{1}H NMR spectra of the MEM-perfused rat heart anaerobically perfused with sodium nitrite for 30 min with spectra of solutions of 1 mM high-spin ferric myoglobin and of 1 mM ferric myoglobin nitrite. Middle spectrum shows the spectrum of Fig. 1 *e* with expanded scale. 30% of the intracellular myoglobin is still in the deoxy form with its resonance at 76 ppm (- - - - -). The rest of the intracellular myoglobin is converted to ferric myoglobin or its nitrite derivative, as indicated by the additional resonances near 50, 68, 81, and 87 ppm. Note that the deoxymyoglobin peak appears in a region where the spectra of both ferric myoglobin and ferric nitrite myoglobin are clear of spectral features. Horse met-myoglobin (1.0 mM, Sigma Chemical Co.) dissolved in 0.05 M Na-Hepes, pH 7.4 containing 0.5 mM EDTA was used to measure the top spectrum of high-spin ferric myoglobin. Sodium nitrite in large excess (200 mM) was added to this sample to convert ferric myoglobin to the nitrite derivative whose NMR spectrum is shown in the bottom trace.

are very similar to the spectrum of met-myoglobin reported by Bondoc and Timkovich (1989); however, the spectrum of the nitrite-treated sample is somewhat different, probably because of differing pH conditions. Nitrimyoglobin was prepared at pH 5.5 in the experiments reported by Bondoc and Timkovich (1989) while our solutions were maintained at pH 7.4.

Fig. 3 shows that deoxygenation of myoglobin in the MEM-perfused heart increased reversibly with increased beating of the heart in the presence of β agonists. Here the initial spectrum (Fig. 3, *trace a*), in which the medium was equilibrated with 95% O_2, 5% CO_2, again showed no deoxymyoglobin signal. When the oxygen content of the gas mixture was reduced to 15% O_2 while maintaining 5% CO_2, 31% deoxymyoglobin was observed (Fig. 3, *trace b*). The addition of 50 nM norepinephrine, an inotropic agent, while maintaining the same flow rate and perfusate oxygenation, caused the heart rate to increase from 185 to 400 beats per min (bpm). The deoxymyoglobin level visibly increased, as expected, given that the increased work required a higher rate of oxygen uptake to meet the demand for more ATP produced by oxidative phosphorylation (Fig. 3, *trace c*). One h

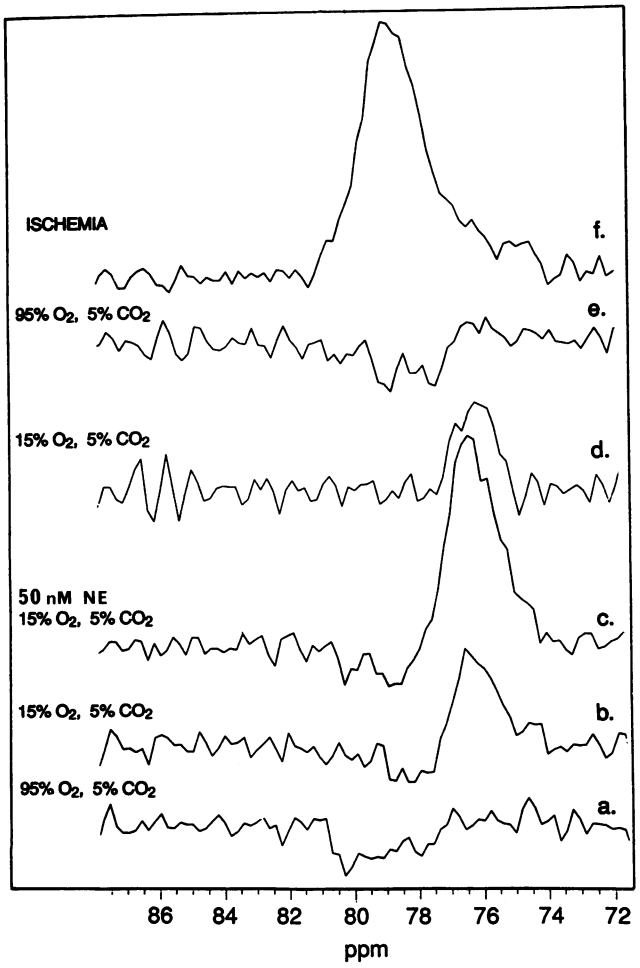

FIGURE 3 ^{1}H NMR spectra of an isovolumic MEM-perfused rat heart showing the reversible effect of perfusate deoxygenation and heart beat stimulation on the deoxygenation of intracellular myoglobin. *Trace a* shows the fully oxygenated control; myoglobin deoxygenation was vanishingly small, and heart rate was 400 bpm. *Trace b* shows that decreasing oxygenation of the perfusion medium to 15% oxygen from 95% decreased intracellular P_{O2} and increased deoxymyoglobin to 31%. The heart rate was 185 bpm. The addition of 50 nM norepinephrine (NE) increased the heart rate from 185 to 400 bpm, which would be expected to increase the oxygen consumption of the heart. *Trace c* shows that the deoxymyoglobin resonance increased to 77% with norephinephrine indicating that the intracellular P_{O2} decreased, (to 1 torr from 6 torr). One h later, the heart rate dropped again to 185 bpm, and deoxymyoglobin decreased to 20% (*trace d*, P_{O2} = 10 torr) comparable to that observed before norepinephrine treatment at this level of medium oxygenation 2 h earlier (*trace b*). Increasing medium oxygenation to 95% oxygen again lowered the deoxymyoglobin resonance to negligible levels, even though heart rate had dropped to 130 bpm (*trace e*). The final no-flow ischemia spectrum is shown in *trace f*.

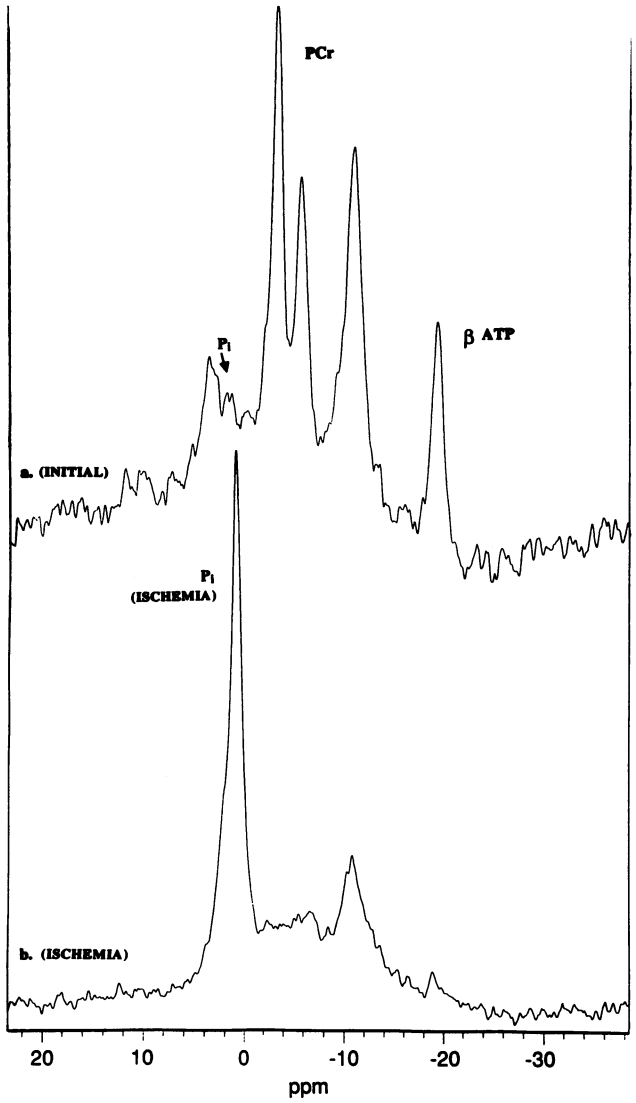

FIGURE 4 ^{31}P NMR spectra of the isovolumic blood-perfused heart. Data were acquired using the parameters described in Materials and Methods with the same heart whose ^{1}H NMR spectra are shown in Fig. 6. The initial spectrum (*a*) was acquired during control conditions before the acquisition of ^{1}H NMR spectra. The final ^{31}P spectrum (*b*) was acquired during no-flow ischemia just after acquisition of the final ^{1}H spectrum. The inorganic phosphate (P_i), phosphocreatine (PCr), and ATP (βATP) resonances are labeled.

later the heart rate dropped to 185 bpm, and 20% deoxymyoglobin was observed, demonstrating that the effects of pacing were reversible (Fig. 3, *trace d*). When the oxygen content of the gas mixture was increased to 95% the deoxymyoglobin resonance intensity was negligible (Fig. 3, *trace e*). Finally the flow was stopped, the heart rate dropped to 0, and myoglobin deoxygenation increased after the first 10 min of ischemia and reached maximal intensity in the subsequent 10 min (Fig. 3, *trace f*). At this point the resonance was considerably downfield shifted because of the expected temperature drop.

We also performed a series of studies with the blood-perfused heart, using washed beef red blood cells at ~45% hematocrit. A typical ^{31}P NMR spectrum of a well-oxygenated blood-perfused heart is shown in Fig. 4 *a* (*upper spectrum*). This spectrum validates the integrity of the blood-perfused beating heart model, because it shows physiological low P_i levels, high phosphocreatine, and ATP levels with pH_i of 6.9. The ischemic heart at the end of a ^{1}H NMR experiment (Fig. 4 *b*, *lower spectrum*) showed negligible phosphocreatine and ATP peaks and high P_i, and the pH_i had dropped to 6.2. Thus ^{31}P NMR is a sensitive assay of compromised energy reserves, although it is not as sensitive to more subtle changes in oxygenation, such as mild to moderate hypoxia (Kreutzer and Jue, 1991).

In well-perfused hearts we have found that any ^{1}H resonance intensity due to deoxyhemoglobin is negligible and does not interfere with the calculation of % deoxymyoglobin. This is not the case in poorly perfused hearts exhibiting partial ischemia with visible pooling of blood in the blood vessels. Under these conditions resonances assigned to the β and α subunits of deoxyhemoglobin (La Mar et al., 1980; Kreutzer et al., 1993) appear in the ^{1}H NMR spectrum at about 74 ppm and 62 ppm, respectively, upfield of the deoxymyoglobin signal (see Fig. 5). Thus, inadequate perfusion is readily detected. Further experiments were not performed on hearts that exhibited deoxyhemoglobin resonances.

Blood has an increased oxygen capacity compared with MEM, and the oxygen affinity of beef red blood cells is similar to that of human red blood cells (Fronticelli and Bucci, 1994). We have measured the P_{50} of the bovine blood used in these studies and find it is 25 torr at 37°C. Fig. 6 shows that the deoxymyoglobin signal in the blood-perfused heart is also well resolved and responds to changes in oxygenation of the perfusing blood. Decreasing the oxygenation of the blood while maintaining heart rate resulted in increased myoglobin deoxygenation. The spectra shown are

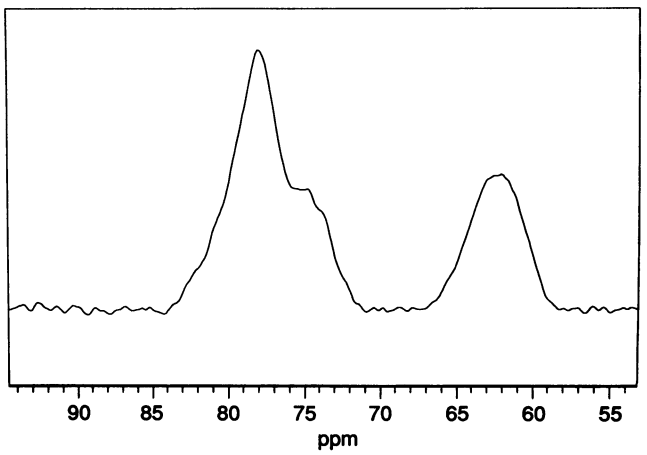

FIGURE 5 ^{1}H NMR spectrum of a blood-perfused rat heart showing the presence of deoxyhemoglobin resonances resulting from a flawed perfusion. The resonances at ~74 and 62 ppm represent deoxyhemoglobin signals from blood that has pooled in the myocardium. By comparison with the proton NMR spectrum of deoxyhemoglobin (La Mar et al., 1980) we attribute the resonance at 74 ppm to the β chains and that at 62 ppm to the α chains of hemoglobin. The ischemic deoxymyoglobin peak at 76 ppm overlaps with the β chain deoxyhemoglobin peak.

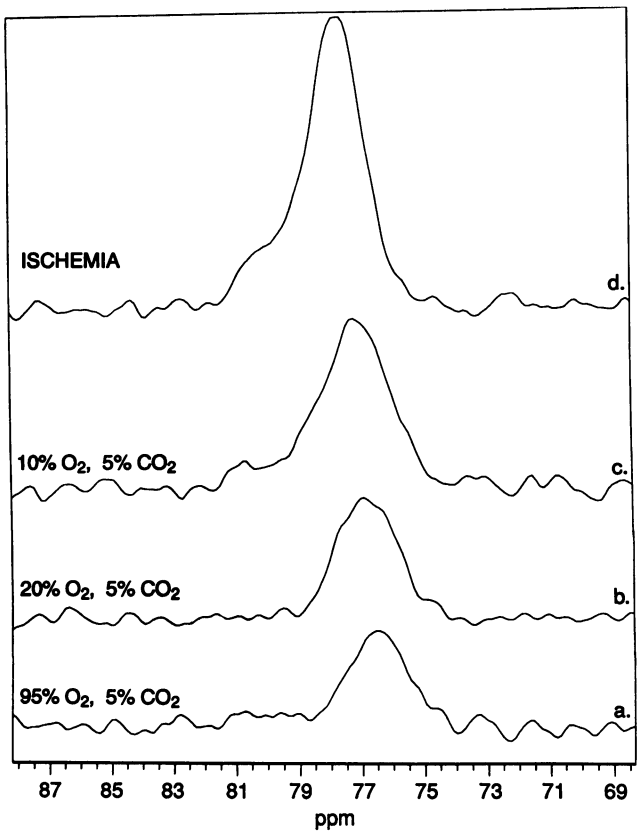

FIGURE 6 ^{1}H NMR spectra of an isovolumic blood-perfused rat heart showing the effect of changing perfusate oxygenation on the deoxymyoglobin resonance. Percent deoxymyoglobin, measured from the area under the peak, was 30% with 95% O_2 (*trace a*; heart rate = 138 bpm), 38% with medium equilibrated with 20% O_2 (*trace b*; heart rate = 132 bpm), and 60% with 10% O_2 (*trace c*; heart rate = 126 bpm). Although the heart rate remained relatively constant, myoglobin deoxygenation increased slightly when the blood was equilibrated with 20% oxygen. Decreasing the medium oxygen pressure to 10% oxygen significantly increased myoglobin deoxygenation indicating a drop of intracellular oxygen pressure from 5 to 2 torr. *Trace d* shows the final ischemic spectrum with 100% of the intracellular myoglobin in the deoxy form.

from one representative heart. In a series of experiments the percent myoglobin deoxygenation was 24.4 ± 2.1% (n = 5) with 95% oxygen equilibration, and 30.0 ± 1.5% (n = 9) with 20% oxygen equilibration of the perfusing blood. These values are significantly different ($p \leq 0.01$) and demonstrate the precision of the ^{1}H NMR method in measuring differences of a small magnitude within the same heart and between different hearts. Decreasing blood oxygenation by equilibration with 10% O_2, 5% CO_2 significantly increased the reduction of intracellular myoglobin to 55.5 ± 4.5% of total n = 2. This value is significantly different from that observed with 95% oxygen equilibration ($p \leq 0.005$) or 20% oxygen equilibration ($p \leq 0.0001$).

Fig. 7 shows representative spectra of a blood-perfused heart during electrical pacing. In this heart, as in others, there was no significant change in myoglobin deoxygenation when pacing was varied progressively from 60 to 252 bpm, at a given flow rate. Furthermore, we observed no correlation between myoglobin deoxygenation and heart rate R^2 = 0.00058 (see Fig. 8) when heart rate was varied from 60 to 480 bpm (n = 26). This suggests that the flux of oxygen from the red blood cells to the mitochondria keeps pace with the rate of oxygen uptake so that the steady-state level of myoglobin deoxygenation is not changed during pacing. In contrast, twofold stimulation of the heart rate (by norepinephrine) in saline-perfused hearts resulted in reversibly increased myoglobin deoxygenation. The saline perfusate has a much lower oxygen capacity, which may account for the greater response of tissue oxygenation to increased stimulation.

We use intracellular myoglobin deoxygenation as an endogenous probe of intracellular oxygen pressure. The Hill equation with n = 1 describes the equilibrium between oxygen and myoglobin:

$$Y/1 - Y = kP_{O2},$$

where Y is the fractional saturation of myoglobin with oxygen, k is a constant for the system, and P_{O2} is the partial

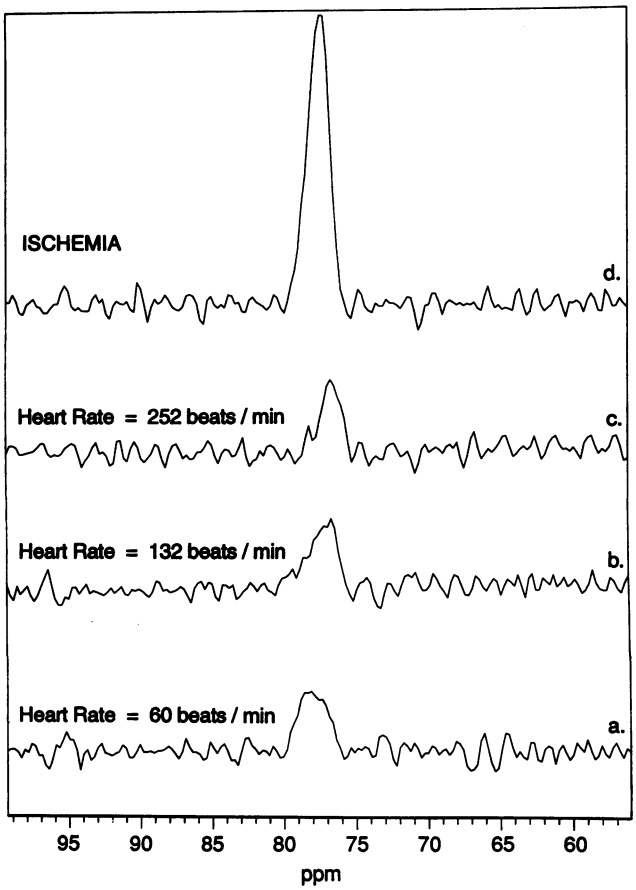

FIGURE 7 The effect of increased electrical pacing on the ^{1}H NMR spectra of an isovolumic blood-perfused rat heart. In this representative experiment, the myoglobin was 27%, 28%, and 22% deoxygenated as pacing was increased fourfold from 60 bpm (*trace a*), to 132 bpm (*trace b*), to 252 bpm (*trace c*), respectively. Accordingly, there was no significant difference in intracellular oxygen pressure (average 7 torr) during a fourfold increase in pacing. The final spectrum of no-flow ischemia is shown in *trace d*.

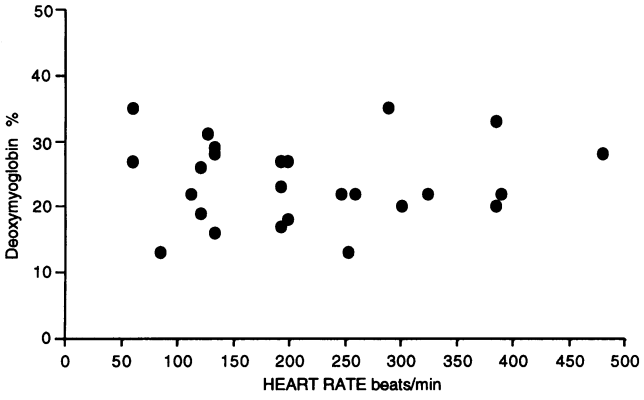

FIGURE 8 Scattergram showing the effect of increasing heart rate on deoxymyoglobin level in blood perfused hearts. There is no correlation between heart rate, over an eightfold range, and % myoglobin deoxygenation. The correlation coefficient $R^2 = 0.00058$ ($n = 25$ observations, six hearts).

pressure of oxygen in the intracellular myoglobin-containing compartment. The P_{50} for rat heart myoglobin in isolated heart cells is 2.3 torr at 37°C, and 1.3 torr at 30°C (Wittenberg and Wittenberg, 1985). Here, we use a value of $P_{50} = 2.5$ torr. The average value of myoglobin deoxygenation in blood-perfused paced rat heart is 23.9 ± 1.2% ($n = 26$) with a range of values from a low of 13% to a high of 35%. Therefore Y, the average fractional saturation of myoglobin with oxygen is 1.00 – 0.24 = 0.76, and the calculated intracellular P_{O2} of the beating blood- perfused heart has an average value of 8 torr with a range from 4.6 to 16.7 torr under our conditions. At this intracellular P_{O2} myoglobin functions in facilitated diffusion, and the measurement of myoglobin oxygenation is exquisitely sensitive to changes in P_{O2}.

Although there was no correlation ($R^2 = 0.063$) between flow rate (0.3 – 4.0 ml/min) and % deoxymyoglobin (Fig. 9) there was a trend toward higher % deoxymyoglobin at the lower flow rates, and it is possible that less deoxymyoglobin would be observed at flow rates greater than 4.0 ml/min. At

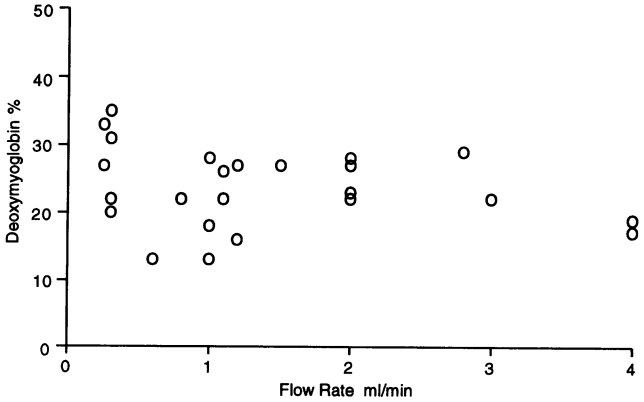

FIGURE 9 Scattergram showing the effect of increasing flow rate on deoxymyoglobin level in blood-perfused hearts. There is no correlation between flow rate, from 0.3 to 4.0 ml/min, and % myoglobin deoxygenation. The correlation coefficient $R^2 = 0.063$ ($n = 24$ observations, six hearts).

similar (low) flow rates (1 ml/min) and low heart rate (60 bpm), there was no difference in myoglobin deoxygenation of a blood-perfused heart (30%) compared with that of an MEM-perfused heart (27%). Under these conditions the capacity of MEM is sufficient to deliver oxygen by simple diffusion at a rate high enough to meet the low oxygen demand. We did observe a flow rate dependence of myoglobin deoxygenation in saline-perfused hearts similar to that reported by Kreutzer and Jue (1991). At flow rates greater than 3.0 ml/min the deoxymyoglobin resonance was not observed in saline-perfused hearts (data not shown). Our finding that myoglobin is partially deoxygenated in the blood-perfused rat heart is in agreement with the results of Gayeski and Honig (1991) showing that myoglobin is close to half saturation with oxygen in working blood-perfused hearts in situ even at low work loads.

SUMMARY AND CONCLUSIONS

We show that noninvasive NMR spectroscopy can be used to measure intracellular myoglobin deoxygenation and thereby sensitively probe oxygenation of the sarcoplasm in the beating heart. Cellular oxygenation was reversibly decreased during hypoxic perfusion conditions, demonstrating that the measurement sensitively reflects changing oxygen levels in the cells. Perfusion with nitrogen-equilibrated medium resulted in a large increase in myoglobin deoxygenation, and subsequent addition of sodium nitrite resulted in the disappearance of the deoxymyoglobin signal as expected upon conversion of myoglobin to ferric and ferric nitrite myoglobin.

We demonstrate that ^{1}H NMR can be used to probe oxygenation of the sarcoplasm in the beating blood-perfused heart without interference from deoxyhemoglobin resonances. With this technique, we have shown that myoglobin is partially deoxygenated in the blood-perfused beating rat heart. Furthermore we show that myoglobin deoxygenation does not change when heart rate is varied over a range from 60 to 480 bpm. This is in agreement with the findings of Gayeski and Honig (1991) using a completely different acute freeze-clamping technique. The data suggest that the intracellular P_{O2} of myoglobin-containing blood-perfused cells is maintained remarkably constant.

We gratefully acknowledge the expert technical assistance of Laura Cipriani. This work was supported in part by National Institutes of Health grants HL 45686 (LAJ) and HL 19299 (BAW).

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