Carbon monoxide inhibition of regulatory pathways in myocardium

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Glabe, Alan, Youngran Chung, Dejun Xu, and Thomas Jue. Carbon monoxide inhibition of regulatory pathways in myocardium. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H2143–H2151, 1998.—The 1H nuclear magnetic resonance (NMR) myoglobin (Mb) Val E11 signal provides a unique opportunity to assess the functional role of Mb in the cell. On CO infusion in perfused myocardium, the MbO2 signal at –2.76 parts per million (ppm) gradually disappears, whereas the corresponding MbCO signal emerges at –2.26 ppm, reflecting the state of Mb inhibition. Up to 76.8% MbCO saturation, myocardial O2 consumption (MVO2) remains constant, whereas the rate-pressure product (RPP) has already dropped to 92% of the control level. At 87.6% MbCO saturation, the lactate formation rate has increased by a factor of two, and MVO2 begins to decline. However, the ratio CO/O2 is still 1/10, well below the inhibition threshold for cytochrome oxidase activity. The MVO2 decline in the face of an adequate O2 supply and an unperturbed high-energy phosphate level implies that Mb may play a role in directly regulating respiration, mediated potentially by a shift in NADH/NAD.

Although nitrite inhibits Mb, nitrite also directly affects myocardial function.

myoglobin; nuclear magnetic resonance; respiration; oxidative phosphorylation

THE PRESENCE OF MYOGLOBIN (Mb) only in myocytes has always raised unsettling questions about its functional role (2, 28). Given the orthodox view of Mb as an O2 storage protein ready to compensate any cellular O2 deficit or as a facilitator of O2 diffusion, one might expect hypoxic-sensitive brain tissue to sequester the protein. Brain tissue does not, and its absence raises the possibility that Mb may have other functions, which recent experimental observations have begun to intimate: under oxygenation conditions that far exceed mitochondrial demand, nitrite or CO inhibition of Mb function still produces a decline in myocardial O2 consumption (MVO2) and phosphocreatine (PCr) (10, 13, 26). Under such conditions, Mb should presumably contribute insignificantly to facilitating O2 diffusion, and respiration is not O2 limited. Yet, if PCr level still declines, it would suggest that Mb inactivation has also removed a role for Mb in directly modulating respiration.

Much of the supporting data for a direct role of Mb in regulating respiration originates from CO or nitrite inhibition studies of myocytes. CO binds more tightly to the heme Fe than O2 does, and nitrite oxidation of the heme iron from the physiological 2+ state [Fe(II)] to the 3+ state [Fe(III)] prevents any O2 binding, because O2 does not bind to Fe(III) heme. The nitrite experiments in perfused myocardium, however, have produced equivocal results (6, 9, 23). In a recent 1H nuclear magnetic resonance (NMR) study that has followed the extent of nitrite inhibition of Mb in myocardium, infused nitrite concentration must exceed 10 mM before any noticeable Mb oxidation appears (6). The stoichiometry is much greater than the stoichiometry previously reported for Mb inhibition in vivo and far greater than the expected stoichiometric amount required for the corresponding in vitro reaction (6, 9, 10, 13, 21, 23). Although the rate-pressure product (RPP) and PCr levels progressively decline with increasing nitrite levels, MVO2 is relatively constant and even rises slightly. The results are in contrast to the myocyte observations and raise the question of whether nitrite acts directly on cellular function or acts indirectly by inhibiting Mb function and whether the disparity arises from any functional differences in the model systems.

Separating the contribution from Mb inactivation and nitrite is possible with CO experiments, because CO binds more tightly to the heme than O2 does and inhibits the nitrite oxidation of the heme Fe(II) to Fe(III) state (2). Moreover, the CO inactivation experiments can yield another perspective into the functional role of Mb. If Mb plays a significant role in directly regulating O2 consumption or oxidative phosphorylation, then CO inactivation of Mb, under conditions when O2 supply is ample and the Mb role in facilitated O2 diffusion is minimal, should still produce a physiological response that will appear to signal O2 limitation.

Measuring the extent of CO binding to Mb in the myocardium is then a crucial step. We report herein that the distinct MbCO signal of the γ-CH3 Val E11 at –2.26 parts per million (ppm) is detectable in the myocardium, separated from the corresponding MbO2 signal at –2.76 ppm (15, 20). Increasing the partial pressure of CO (Pco) in the perfusate enhances the MbCO signal intensity and at the same time depresses the MbO2 signal intensity.

The physiological and metabolic impact of CO in the cell is somewhat unexpected. Up to 80% of MbCO saturation, the MVO2 remains constant, whereas RPP is depressed by 10%. The 31P NMR spectra reveal that PCr, ATP, and Pi levels are not significantly disturbed. Although pH is constant, lactate formation rate has increased by a factor of two. At the CO concentration to saturate 80% MbCO, cytochrome oxidase activity should not be impaired, yet MVO2 begins to decline. On transient infusion of 50 mM nitrite in the presence of CO, MbCO is not oxidized; yet, RPP and lactate level shift dramatically. The results are consistent with a potential direct role of Mb in regulating respiration and a direct route for nitrite action.

MATERIALS AND METHODS

Animal preparation and heart perfusion. Male Sprague-Dawley rats (350–400 g) were anesthetized by an intraperito-
CO inhibition of myoglobin and respiratory regulation

neural injection of pentobarbital sodium (60 mg/kg) and heparinized (1,000 U/kg body wt). The heart was quickly isolated and placed in an ice-cold buffer solution until aortic cannulation. It was then perfused in a retrograde mode, as prescribed by a modified Langendorff model, and was maintained at 35°C with a Lauda MT-3 water bath and temperature-jacketed reservoirs and tubings. A peristaltic pump (Rainin Rabbit) maintained a constant, non-recirculating oxygen perfusion flow of 18 ml/min. A saline-filled latex balloon inserted in the left ventricle monitored the heart rate (HR) and left ventricular pressure (LVP) via a strain-gauge transducer (Statham P23XL) connected to an oscillographic recorder (Gould RS 3200). The balloon volume was adjusted to give an end-diastolic pressure (EDP) of 6–8 mmHg. RPP values were calculated from HR times the LV developed pressure (LVDP).

The perfusion medium was a modified Krebs-Henseleit buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.8 CaCl2, 20 NaHCO3, 1.2 MgSO4, and 15 glucose. The perfusate was first oxygenated with 95% O2-5% CO2 and then passed through a home-built Lucite mixing chamber, comprised of gas-permeable Dow Corning Silastic tubing (ID 0.058 in., OD 0.077 in.) wrapped around a heat exchanger. Different gas mixtures equilibrated with the inflowing perfusate just before it entered the heart. The perfusate passed through both 5-µm and 0.45-µm Millipore filters. The gas equilibration conditions were similar to ones previously reported (5, 14).

Perfusate O2 measurement. The heart was placed in an NMR tube and isolated with a Teflon plug with holes to permit perfusate overflow. Approximately 50% of the perfusate was withdrawn via a polyethylene (PE) catheter inserted close to the pulmonary artery. A Yellow Springs Instrument (YSI) 5300 meter monitored the perfusate O2 concentration with two YSI 5331 O2 electrodes in a temperature-jacketed chamber (one for inflow and the other for outflow perfusate). The remaining 50% of the perfusate exited the chamber above the Teflon plug as an overflow.

Parallel bench experiments determined empirically the O2 loss in the tubing and adjusted the measured PO2 value to reflect the venous value proximal to the heart. In the first set of measurements, the tubing lines from the heart chamber to the O2 electrode were kept as short as possible. Independent measurements determined a small O2 loss per length of tubing in such an arrangement. In the second set of measurements, the tubing length matched the NMR experimental conditions. The results from the two sets of measurements formed a calibration curve that adjusted the observed outflow O2 level to approximate the venous O2 (5, 6, 14). The CO loss was assumed to be similar.

CO infusion protocol. CO was introduced into the perfusion buffer in a stepwise manner. Two flowmeters controlled the flow of 95% O2-5% CO2 and 95% CO-5% CO2 gases, which entered a temperature-jacketed gas-mixing chamber and equilibrated with the perfusate passing through 50 ft of gas-permeable Dow Corning Silastic tubing (ID 0.058 in., OD 0.077 in.). The CO flow rate varied stepwise at 0.0, 0.1, 0.3, 0.5, and 1.0 l/min, while the 95% O2-5% CO2 flow rate remained constant at 5.0 l/min. After correction was made for loss in the tubing, the resulting PCO values at the catheter tip were 0.0, 12.6, 36.3, 58.4, and 107.0 Torr; respectively. The CO2 measurement of perfusate exiting the gas-mixing chamber confirmed that equilibration was essentially complete within 2 min after the CO flow rate was adjusted at each step.

After the last CO infusion step, the heart was reperfused with oxygenated buffer. The RPP and MV2O2 returned to values observed in control hearts, where no CO was introduced. In control hearts the RPP declined −10% over the course of the experiment. In the CO-treated myocardium experiments, the control and the O2 reperfusion data determined the baseline drift, which was consistent with the drift during the control period. All the CO-induced changes in RPP were then normalized against this extrapolated baseline.

Nitrite infusion protocol. In the transient infusion protocol, periods corresponding to a CO infusion, a nitrite infusion, and an O2 reperfusion followed the control interval. During the control period, the myocardium was perfused with nitrite-free, O2-saturated buffer flowing at 18 ml/min. CO was then introduced for 30 min at a PCO sufficient to saturate 86% of the Mb. The buffer was then switched to one containing 50 mM nitrite buffer, which was kept in a separate reservoir, which contained a specific amount of NaNO2 dissolved in O2-saturated perfusate. The Na+ concentration was adjusted to maintain the proper ion balance. After the transient nitrite infusion, reflow with O2-saturated, nitrite-free buffer began at 18 ml/min. During the entire protocol the 31P NMR followed the high-energy metabolite changes. Physiological monitors continuously tracked the EDP, LVDP, HR, and MV2O2.

Lactate measurement. A YSI 2700 Bioanalyzer determined the perfusate lactate concentration. Samples were measured in triplicate, and the analyzer’s linear response was calibrated against a set of standard lactate solutions, ranging from 1 to 20 mM. The membrane current stabilized at <2 nA before any measurement commenced. An additional calibration curve, derived from buffer perfusate at different nitrite concentrations, corrected for any nitrite-dependent interference.

Curve fitting and statistical analysis. Linear regression analysis, using a least-squares method (SigmaPlot, Jandel Scientific), determined the correlation coefficient, slope, and intercept. Errors were noted as standard error. Student’s t-test indicated statistical significance when P < 0.05.

NMR. An AMX 400-MHz Bruker spectrometer recorded 1H/31P signals with a 20-µm 1H-(X) probe, where X represented nuclei from 15N to 31P. A modified binomial pulse sequence suppressed the H2O line and selectively excited the MbO2 and MbCO Val E11 resonances at −2.76 and −2.26 ppm, respectively (5, 15). The 1H 90° pulse was 65 µs, calibrated against the perfusate H2O signal. Observing the MbO2 and MbCO signal required a 40-ms acquisition time and a 45° pulse. The spectral width was set at 8,065 Hz; the data block size was 512. Six thousand transients were averaged for a typical 1H spectrum, requiring 5 min of signal accumulation. The free induction decays (FID) were then zero-filled to 2K and multiplied by an exponential-Gaussian window function, W(t) = exp(−t/a)×exp(−t2/b), where a and b are input constants. A nonlinear spline fit (Bruker UXNMR algorithm), based on zero points set at regions well removed from the peaks of interest (data points at least 5 times the half-height line-width excursion from the peak maximum), then smoothed the baseline. All spectral lines were referenced to the H2O resonance at 4.67 ppm at 35°C. The chemical shift was in turn calibrated against sodium-3-(trimethylsilyl)propionate-2,2,3,3-d4 as 0 ppm. The integrated area of the Val E11 signal at 18 ml/min flow rate was normalized to 100% MbO2 saturation. For the 31P spectra, a typical spectrum utilized a 45° pulse angle, a 0.5-s repetition time, and 512 scans/block (4.3 min). The 31P 90° pulse was 72 µs, calibrated against a 0.1 M phosphate solution. Spectral width was set at 6,494 Hz; the data size was 4 K. FID were apodized with an exponential function to improve the 31P signal-to-noise ratio. The 31P signals were referenced to PCr as 0 ppm and apodized with a 15-Hz exponential function.

PCR, ATP, and P, levels were determined from integrated areas of the PCr, β-ATP, and P, signals, respectively. The areas were then normalized to the control values. The P,
The myocardial response to increasing Pco is reflected in the 1H and 31P spectra (Fig. 1, A and B, respectively). During the control period the 1H NMR spectra from well-oxygenated myocardium, perfused with 95% O2-5% CO2 saturated buffer flowing at 18 ml/min, exhibit a distinct g-CH3 Val E11 signal of MbO2 at 2.76 ppm (Fig. 1A). Under these perfusion conditions, the signal reflects the fully saturated state (5, 14, 15). As the PCO of the perfusate increases, the MbO2 signal decreases, while the corresponding MbCO peak at ~2.26 ppm increases (Fig. 1A, spectra b and c). At PCO of 12.6 Torr the MbO2 signal decreases to 53.5% of control level, while the MbCO signal increases correspondingly (Fig. 1A, spectrum b). Increasing the PCO to 58.4 Torr decreases further the MbO2 signal to 20.3% of control level, while MbCO peak rises to 84.9% (Fig. 1A, spectrum c). On reperfusion with 95% O2-5% CO2 saturated buffer, the MbO2 signal recovers as the MbCO signal falls (Fig. 1A, spectrum d). In contrast the 31P NMR spectra show no response to the infused CO (Fig. 1B, spectra a’-d’).

Throughout a range of Pco, a dynamic equilibrium exists between MbO2 and MbCO with no significant intervening Mb species (Fig. 2A). Changes in the MbCO signal intensity are balanced by corresponding alterations in the MbO2 signal, such that the sum of the MbO2 and MbCO signals is relatively constant, as denoted in Fig. 2A (dotted line). At Pco of 20 Torr, 50% of the Mb has converted to MbCO. A plot of the fractional MbCO/MbO2 vs. Pco/Po2 yields a linear relationship and a partition coefficient of 36, which is consistent with the values from in vitro studies (Fig. 2B).

The in vivo MbCO off-rate kinetics are shown in Fig. 3A. At Pco of 58.4 Torr, 84.9% of intracellular Mb is sequestered as MbCO. On reperfusion with oxygenated buffer, the MbCO declines to 50% of its original intensity within 10 min. After 40 min, the MbCO signal is no longer detectable, whereas the MbO2 signal has returned to its control level. The time for equilibration and dead space volume clearance is <2 min, a time frame sufficient for the PCr signal to recover fully (A Glabe, S. Huang, and T. Jue, unpublished observations).

The MVo2 and RPP response to varying Pco is shown in Fig. 3B. MVo2 remains constant up to 76.8% MbCO.

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**Fig. 1.** 1H and 31P nuclear magnetic resonance (NMR) spectra of myocardium perfused under varying partial pressures of CO (Pco). A: 1H NMR spectra. a) Pco 0 Torr: control spectrum from myocardium perfused with CO-free, O2-saturated buffer flowing at 18 ml/min shows the g-CH3 Val E11 signal of oxymyoglobin (MbO2) appearing at ~2.76 parts per million (ppm), which reflects the fully oxygenated state. b) Pco 12.6 Torr: with 12.6 Torr CO infusion, the 1H spectrum shows a modest decrease in the g-CH3 Val E11 signal of MbO2. However, a signal corresponding to the g-CH3 Val E11 of MbCO emerges at ~2.26 ppm. c) Pco 58.4 Torr: MbO2 signal is now significantly reduced, whereas signal of MbCO at ~2.3 ppm becomes prominent. d) Pco 0 Torr: on reperfusion with CO-free, O2-saturated buffer, MbCO signal intensity decreases, whereas the g-CH3 Val E11 signal of MbO2 recovers its intensity. The MbCO and MbO2 signals are in dynamic equilibrium. Signal intensity loss in 1 peak corresponds to signal intensity gain in the other. B, a’-d’: 31P NMR spectra under conditions that correspond, respectively, to those in A, a-d.
saturation. At 87.6% MbCO saturation, MV\textsubscript{O2} shows a significant decline (34.0 ± 1.3 µmol·min\textsuperscript{-1}·g dry wt\textsuperscript{-1}). In contrast, RPP has already dropped significantly at 53.5% MbCO saturation (27,436 ± 2,483 mmHg/min) and remains at this depressed level up to 87.6% MbCO saturation.

Despite the increasing PCO, the high-energy phosphate signals (ATP, PCr, and P\textsubscript{i}) show no alteration (Fig. 4A). Although pH remains constant, the lactate formation rate has increased sharply to 191% (0.495 ± 0.149 µmol·min\textsuperscript{-1}·g dry wt\textsuperscript{-1}) of control, when MbCO is at 76.8% saturated (Fig. 4B). The metabolic and physiological responses to varying levels of PCO under non-O\textsubscript{2}-limiting conditions are summarized in Table 1.

Nitrite does not significantly oxidize Mb in CO-treated myocardium, as shown in Fig. 5A. Infusion of 50 mM NaNO\textsubscript{2} in the continuing presence of CO, sufficient to saturate 87.6 ± 3.7% MbCO, does not produce any metmyoglobin (metMb) signal at −3.9 ppm nor does it decrease the MbCO signal significantly.

On reperfusion with oxygenated, nitrite- and CO-free buffer, the MbCO signal disappears as the MbO\textsubscript{2} signal recovers (Fig. 5A).

During nitrite perturbation in the presence of CO, some of the high-energy phosphate levels are altered. With only CO infusion, the PCr, ATP, and P\textsubscript{i} levels are constant. Once nitrite is infused, the PCr level declines to 59.5 ± 3.4% of control, whereas P\textsubscript{i} concentration rises to 124 ± 17.6%. ATP concentration, however, remains constant (Fig. 5B). After 30 min of reperfusion, the PCr level reaches 113 ± 2.1% of control, whereas P\textsubscript{i} falls to 41.7% of control.

The pH and lactate formation response to nitrite infusion in the presence of CO is shown in Fig. 6A. pH remains constant at 7.14 throughout the control and CO addition period but declines to 7.07 on NaNO\textsubscript{2} infusion. It recovers to 7.14 after reperfusion with
oxygenated, nitrite-free buffer (Fig. 6A). The lactate formation rate stays constant during the control period (0.184 ± 0.024 µmol·min⁻¹·g dry wt⁻¹) but increases during CO addition to 0.590 ± 0.059 µmol·min⁻¹·g dry wt⁻¹. With NaNO₂ infusion the lactate concentration rises dramatically to 44.6 ± 7.91 µmol·min⁻¹·g dry wt⁻¹. With reperfusion the lactate level declines rapidly and approaches the control level. After 30 min of reperfusion with oxygenated buffer were 30,025 ± 4,423 mmHg/min and 38.2 ± 9.2 µmol·min⁻¹·g dry wt⁻¹, respectively. Mb, myoglobin; PCr, phosphocreatine. *Significantly different from control (P < 0.05, paired t-test).

Table 1. Metabolic response on CO inhibition of Mb

<table>
<thead>
<tr>
<th>Pco₂, Torr</th>
<th>P O₂, Torr</th>
<th>MbCO, %</th>
<th>MbO₂, %</th>
<th>ATP, %</th>
<th>PCr, %</th>
<th>Pi, %</th>
<th>pH</th>
<th>Lactate, µmol·min⁻¹·g dry wt⁻¹</th>
<th>RPP, mmHg/min</th>
<th>Heart Rate, beats/min</th>
<th>MV O₂, µmol·min⁻¹·g dry wt⁻¹</th>
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<td>0.0</td>
<td>642.6</td>
<td>0.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>7.14</td>
<td>0.259 ± 0.089</td>
<td>29.846 ± 1.093</td>
<td>235 ± 10</td>
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<td>12.6</td>
<td>630.4</td>
<td>53.5</td>
<td>57.6</td>
<td>102.4</td>
<td>4.4</td>
<td>95.8 ± 2.9</td>
<td>97.8 ± 4.6</td>
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<td>27.436 ± 2.483*</td>
<td>228 ± 14</td>
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<td>36.3</td>
<td>670.4</td>
<td>76.8</td>
<td>31.9</td>
<td>101.1</td>
<td>4.5</td>
<td>98.6 ± 3.7</td>
<td>96.3 ± 5.5</td>
<td>7.14 ± 0.01</td>
<td>0.495 ± 0.149*</td>
<td>26.625 ± 2.549*</td>
<td>224 ± 15</td>
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<td>58.4</td>
<td>586.2</td>
<td>84.9</td>
<td>20.3</td>
<td>104.4</td>
<td>3.8</td>
<td>100.4 ± 4.2</td>
<td>91.5 ± 4.3</td>
<td>7.14 ± 0.01</td>
<td>0.545 ± 0.128*</td>
<td>27.021 ± 2.751*</td>
<td>218 ± 15</td>
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<td>107.0</td>
<td>539.1</td>
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<td>12.2</td>
<td>98.4 ± 1.9</td>
<td>96.3 ± 2.0</td>
<td>101.1 ± 5.2</td>
<td>7.15 ± 0.01</td>
<td>0.590 ± 0.059*</td>
<td>27.796 ± 1.277*</td>
<td>215 ± 15</td>
<td>34.0 ± 1.3*</td>
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</table>

Values are means ± SE; n = 5. Control is 0.0% MbCO. Values for rate-pressure product (RPP) and myocardial O₂ consumption (MV O₂) after reperfusion with oxygenated buffer were 30.025 ± 4.423 mmHg/min and 38.2 ± 9.2 µmol·min⁻¹·g dry wt⁻¹, respectively. Mb, myoglobin; PCr, phosphocreatine. *Significantly different from control (P < 0.05, paired t-test).
level, but lactate formation rate does not. With CO and nitrite-free, oxygenated buffer, pH recovers to control 7.06, while lactate formation rate rises dramatically. On reperfusion, the lactate level reaches 1.06 ± 0.230 µmol·min⁻¹·g dry wt⁻¹. With 50 mM NaNO₂ infusion, lactate formation rate rises to 117% of control level after 30 min, whereas RPP declines to 39% of control. pH drops to 7.06 as PCr drops to 59.5 ± 3.4% of control. With reperfusion, MVO₂ returns to its control level; however, RPP remains depressed at 74% of control. The metabolic and physiological parameters are listed in Table 2.

**DISCUSSION**

Partitioning of CO and O₂. Analyzing the cellular function of Mb requires a characterization of its ligand as well as oxidation states and entails a correlation with the physiological-biochemical response (5, 6, 14). Although optical techniques can distinguish MbO₂ saturation in vitro, they are not as successful in vivo in discriminating the overlapping MbCO and MbO₂ bands or distinguishing the Fe(II) from the Fe(III) states in a beating heart (6, 22). In contrast the 1H NMR CH₃ Val E11 signal offers a unique opportunity to observe directly the MbO₂, MbCO, and metMb states in the myocardium. The CH₃ Val E11 signal can mark both the intracellular P₀₂ and the Pco. At −2.7 ppm the signal of MbO₂ reflects the oxygenated state and decreases its intensity on deoxygenation (15). With increasing Pco, the MbO₂ signal drops as the corresponding MbCO signal emerges at −2.26 ppm (Fig. 6A). Moreover, the metMb reporter signal at −3.9 ppm reflects any nitrite oxidation of MbCO to the Fe(III) state (6, 16).

At 37°C the intracellular partition coefficient between CO and O₂ in myocardium, P = [MbCO] P₀₂/[MbO₂] Pco, is 36, which is in agreement with the myocyte and solution values of 20–35 (1, 8, 26, 27). Given a reported intracellular [P₀₂]₅₀ of 2.3 Torr at 37°C in myocyte, the corresponding [Pco]₅₀ is then 0.06 Torr (1), where [P₀₂]₅₀ and [Pco]₅₀ refer, respectively, to the P₀₂ and Pco values required to half-saturate Mb. The agreement in the partition coefficient values and the excellent linear relationship, shown in Fig. 2B, support the notion that Mb, O₂, and CO are in a near-equilibrium state. Figure 2A confirms a dynamic equilibrium between MbO₂ and MbCO and indicates no significant contribution from any intermediate Mb state.

The in vivo partition coefficient also indicates that in perfused heart, the presence of a vasculature does not discriminate significantly the CO from the O₂ delivery or transport to the cell. Any vasculature-to-cell or cytosol-to-mitochondria gradient would be identical for CO as well as O₂.

Table 2. Metabolic response to nitrite in MbCO myocardium

<table>
<thead>
<tr>
<th></th>
<th>MbCO, %Control</th>
<th>ATP, %Control</th>
<th>PCr, %Control</th>
<th>Pi, %Control</th>
<th>pH</th>
<th>Lactate, µmol·min⁻¹·g dry wt⁻¹</th>
<th>RPP, mmHg/min</th>
<th>Heart Rate, beats/min</th>
<th>MVO₂, µmol·min⁻¹·g dry wt⁻¹</th>
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<tr>
<td>Control</td>
<td>0.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>7.14±0.00</td>
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<td>29,760±1,444</td>
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<td>36.8±1.7</td>
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<td>CO</td>
<td>86.1±3.7</td>
<td>98.4±1.9</td>
<td>96.3±2.0</td>
<td>101.1±5.2</td>
<td>7.15±0.00</td>
<td>0.590±0.059*</td>
<td>27,796±1,198*</td>
<td>260±2</td>
<td>34.0±1.3*</td>
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<td>Nitrite</td>
<td>79.1±7.2</td>
<td>92.1±2.5†</td>
<td>59.5±3.4†</td>
<td>124.3±17.6†</td>
<td>7.06±0.021</td>
<td>44.6±7.91†</td>
<td>11,487±1,128*</td>
<td>186±19</td>
<td>43.1±1.3†</td>
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<tr>
<td>Reperfusion</td>
<td>18.2±10.9*</td>
<td>87.2±2.4*</td>
<td>113.1±2.1†</td>
<td>41.7±4.3*</td>
<td>7.15±0.01</td>
<td>1.06±0.230*</td>
<td>21,903±2,928*</td>
<td>242±4</td>
<td>36.5±1.9</td>
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Values are means ± SE; n = 6. Significantly different (paired t-test, P < 0.05): *from control, †from CO.
Critical O$_2$ level. The Mb and cytochrome oxidase have contrasting ligand binding affinities for O$_2$ and CO. For CO the Mb [Pco]$_{50}$ is 0.06 Torr, whereas cytochrome oxidase [Pco]$_{50}$ is ~0.1 Torr. For O$_2$ the cytochrome oxidase [Pco]$_{50}$ is ~10 times lower than the corresponding Mb [Pco]$_{50}$ (19, 30). A similar conclusion emerges on comparison of the inhibition ratio R = CO/O$_2$, which will produce 1:1 binding of CO:O$_2$ to the protein. For Mb the reported ratio is 0.025–0.04, whereas for cytochrome oxidase it is 5–15 (7, 8, 25, 29).

At the Mb [Pco]$_{50}$, CO binds insignificantly to cytochrome oxidase and therefore does not perturb the MV˙O$_2$ or the PCr level. At 87.6% MbCO saturation, MV˙O$_2$ begins to decline significantly; yet, CO/O$_2$ is only 0.025–0.04, well below the 6/1 required to detect any decline in respiration arising from CO inhibition of cytochrome oxidase (7, 8, 27). It would appear that the drop in MV˙O$_2$ does not arise from any direct CO inhibition of cytochrome oxidase.

Even though Mb has a higher CO affinity than cytochrome oxidase, hemoglobin (Hb) has a higher affinity than Mb. The contrasting affinities cast a critical perspective on CO disposal during heme catabolism and CO clearance (8). If Mb in the myocardium has a resting PO$_2$ of 2.3 Torr, as some investigators have suggested, then the critical PCo is 0.36 Torr, derived from the equation PCo = ([MbCO/MbO$_2$] x (PO$_2$/P) = (8.49/15.1) x (2.3/36) = 0.36 Torr. At the critical PCo respiration begins to show inhibition (1, 27). Given the Mb CO/O$_2$ partition coefficient of 240 and an arterial PO$_2$ of 100 Torr, the corresponding ratio HbCO/HbO$_2$ must reach 0.86 in the arterial blood before the critical MbCO saturation level is achieved in the myocyte. At venous blood PO$_2$ of 15 Torr, which approximates the capillary PO$_2$, HbCO/HbO$_2$ is 5.8. However, when MbCO saturation reaches 76%, corresponding to a PCo of 0.2 Torr, contractile function has already decreased. The corresponding values of the ratio HbCO/HbO$_2$ are 0.48 and 3.2 in the arterial and venous blood, respectively. For O$_2$ the ligand affinities increase from Hb to Mb to cytochrome oxidase. Compared with CO ligand affinities, these O$_2$ ligand affinities are in reverse order, consistent with the physiological function of respiration and CO disposal.

Intracellular ligand kinetics. Although the MbCO/MbO$_2$ partition coefficient in vivo is identical to the one in vitro and supports a nonselective CO/O$_2$ transport into the cell, the CO off-rate can reveal insight into the property of Mb in the cell. In particular, the difference in Mb ligand binding properties in solution vs. in the cell remains an open question. During reperfusion with oxygenated buffer, MbCO level drops to 50% of its original level within 10 min, indicating an apparent first-order rate constant (k$_{off}$) of 1.2 x 10$^{-3}$ s$^{-1}$ at 37°C. The in vivo k$_{off}$ value is somewhat lower than the in vitro value of 1.7–4 x 10$^{-2}$ s$^{-1}$ at 22°C. Additional experiments will be required to determine the origin of the MbCO kinetics, which may indicate the presence of an unidentified cellular effector (11, 18).

CO and contractile energy coupling. With CO, 76.8% saturation of MbCO does not significantly impair MV˙O$_2$. Yet, the lactate formation rate has increased by a factor of two, well below the reported value of the ratio CO/O$_2$ required to inhibit cytochrome oxidase (8, 19, 22). No shift in MV˙O$_2$ appears until the MbCO saturation reaches 87.6% PCr, ATP, pH, and P$_i$ levels still remain constant. These observations are not completely consonant with the myocyte results, which note a decline in both MV˙O$_2$ and PCr above a 40% MbCO saturation threshold (6, 10, 13, 26).

Quite clearly, a highly energized cellular state, as reflected by the $^{31}$P spectra, does not prevent a decline in contractile function. The CO-induced response appears in two phases. In the initial phase, a drop in the developed pressure contributes to the RPP decline, which shows no dose-dependent response to MbCO saturation. At 53.5% MbCO saturation, RPP has already declined significantly from 29,846 ± 1,093 to 27,436 ± 2,483 mmHg/min but does not decline further as MbCO saturation increases to 76.8%. The response curve suggests that CO interacts independently of Mb to reduce contractile pressure. Although the developed pressure has fallen, oxidative phosphorylation, as reflected in MV˙O$_2$ and PCr level, still appears normal.

The drop in RPP without a concomitant alteration in MV˙O$_2$ might suggest a potential uncoupling in oxidative phosphorylation, which is mediated by Mb but is independent of nitrite or heme oxidation. However, the contractile function falls at very low MbCO saturation, suggesting a Mb-independent CO effect. In the low-PCo regime the decrease in contractile function may arise from a CO interaction with the heme protein, guanylyl cyclase. The research literature has substantiated that NO binding to guanylyl cyclase will trigger a reduction in myocardial contractile function (3, 12). However, whether CO can mediate the same effect is unclear. Certainly, studies have suggested that CO can affect guanylyl cyclase (17, 24). However, other investigators have contested the conclusion and have also argued that the nonphysiological conditions of many experiments raise questions about the significance of the CO effect (4). Our observation of an MbCO-independent RPP decline in the initial phase of CO infusion is consistent with a CO interaction with guanylyl cyclase. Additional work is required to substantiate this hypothesis.

CO and mitochondrial energy coupling. In the second phase of the CO-induced effect, lactate formation rises dramatically despite constant energy production and utilization. Lactate oxidation is assumed to be constant. Above 53.5% MbCO saturation, MV˙O$_2$, PCr, and ATP remain unaltered, whereas RPP maintains its depressed, but steady-state, level. The lactate formation rate appears to increase as a function of MbCO saturation in a dose-dependent manner and implicates potentially the presence of anaerobic ATP production. Consistent with such a view is an enhanced glycolytic ATP production to meet an energy deficit, which can arise if the ratio P/O has shifted. From the MV˙O$_2$ and the $^{31}$P spectra, oxidative phosphorylation activity appears normal. Under all experimental conditions, the cellular PCo is insufficient to arrest the cytochrome
oxidase activity. This view of Mb interaction is consistent with previous CO myocyte studies, which have suggested that Mb may play a direct role in regulating respiration (26).

Certainly another interpretation might posit that the cytosolic NADH has risen without a concomitant shift in oxidative phosphorylation. The mechanism underlying such an enhanced formation as a function of CO concentration or Mb inactivation is uncertain. Clearly, additional experiments must measure directly the ATP/Pi flux in order to establish the presence of any alteration in oxidative phosphorylation.

An additional perspective also arises from analyzing the sequence of the physiological and biochemical events on CO perturbation. The sequence shows a striking similarity to the myocardial response profile as the O2 on CO perturbation. The mechanism underlying such an enhanced formation as a function of CO concentration or Mb inactivation is uncertain. Clearly, additional experiments must measure directly the ATP/Pi flux in order to establish the presence of any alteration in oxidative phosphorylation.

The cellular-metabolic response is quite similar to the nitrite-perfused myocardium. The results are consistent with a direct Mb role in regulating respiration. However, the experiments have not quantitated the relationship between the extent of Mb inhibition and the cellular response, and these studies have not convincingly established any dose-dependent response. Moreover, these studies have not removed the doubt that perhaps nitrite itself, not Mb, is mediating the interaction. The present study has utilized the 1H NMR Val E11 signal of Mb to map the extent of CO inhibition in order to test the hypothesis that Mb has a direct regulatory role. It has established the NMR methodology to investigate the role of Mb in the cell with CO and nitrite inhibition. Indeed the O2/CO partition coefficient agrees with the in vitro value and indicates nonselective transport.

In the CO inhibition experiments, the response occurs in two phases. In the first phase, at PCO well below the level required to saturate MbCO at 53.5%, contractile function drops to a steady-state level, which is ~90% of control. No further decline is observed on increasing the cellular PCO in the experimental protocol. Above 53.5% MbCO saturation, lactate formation begins to rise and is enhanced by a factor of two when MbCO is 84.9% saturated. However, MV\(\dot{O}_2\) is constant until MbCO reaches 84.9%, whereas the high-energy phosphate levels are still unperturbed. Under these experimental conditions, Mb does not participate significantly in facilitating O2 diffusion in the myocyte.

In the first phase of CO interaction, the drop in contractile function is independent of MbCO saturation and is postulated to involve guanylyl cyclase interaction. When MV\(\dot{O}_2\) declines, the ratio CO/\(\dot{O}_2\) is still 1/10, well below the 6/1 ratio required to inhibit cytochrome oxidase activity. In the second phase, when PCO is insufficient to inhibit cytochrome oxidase activity and in face of normal oxidative phosphorylation, as reflected in the constant high-energy phosphate signals, a drop in MV\(\dot{O}_2\) would indeed be consistent with Mb having a direct role in modulating respiration. The mechanism of the postulated Mb interaction may involve a shift in the redox poise (NADH/NAD), because the lactate formation rate responds first to the increasing level of PCO.

Although nitrite will also inhibit Mb function by oxidizing Mb from Fe(II) to Fe(III), the experimental data are not as strong as the CO data in supporting a direct Mb role. With MbCO, nitrite can no longer oxidize Mb readily. Yet, the infusion of 50 mM nitrite in myocardium with 86.1% MbCO saturation produces neither an alteration in the MbCO signal intensity nor a Mb signal but elicits a set of physiological metabolic responses similar to the nitrite-perfused myocardium. The results are consistent with a direct role of nitrite itself in mediating the set of cellular responses. Both the CO and nitrite experimental re-
results have now set up a basis for continuing study of Mb function in the cell.

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