Implication of CO inactivation on myoglobin function

Youngran Chung, Shih-Jwo Huang, Alan Glabe, and Thomas Jue

Department of Biochemistry and Molecular Medicine, University of California Davis, Davis, California

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Chung, Youngran, Shih-Jwo Huang, Alan Glabe, and Thomas Jue. Implication of CO inactivation on myoglobin function. *Am J Physiol Cell Physiol* 290: C1616–C1624, 2006. First published January 18, 2006; doi:10.1152/ajpcell.00360.2005.—Myoglobin (Mb) has a purported role in facilitating O2 diffusion in tissue, especially as cellular PO2 drops or the respiration demand increases. Inhibiting Mb with CO under conditions that accentuate the facilitated diffusion role should then elicit a significant physiological response. In one set of experiments, the perfused myocardium received buffer with decreasing PO2 (225, 129, and 64 mmHg). Intracellular PO2 declined, as reflected in the 1H NMR Val E11 signal of MbO2 (67%, 32%, and 18%). The addition of 6% CO further reduced the available MbO2 (11%, 9%, and 7%), as evidenced by the decline of the MbO2 Val E11 signal intensity at −2.76 ppm. In a second set of experiments, electrical stimulation increased the heart rate (300, 450, and 540 beats/min) and correspondingly the O2 consumption rate (MV˙O2). Intracellular PO2 also declined, as reflected in the slight drop in the MbO2 signal (100%, 96%, and 82%). MV˙O2 increased (100%, 114%, and 165%). The addition of 3% CO in the stimulated hearts further decreased the available MbO2 (46%, 44%, and 29%). In all cases, CO inactivation of Mb does not induce any change in the respiration rate, contractile function, and high-energy phosphate levels. Moreover, the MbCO/MbO2 partition coefficient shifts dramatically from its in vitro value during hypoxia and increased work. The observation suggests a modulation of an intracellular O2 gradient. Overall, the experimental observations provide no evidence of a facilitated diffusion role for Mb in perfused myocardium and implicate a physiologically responsive intracellular O2 gradient.

nuclear magnetic resonance; respiration; carbon monoxide; myocardium; oxidative phosphorylation

The presence of myoglobin (Mb) in vertebrate cardiac muscle and skeletal muscle has always posed questions about its functional role (45). In vitro experiments have provided evidence to support the role of Mb in storing O2 and facilitating O2 transport in the cell. In marine animals, the high concentration of Mb can certainly buffer tissue hypoxemia during a dive or apnea (18, 34). In high-altitude adaptation, enhanced Mb expression does increase the O2 depot (15, 41). Many studies (i.e., Ref. 48) have presented data to support the capacity of Mb to serve as a cellular O2 reserve. Yet, in the spontaneously beating rat heart, Mb can prolong normal heart function for only a few seconds (3). Even without any Mb, the Mb-knockout mouse model shows no physiological impairment (12, 17).

In addition to O2 storage, the current physiology paradigm ascribes to Mb an O2-facilitated diffusion role. In contrast to the low solubility of O2, the high O2 carrying capacity of Mb confers an advantage in transporting O2 from the sarcolemma to the mitochondria, especially under low cellular O2 environment (45, 46). Indeed, in vitro studies have confirmed that O2 diffuses faster in Mb solution than in Mb-free solution, leading to the idea that Mb can facilitate intracellular O2 delivery in blood-perfused tissue, where cellular O2 operates in a low PO2 environment. For Mb to compete effectively with free O2; however, Mb must exhibit sufficiently rapid translational mobility in the cell (21). Photobleaching experiments with an isolated fiber model, however, have revealed a low diffusion coefficient, indicating <25% Mb contribution to the overall O2 transport (23, 32, 33). Rotational diffusion measurements in respiring tissue have also raised questions about the contribution of Mb-facilitated O2 transport (39, 43).

Nevertheless, based on the Mb theory of facilitated diffusion, any acute inactivation of Mb should produce a noticeable alteration in respiration or oxidative phosphorylation. The predicted physiological response provides then an experimental basis to test the theory.

Early experiments inactivated Mb with CO or nitrite oxidation in tissue and detected an altered metabolic response. These experiments could not verify the extent of Mb inhibition or any direct interaction of CO or nitrite, especially on cytochrome oxidase (8, 40). Because the 1H NMR can now discriminate the distinct MbCO signal of the γ-CH3 Val E11 at −2.26 ppm from the corresponding MbO2 signal at −2.76 ppm in the myocardium, it provides a means to measure the intracellular partial pressure of CO (PCO), partial pressure of O2 (PO2), and the extent of Mb inactivation with CO. Indeed, as intracellular PCO rises, the MbCO signal intensity increases, whereas the MbO2 signal intensity decreases correspondingly. In the spontaneously beating heart, 77% of Mb inactivated with CO (6% CO in buffer) induces no significant change in respiration, contractile function, or high-energy phosphate state (16).

With an abundant source of buffer O2, the perfused heart might not mimic the in situ environment in blood-perfused tissue, especially as O2 demand begins to increase (19). The present study addresses the question of Mb function by investigating the physiological and biochemical response to Mb inactivation under experimental conditions that accentuate the hypothesized role of Mb in facilitated diffusion. As cellular PO2 decreases or as workload increases, the Mb contribution to facilitated O2 transport should increase correspondingly. Inactivating Mb should lead then to a marked change in respiration or contractile function. The experimental observations, however, show that under all conditions, Mb inactivation does not alter either the myocardial O2 consumption rate (MV˙O2) or high-energy phosphate levels, such as phosphocreatine (PCr) and ATP or the rate-pressure product (RPP). The undetectable physiological response during Mb inactivation under conditions that favor Mb-facilitated diffusion appears incongruent.

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with the hypothesis that Mb has a significant role in facilitating O2 transport in the cell.

The CO inhibition experiments also yield insight into an intracellular O2 gradient that responds to cellular energy demand. Introducing CO in the buffer alters the extracellular Pco/Po2 ([Pco/Po2]o) ratio. If the cell does not consume O2, passive diffusion transport should equalize the ratios of intracellular Pco/Po2 ([Pco/Po2]i) and [Pco/Po2]o. A plot of [Pco/Po2]i vs. [MbCO]/[MbO2], however, reveals a partition coefficient during hypoxia and increased work, which deviates diametrically from the expected in vitro value. During hypoxia, it decreases; during increased work, it increases. That change in partition coefficient is consistent with an alteration in the intracellular O2 gradient (38, 39). The study sheds light on the function of Mb and the response of an intracellular O2 gradient in vivo (1, 9).

MATERIALS AND METHODS

Animal preparation and heart perfusion. The rat heart perfusion method has been previously described (6, 7). Male Sprague-Dawley rats (350 – 400 g) were anesthetized with pentobarbital sodium (60 mg/kg ip) and heparinized (1,000 U/kg body wt). The heart was isolated and perfused with a modified Langendorff system that was maintained at 35°C. A peristaltic pump (Rainin Rabbit) delivered a constant, nonrecirculating perfusion flow of 18 ml/min.

A saline-filled latex balloon (1,900 mg/kg ip) and heparinized (1,000 U/kg body wt) was attached to the right atrium, and the perfusate was withdrawn via a polyethylene catheter inserted close to the pulmonary artery. A meter (model 5300, YSI) monitored the perfusate O2 concentration with two O2 electrodes (model 5331, YSI) in a temperature-jacketed chamber (one for inflow and the other for outflow perfusate). The resulting 50% of the perfusate exited the chamber above the Teflon plug as an overflow. Heart O2 consumption was calculated from the difference in the perfusate O2 content (inflow – outflow) and the perfusion flow rate.

Perfusate lactate measurement. A YSI 2700 bioanalyzer determined the perfusate lactate concentration (7). Samples were measured in triplicate, and the analyzer linearity was calibrated against a set of standard lactate solutions, ranging from 0.01 to 20 mM. The baseline concentration settled at <2 nA before any measurement commenced. An assay of lactate concentration in a defined effluent volume at specific time points and divided by the value of the perfusion flow rate led to average lactate production rate.

NMR. An AMX 400-MHz Bruker spectrometer recorded 1H/31P signals with a 20 mm 1H-(X) probe, where X represents nuclei from 15N to 31P. A modified 1,331 binomial pulse sequence suppressed the H2O line and selectively excited the MbO2 and MbCO Val E11 resonances at –2.76 and –2.26 ppm (7). The 1H 90° pulse was 65 μs, calibrated against the perfusate H2O signal. Observing the oxygen- and CO-Mb signals required a 40-ns 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 (FIDs) were then zero filled to 2K and multiplied by an exponential Gaussian function. 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) smoothed the baseline. All spectral lines were referenced to the H2O resonance at 4.67 ppm at 35°C. The integrated area of the Val E11 signal at 18 ml/min flow rate was normalized as 100% MbO2 saturation.

For the 31P spectra, a typical spectrum utilized a 45° pulse angle, a 0.5-s repetition time, and 512 scans per 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 4K. FIDs were apodized with an exponential function to improve the signal-to-noise ratio and the signals were referenced to PCr as 0 ppm.

PCCr, ATP, and P levels were determined from integrated areas of the PCR and β-ATP, and P, signals, respectively. The areas were then normalized to the prehypoxic (or pre-high-workload) baseline values. The P, chemical shift reflects pH, which was estimated from the equation

\[ \text{pH} = pK + \log \left( \frac{\Delta_A - \Delta_B}{\delta_0 - \delta_B} \right) \]

where \( pK = 6.9 \), \( \Delta_A = \Delta_{ppm} \text{ of } [\text{HPO}_4^{2-}] \) at 3.290 ppm, \( \delta_B = \delta_{ppm} \text{ of } [\text{HPO}_4^{2-}] \) at 5.805, and \( \delta_0 = \delta_{ppm} \text{ of observed } P_i \) (see Ref. 2).

Curve fitting and statistical analysis. Linear regression analysis, using the least-squares method (SigmaPlot, Jandel Scientific), determined the correlation coefficient, slope, and intercept. Values are

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CO INACTIVATION ON MYOGLOBIN FUNCTION

C1617

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Under 64 mmHg O2 and 38.5 mmHg CO, the myocardium exhibits a predominant MbCO peak at 2.26 ppm. The residual MbO2 peak appears at 2.76 ppm and reflects ~10% of the total Mb pool. Trace A, under perfusion with only O2, MbO2 begins converting to MbCO. Trace B, upon perfusion with only O2, MbO2 begins converting to MbCO. Trace C, when O2 displaces completely the CO in MbCO, only MbO2 signal at 2.76 remains. A dynamic equilibrium exists between MbO2 and MbCO.

Table 1. Metabolic response with and without CO inhibition of Mb during hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Hypoxia I (225 mmHg O2)</th>
<th>Hypoxia II (129 mmHg O2)</th>
<th>Hypoxia III (64 mmHg O2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypoxia (38.5 mmHg CO)</td>
<td>Control</td>
</tr>
<tr>
<td>MbO2</td>
<td>100</td>
<td>67.2±6.0*</td>
<td>100</td>
</tr>
<tr>
<td>MbCO</td>
<td>0</td>
<td>77.8±5.6†</td>
<td>0</td>
</tr>
<tr>
<td>MVO2</td>
<td>100</td>
<td>44.1±3.8*</td>
<td>100</td>
</tr>
<tr>
<td>Outflow PO2, mmHg</td>
<td>226±25</td>
<td>48±4.1*</td>
<td>268±12</td>
</tr>
<tr>
<td>RPP, mmHg/min</td>
<td>30,467</td>
<td>16,249±515*</td>
<td>16,543±797*</td>
</tr>
<tr>
<td>PCR</td>
<td>41.0</td>
<td>175.9</td>
<td>41.0</td>
</tr>
<tr>
<td>%control</td>
<td>100</td>
<td>83.8±5.7*</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td>87.8±14.5</td>
<td>100</td>
</tr>
<tr>
<td>P, %control</td>
<td>100</td>
<td>112.6±27.5</td>
<td>100</td>
</tr>
<tr>
<td>Lactate, μmol\·min\·g dry wt\·1-1</td>
<td>7.15±0.01</td>
<td>7.12±0.02</td>
<td>7.13±0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. CO, carbon monoxide; MVO2, O2 consumption; RPP, rate-pressure product; PCR, phosphocreatine; I–III, workloads. For Hypoxia + CO, the CO concentration for each protocol was 6% of total gas mixture. When CO was introduced, N2 was reduced accordingly to maintain a constant PO2 level. MVO2 with no CO and under perfusion at PO2 of 225, 129, and 64 mmHg (35%, 20%, and 10% of control PO2). MVO2 is normalized to the control, normoxic values (30.7 ± 2.4, 28.9 ± 0.6, and 30.9 ± 1.8 μmol·min\·g dry wt\·1) and is expressed as % control. *P < 0.05, significantly different from control based on paired t-test; †P < 0.05, significant difference between hypoxia and hypoxia + CO based on paired t-test.
decline. Despite marked changes in Mb and cellular parameters induced by hypoxia, CO inactivation of Mb produces no further alteration in respiration, contractile function, and energy state. Upon reperfusion with CO-free normoxic buffer, MbO2 concentration recovers to the control state level, consistent with previously reported kinetics (16). Table 1 lists the complete set of experimental data.

With electrical stimulation, the O2 demand increases with the rising workload. At the highest workload (III), the heart, contracting at 540 beats/min, elicits a 65% increase in MV˙O2, with respect to the MV˙O2 observed at workload I at 300 beats/min. RPP rises to 213% of control; MbO2 falls to 82% of the control level; PCr declines to 89% (Table 2). These observations agree with a previous report (4). At 450 beats/min, the myocardium increases its MV˙O2 by 14% and its RPP by 25%. Figure 3 shows the linear inverse relationship between MV˙O2 and MbO2. As MV˙O2 increases with workload, the intracellular PO2 declines. The graph yields the linear relationship: \( y = -25.6x + 100.1 \) (y = %MbO2 saturation and x = fractional increase in MV˙O2). According to the graph, when MV˙O2 increases by 100%, MbO2 declines to 74.5%.

At workload III, introducing 3% CO converts 64% of the steady-state MbO2 to MbCO. Only 29% of the Mb pool now remains as MbO2. The CO inactivation of 71% of the Mb pool, however, does not evoke any alteration in respiration, contractile function, and energy state (see Table 2 and Fig. 4).

The lactate formation shows a significant increase during hypoxia and increased work (see Fig. 5). During hypoxia, the lactate formation rate increases from its control level of 1.6 to 33 μmol·min⁻¹·g dry wt⁻¹ in level III hypoxia. The addition of 6% CO at a constant PO2 in all hypoxia conditions does alter significantly the lactate formation rate (see Fig. 5A). Similarly, with increased work, lactate formation increases. At 540 beats/min (workload III), lactate rises to 13.8 μmol·min⁻¹·g dry wt⁻¹, nine times above the basal workload I. With the addition of CO, the lactate production also shows no statistically significant change (see Fig. 5B).

### Table 2. Metabolic response with and without CO inhibition of Mb at different workloads

<table>
<thead>
<tr>
<th>CO⁻ Workloads, in beats/min</th>
<th>CO⁺ Workloads, in beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (300)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>MbO2</td>
<td>100</td>
</tr>
<tr>
<td>MbCO</td>
<td>NA</td>
</tr>
<tr>
<td>MVO2</td>
<td>100</td>
</tr>
<tr>
<td>Outflow PO2</td>
<td>293±8.4</td>
</tr>
<tr>
<td>RPP</td>
<td>24,000±1,098</td>
</tr>
<tr>
<td>PCr</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>Pi</td>
<td>100</td>
</tr>
<tr>
<td>PH</td>
<td>7.16±0.006</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.52±0.43</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. NA, not applicable. MV˙O2 without CO (CO⁻) and with CO (CO⁺) experiments are normalized to the 28.8 ± 1.5 and 26.4 ± 0.9 μmol·min⁻¹·g dry wt⁻¹, the observed, control values at workload I (baseline).

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The analysis of PCO/PO$_2$ vs. MbCO/MbO$_2$ under different hypoxia and workstate conditions utilizes the equation

$$k_{CO} \frac{[MbCO]}{[Mb][CO]}; \quad k_{O2} \frac{[MbO2]}{[Mb][O2]}$$

$$\frac{PCO}{PO_2} = K \frac{[MbCO]}{[MbO2]}; \quad K = \frac{k_{CO}}{k_{O2}}$$

where the partition coefficient ($K$) is the ratio $k_{O2}$ and $k_{CO}$ for Mb, respectively. Under control condition the partition coefficient is 36. During hypoxia, the coefficient falls to 22, whereas during increased work, the coefficient rises to 65. Table 3 summarizes the partition coefficient values.

**DISCUSSION**

Physiological response to Mb inactivation during hypoxemia. The present study has used 6% CO to inactivate 78–90% Mb, as reflected in $^1$H NMR signal intensity of MbCO and MbO$_2$ in the perfused rat myocardium. Because of the contrasting Mb and cytochrome oxidase CO binding affinity, 6% CO will bind primarily to Mb. Experiments have confirmed that supposition. Up to 9% CO, Mb becomes almost completely inhibited, but cytochrome oxidase activity remains unaffected. These in vitro studies show the cytochrome oxidase and myoglobin inhibition ratio, $R$ = PCO/PO$_2$, required to produce 1:1 cytochrome oxidase-CO: cytochrome oxidase-O$_2$ and MbCO:MbO$_2$, differs significantly: 5–15 for cytochrome oxidase and 0.025–0.04 for Mb (31, 49).

Above 9% CO, the rising PCO/PO$_2$ will introduce a cellular hypoxemia, as the fractional amount of O$_2$ falls. CO can directly inhibit cytochrome oxidase. As a consequence, infusing a perfusate buffer >9% CO would confound any data interpretation because hypoxemia and CO inactivation of Mb can both lead to the observed physiological response. Indeed, experiments have already demonstrated that up to PCO of 58 mmHg or 9% CO in the buffer at the aorta catheter tip, no significant change occurs in respiration or high-energy phosphate levels in spontaneously beating heart. Above 9% CO, however, MVO$_2$ begins to drop sharply (16).

In the present study, the introduction of 6% CO (39 mmHg) under declining fraction of PO$_2$ (225, 129, and 64 mmHg) avoids cytochrome oxidase inhibition with PCO/PO$_2$ ratios of 0.2, 0.3, and 0.6, respectively. Because the reference experiment condition substitutes N$_2$ for CO, the protocol has maintained a constant relative PO$_2$ in both the CO and CO-free experiments and has factored out any hypoxia contribution.

Under increasing hypoxia conditions, which should accentuate the role of Mb in facilitating the diffusion of O$_2$, Mb inactivation induces no significant change in respiration, bioenergetics, and contractile function.

### Table 3. Partition coefficients for MbCO/MbO$_2$

<table>
<thead>
<tr>
<th>Hypoxia, mmHg O$_2$</th>
<th>Work, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>225</td>
</tr>
<tr>
<td>[PCO/PO$_2$]$_i$</td>
<td>0.17</td>
</tr>
<tr>
<td>MbCO/MbO$_2$</td>
<td>6.82</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td>39.8</td>
</tr>
</tbody>
</table>

Partition coefficient, $K$, is defined by the equation

$$K = \frac{k_{CO}}{k_{O2}} = \frac{MbCO}{MbO_2};$$

$$K = \frac{[MbCO]}{[MbO2]}.$$
Physiological response to Mb inactivation during increased work. From an alternative vantage, increasing the O2 demand during high workload provides an alternative model to test the contribution of Mb-facilitated O2 diffusion. With electrical stimulation, the heart rate increases from 300 to 540 beats/min and elicits a rise in MV02 and RPP of 65% and 213%, respectively. As work increases, MV02 increases correspondingly. At the highest workload (III), MV02 increases by 65% in response to the 213% increase in RPP. In the perfused myocardium, the increased work triggers MbO2 saturation to fall 18%, whereas PCR declines 11%, consistent with a previous report (4). Introducing 3% CO in the perfusate at each work state inactivates Mb and decreases further the available MbO2 from the control level of 82% to 46%, 44%, and 29%. Even with only 29% of the Mb pool available as MbO2, the cell still exhibits no relative change in respiration, contractile function, or high-energy phosphate level. Under increased work conditions, Mb inactivation also does not elicit any observable physiological response.

Comparison of findings with nitrite inactivation. The study’s results disagree with several previous findings, which show Mb inactivation with nitrite, peroxide, or CO alters physiological function (40, 47). In one approach, nitrite or peroxide oxidation converts physiological Fe(II) to Fe(III) state (metMb), which can no longer bind O2. In vitro, nitrite readily oxidizes Mb. In the early Mb oxidation studies, the investigators assumed that oxidation should proceed according to in vitro stoichiometry. However, the experiments could not confirm the extent of Mb oxidation in vivo. Subsequent 1H NMR experiments have shown that in perfused heart, nitrite oxidation does not follow the in vitro stoichiometry. Even with a 350-fold stoichiometric excess of nitrite to Mb, only a fraction of Mb converts to metMb (7). The contrasting in vitro vs. in vivo Mb oxidation stoichiometry arises presumably from the presence of a robust cellular metMb reductase activity, which defends against Mb oxidation and minimizes the nitrite oxidation kinetics. Moreover, because nitrite can exert a direct effect on myocardial function, independent of Mb inactivation, any data interpretation must discriminate the direct nitrite vs. Mb inactivation effect. Indeed, nitrite can directly impair the contractile and respiratory function of the heart (16). A similar concern encumbers the peroxide oxidation approach (47). Inactivating Mb by forming metMb yields inconclusive results, unless the experiments can confirm the extent of Mb oxidation, corroborate the in vivo oxidation stoichiometry, and account for any direct nitrite or peroxide effect.

Comparison of findings with other CO inactivation experiments. Alternatively, studies have used CO to inhibit Mb function by competitive binding to the heme. Early studies also could not measure the extent of MbCO saturation in vivo and relied on in vitro experimental results to guide the CO infusion amount. 1H NMR techniques can follow the distinct CH3 Val E11 signal of MbO2 at −2.76 ppm and the corresponding MbCO signal appears at −2.26 ppm and show a dynamic equilibrium between MbO2 and MbCO with no detectable intermediate. Consistent with the present study’s results, up to 76.8% MbCO inactivation, the spontaneously beating myocardium shows no significant alteration in respiration or high-energy phosphate levels (16).

However, the present study’s observations differ significantly from those in a recent report (29). In that report, the constant pressure perfused mouse hearts receiving 20% CO showed about a 25% increase in the venous Po2 and a 10% drop in the left ventricular developed pressure. At 20% CO in the buffer, Po2 decreases sharply, the myocardium will face hypoxemia, and CO can now significantly inhibit cytochrome oxidase activity. Given the complicating contribution from hypoxemia and direct CO binding to cytochrome oxidase, ascribing the physiological response to only Mb inactivation appears moot.

Unfortunately, the published work did not provide spectroscopic evidence to substantiate the extent of MbCO inhibition nor experimental data to permit a verification of the statistical significance in the reported small left ventricular developed pressure drop, which then gives rise to the calculated work function, RPP. Moreover, the authors inferred that the 25% increase in the venous Po2 reflected a decrease in MV02. Without any flow data in the report, the reader can only infer O2 extraction and not MV02. The distinction between O2 extraction and O2 consumption takes on a special significance, because CO can induce vasodilatation and therefore enhance flow to yield an increased venous Po2 without an actual change in MV02. These concerns raise questions about a potential difference between the mouse and rat perfused heart models. A definitive comparative analysis must then await further experiments.

MbO2 saturation in tissue. As cellular Po2 declines, the Mb-facilitated diffusion theory predicts that the O2 carrying capacity of Mb, in contrast to the low solubility of free O2, begins to dominate O2 transport from the sarcolemma to the mitochondria (46). The theory contains then two implicit assumptions: 1) in the cell, the O2 supply must only partially saturate Mb; and 2) Mb must have sufficient translational mobility to compete with free O2 diffusion (D0).

Investigators have debated the oxygenation state of Mb in tissue. Recent optical experiments have contended that normoxic perfused myocardium only receives O2 to saturate only 72% MbO2 (10, 36). These optical results appear to conflict with the NMR observations, which support a much higher level of MbO2 saturation in perfused heart, in situ myocardium, and in situ skeletal muscle (16, 25, 28). Even under elevated work states, the in situ myocardium spectra do not exhibit any partially saturated MbO2 (26, 27, 50). Resting human skeletal muscle also does not reveal partially saturated MbO2 (30). So far, experimental evidence provides no definitive support for the corollary assumption of a partially saturated MbO2 in blood-perfused tissue.

Mb translational mobility. Any Mb contribution to intracellular O2 transport depends on a competitive Mb translational diffusion (Dmb) relative to D0. A low diffusion coefficient of Mb in the cell would argue against a significant role for Mb in facilitating O2 diffusion because D0 will overcome the O2 carrying capacity advantage of Mb. Measuring the Dmb in respiring tissue has posed an imposing technical challenge. Isolated muscle fiber and model experiments, however, have now estimated a Dmb from 1−23 × 10−7 cm2/s (11, 33, 35). Although these model experiments do not necessarily reflect the Mb mobility in respiring, in situ tissue, they do yield estimates of diffusion coefficients that attributes only a 25% Mb contribution to the overall O2 flux (33).

With the use of a cell boundary Po2 (estimated from the MbO2 shown in Table 1), the reported Dmb (1.7 × 10−7 cm2/s), Mb concentration of 0.2 mM, Mb PsO2 of 3.8 mmHg,
and a Krogh’s diffusion constant for $D_0$, in muscle of $0.76 \times 10^{-12}$ mol/(cm·min$^{-1}$·torr$^{-1}$) yields a basis to determine an estimate of the contribution of Mb facilitated O$_2$ vs. free O$_2$ transport, as the O$_2$ level falls under the present hypoxia levels I, II, and III. The analysis predicts that the Mb contribution to O$_2$ flux, transport should increase from 22% to 46% to 57%, respectively (21, 23). Relative to the reference condition, where MbO$_2$ is 67%, 32%, and 18% saturated, introducing CO further inactivates Mb and decreases the MbO$_2$ pool to 11%, 9%, and 7% of the control value. Because Po$_2$ remains constant, the free O$_2$ contribution does not change. Relative to MV$_{O2}$ in hypoxia I-III, CO inactivation of Mb should decrease MV$_{O2}$ by 20%, 38%, and 40%. The use of a higher value of $D_{Mb}$, as required by the facilitated diffusion theory, would decrease the MV$_{O2}$ even further as Mb becomes inactivated. In all cases, Mb inactivation under different hypoxia states, which accentuate the role of Mb-facilitated O$_2$ diffusion, induces no significant change in respiration.

**Capillary to cell O$_2$ gradient.** The rise in MV$_{O2}$ with work must also rely on a modulation the O$_2$ gradient from the capillary to the mitochondrial. Unlike the in situ blood perfused or constant pressure perfused myocardium, the constant flow perfused myocardium does not autoregulate its coronary flow and must depend upon convective or diffusive flow to respond to the increased O$_2$ demand. An increase in the O$_2$ gradient or shrinkage of the capillary to cell distance would enhance the diffusion and consequently the O$_2$ delivery. That gradient alteration should enhance the diffusion driving force for O$_2$ delivery. Indeed, the fall in the MbO$_2$ signal intensity reflects a lower intracellular Po$_2$, and therefore, an increase in the O$_2$ gradient as MV$_{O2}$ rises. Although MbO$_2$ saturation decreases with work, the change in O$_2$ gradient from the vasculature to the cell cannot account for the observed 65% rise in MV$_{O2}$ (4).

**Intracellular O$_2$ gradient.** In addition to the capillary to cell gradient, researchers have proposed the presence of an intracellular O$_2$ gradient from the sarcolemma to the mitochondria. Much controversy still surrounds the presence and significance of any intracellular O$_2$ gradient (2, 39). Partition coefficient analysis, however, appears to support the existence of an intracellular O$_2$ gradient and gives insight into its responses during hypoxia and increased energy demand. During CO inactivation of Mb, initial experiments assume that [Pco/Po$_2$]E approximates [Pco/Po$_2$]I. As a result, the solution state and cellular partition coefficient values for Mb should agree. Indeed, spontaneously beating, normoxic myocardium and solution state values show good agreement (16). However, because Po$_2$ decreases under different hypoxia protocols, the experimental results reveal a declining partition coefficient ($K$) for [Pco/Po$_2$]I, reaching 22 at hypoxia III, well below the expected solution state of 36 for [Pco/Po$_2$]E. In contrast, as work increases, the $K$ for [Pco/Po$_2$]I rises to 69 in workstate III. The assumption of an equivalent [Pco/Po$_2$]E and [Pco/Po$_2$]I no longer holds under all physiological conditions.

Because the Mb concentration remains constant, any deviation in the partition coefficient from the solution state value implies a change in the available intracellular O$_2$ or CO, which then alters the relationship between [Pco/Po$_2$]E and [Pco/Po$_2$]I vs. MbCO/MbO$_2$. The cell, however, does not consume CO. Only the O$_2$ level can change in response to physiological conditions. As the cell increases its energy demand or faces hypoxia, it can modulate both the O$_2$ concentration as well as the O$_2$ gradient from the sarcolemma to mitochondria.

During hypoxia, the overall O$_2$ supply decreases. However, the decline in [Pco/Po$_2$] ratio relative to [Pco/Po$_2$]E implies either a rise in the intracellular Po$_2$ relative to the extracellular Po$_2$ or a flattening of the intracellular O$_2$ gradient. Because O$_2$ consumption modulates the intracellular Po$_2$, the decreased MV$_{O2}$ during hypoxia could potentially raise the [Pco/Po$_2$] [Pco/Po$_2$]E ratio, which leads then to a decrease in the partition coefficient. From this vantage, during hypoxia the intracellular O$_2$ supply exceeds the falling demand. Consequently, during hypoxia III, the partition coefficient drops 1.6 times below the corresponding solution state value. However, the experimental protocol maintains a constant Po$_2$ during CO inactivation, and Mb appears to contribute insignificantly to O$_2$ transport. The change in that the partition coefficient appears more in line with an alteration in the intracellular O$_2$ gradient. Flattening the gradient will yield a higher integrated Po$_2$ across the cell. As a result, the [Pco/Po$_2$]E will decrease the observed partition coefficient.

In contrast, during work, the intracellular Po$_2$ decreases or the intracellular O$_2$ gradient steepens, relative to [Pco/Po$_2$]E, to effectively increase the [Pco/Po$_2$]E. At work level III, [Pco/Po$_2$]E has increased by 1.9, consistent with the enhanced MV$_{O2}$. Steepening the gradient will yield a lower integrated Po$_2$ across the cell and a rise in the partition coefficient. Increased work will steepen the intracellular O$_2$ gradient, whereas hypoxemia will flatten it. The partition coefficients will increase and decrease correspondingly, as observed.

Accommodating changes in the intracellular O$_2$ gradient imposes certain cellular conditions. In the normoxic myocardium, the Po$_2$ at the mitochondria cannot approximate zero, based on the presumed cytochrome oxidase $K_m$ for O$_2$ of 0.1 mmHg (49). Assuming a near-zero Po$_2$ at the mitochondria under control condition would preclude the cell from adjusting its intracellular O$_2$ gradient to meet any increased energy demands with rising work. Such a viewpoint would also suggest that O$_2$ does not limit respiration in a range of myocardial workstates. As work increases, however, the gradient will steepen and reach a maximum value, corresponding to the $V_{O_2\,max}$. From this vantage, O$_2$ may indeed become limiting at $V_{O_2\,max}$.

**Partition coefficient analysis and O$_2$ affinity of Mb.** Alternately the deviation in the [Pco/Po$_2$]E during hypoxia and work can arise from an alteration in the Mb affinity for O$_2$ and CO. All analyses have assumed that Mb behaves identically in solution and in the cell, partly because no experiments have measured directly the O$_2$ binding kinetics in the cell. An effector could alter the Mb affinity for O$_2$, even though such an interaction would challenge the classic definition of a multi-subunit protein capable of responding to an allosteric effector. Indeed, one study (14) has proposed that lactate at pH 6.5 can modulate the Mb affinity for O$_2$. Altering the Mb binding affinity for O$_2$ would certainly change the partition coefficient analysis. So far, no experiments have corroborated the lactate modulation of Mb function. Moreover, the present experiments, under the most severe hypoxia, produce only a pH drop from 7.15 to 7.05, although lactate production rises from 1.6 to 33 $\mu$mol·min$^{-1}$·g dry wt$^{-1}$. Lactate level also rises from 1.4 to 12 as work increases from control to work state III. The increased lactate level in both the hypoxia and enhanced work experiments would then induce both a drop and a rise in
increased work conditions, which accentuate the role of Mb in experimental data have now presented evidence for a recon-
produces no alteration in the MV˙O2, contractile function, and shows a marked difference between the [PCO/PO2]E and [PCO/
V˙O2 rises as O2 falls during contraction, and the resting cellular gradient steepens as O2 demand increases. Given such a view-
distance adapt rapidly to match respiration needs. The obser-
intact lungs, populations, subsarcolemmal and intermyofibrillar mitochondria, and controversy still surrounds the characterization of the cell solution state (37, 44). Given a mitochondria reticulum, subpopulations of mitochondria, and uncertain solution state character, the present study cast only a simple perspective on the regulation of oxidative phosphory-
Perspective on barrier to O2 diffusion. As cellular energy demand increases or decreases, blood flow and capillary to cell distance adapt rapidly to match respiration needs. The observation has spawned the supply side view of O2 delivery regulates VO2 (42). From this vantage, the present study suggests that an intracellular O2 gradient can pose a potential barrier to O2 flow from the sarcotubule to the mitochondria, especially in light of the insignificant role of Mb in facilitating O2 diffusion. Any regulatory intracellular O2 gradient, how-
ever, requires that the PO2 at the mitochondria does not approx-
approximate zero under the basal state, because increased O2 demand would require a steepening of the gradient to increase the O2 flux. With a baseline PO2 of 0, a gradient steepening cannot occur within a simple diffusion model.

From another vantage, a strict supply side regulation of VO2 does not easily account for in skeletal muscle observations: O2 appears uncoupled with VO2 at the initiation of contraction, VO2 rises as O2 falls during contraction, and the resting cellular PO2 fully saturates Mb (5, 30). A demand side regulation of VO2 must also exist. The present report and other recent experimental data have now presented evidence for a recon-
reconstruction of muscle bioenergetics paradigm within a biochem-
In conclusion, the study has examined the impact of CO inactivation of Mb in perfused myocardium under hypoxia and increased work conditions, which accentuate the role of Mb in facilitating O2 diffusion from the sarcotubule to the mitochondria. Under all experimental conditions, CO inactivation of Mb produces no alteration in the MV˙O2, contractile function, and bioenergetics. The experimental data do not reveal evidence to support the currently formulated hypothesis that envisions a significant role for Mb in facilitating O2 transport in the cell.

In the CO inactivation experiments, the partition coefficient analysis yields insight into the intracellular O2 gradient. It shows a marked difference between the [PCO/PO2]E and [PCO/ PO2]H during hypoxia and increased work. The relative change in [PCO/PO2]H agrees with the interpretation that the intracellular O2 gradient has modulated in response to energy demand. During hypoxia the intracellular O2 gradient flattens as O2 demand decreases; during increased work the intracellular O2 gradient steepens as O2 demand increases. Given such a viewpoint, the intracellular O2 gradient cannot adapt, if under control condition, the PO2 at the mitochondria already ap-
approaches zero, consistent with the low Km of cytochrome oxidase, and the maximum MVo2 will correspond to the steepest gradient. Lactate does not appear to play any signifi-
cant role in modulating Mb binding affinity.

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