Published in final edited form as:

Nitric Oxide. 2008 June ; 18(4): 296–302.

Rate of Nitric Oxide Scavenging by hemoglobin bound to haptoglobin

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Abstract

Cell-free hemoglobin, released from the red cell, may play a major role in regulating the bioavailability of nitric oxide. The abundant serum protein haptoglobin, rapidly binds to free hemoglobin forming a stable complex accelerating its clearance. The haptoglobin gene is polymorphic with two classes of alleles denoted 1 and 2. We have previously demonstrated that the haptoglobin 1 protein-hemoglobin complex is cleared twice as fast as the haptoglobin 2 protein-hemoglobin complex. In this report we explored whether haptoglobin binding to hemoglobin reduces the rate of nitric oxide scavenging using time-resolved absorption spectroscopy. We found that both the haptoglobin 1 and haptoglobin 2 protein complexes react with nitric oxide at the same rate as unbound cell-free hemoglobin. To confirm these results we developed a novel assay where free hemoglobin and hemoglobin bound to haptoglobin competed in the reaction with NO. The relative rate of the NO reaction was then determined by examining the amount of reacted species using analytical ultracentrifugation. Since complexation of hemoglobin with haptoglobin does not reduce NO scavenging, we propose that the haptoglobin genotype may influence nitric oxide bioavailability by determining the clearance rate of the haptoglobin-hemoglobin complex. We provide computer simulations showing that a two-fold difference in the rate of uptake of the haptoglobin hemoglobin complex by macrophages significantly affects nitric oxide bioavailability thereby providing a plausible explanation for why there is more vasospasm after subarachnoid hemorrhage in individuals and transgenic mice homozygous for the Hp 2 allele.

Keywords

Nitric Oxide; hemoglobin; haptoglobin; kinetics; time-resolved absorption spectroscopy; analytical centrifugation

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Introduction

Significant pathology is associated with cell-free hemoglobin (Hb)¹. Enhanced nitric oxide (NO) scavenging is one deleterious consequence of the presence of cell-free hemoglobin [1]. Nitric oxide scavenging by hemoglobin is accomplished primarily via the dioxygenation reaction involving oxygenated Hb

where HbO₂ represents oxygen bound Hb and MetHb refers to methemoglobin with a ferric (oxidized) heme. This reaction occurs at a nearly diffusion-limited rate of $5\text{--}6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [2–5]. NO binding to deoxygenated Hb occurs at a similar rate. Due to its ability to enter the red blood cell free zone, and extravasate, even as little as one micromolar cell-free hemoglobin can significantly decrease NO bioavailability even in the background of normal levels of red cell encapsulated hemoglobin (10 mM in heme)² [6].

Haptoglobin (Hp) is a plasma protein that binds to Hb with a very high affinity ($K_d \sim 10^{-12} \text{ M}$) [7–9]. The Hp gene is polymorphic with two common alleles, denoted 1 and 2, present at the haptoglobin locus at chromosome 16q22. The protein products of the Hp 1 and 2 alleles differ markedly in their biochemical and biophysical properties. The monomeric protein product of both Hp alleles can bind one Hb $\alpha\beta$ dimer [10,11]. The Hp monomeric protein is found as a multimer in serum whose stoichiometry is Hp genotype dependent. In individuals homozygous for the Hp 1 allele, only Hp dimers are found (MW 98kD) while in individuals homozygous for the Hp 2 allele only large cyclic Hp multimers are found (MW 160-500 kD) (3–10 Hp monomers per Hp multimer) [10,11]. Once bound, the Hb bound Hp can be cleared via the monocyte/macrophage haptoglobin-hemoglobin scavenger receptor CD163 [11,12].

Haptoglobin genotype has been linked to diseases in which NO is known to play a key role [8,10,13–15]. Cerebral artery vasospasm is a common complication of subarachnoid hemorrhage thought to be mediated in large part by the scavenging of NO by Hb released from extravasated red blood cells [16]. Borsody et al. found that patients with the Hp 2 allele are significantly more likely to develop cerebral artery vasospasm following subarachnoid hemorrhage (SAH) than patients with those who have only the Hp 1 allele [15]. Furthermore, mice transgenic for Hp 2 allele demonstrate markedly greater vasospasm after experimentally induced SAH than mice with the Hp 1 allele [17]. One proposed mechanism for the association between the Hp genotype and vasospasm after SAH was that NO scavenging ability may be Hp genotype dependent because the Hp genotype determines the rate at which Hb is cleared. Hp genotype dependent differences in NO scavenging ability could be due to the rate of uptake of Hp-Hb complex by macrophages wherein complexes with Hp 1 protein are found to be cleared 2–3 times as fast as those with the Hp 2 protein (half-life in vivo of the Hp 1-Hb complex is approximately 18 minutes while the half-life of the Hp 2-Hb complex is approximately 50 minutes) [18,19]. Alternatively, the complexes themselves may have intrinsically different reactivities with NO.

In this study we measured the rate of NO scavenging by Hb alone and by Hb-Hp 1 and Hb-Hp 2 complexes via the dioxygenation reaction under aerobic conditions using time-resolved absorption. We also employed competition experiments where the rates of NO scavenging were deduced from the amount of MetHb formed when cell-free and Hp bound Hb competed for NO. Both methodologies consistently demonstrated no difference in the rates found with

¹The abbreviations used are: Hb: hemoglobin, NO: nitric oxide, Hp: haptoglobin, Hct: hematocrit, SAH: subarachnoid hemorrhage.

²In this paper all hemoglobin concentrations are reported in terms of a per heme basis. One millimolar hemoglobin in heme is equivalent to 250 μM in hemoglobin tetramers. One g/dL of hemoglobin is 625 μM in heme. Concentrations of haptoglobin are reported in terms of haptoglobin $\alpha\beta$ dimers.

free Hb or Hb bound to either Hp 1-1 or Hp 2-2, suggesting that different uptake rates by macrophages of the Hp 1 and Hp 2-Hb complexes could have a profound effect on NO bioavailability.

Experimental Procedures

Reagents

Hemoglobin was purified from whole blood drawn from healthy human volunteers as described previously [20]. Briefly, red blood cells were washed, lysed by being placed in distilled water, and hemoglobin was separated from the membrane fraction by sedimentation. Hemoglobin was stored by pelleting in liquid nitrogen. Haptoglobin was purified from human serum by monoclonal antibody affinity chromatography [18]. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Sample preparation and analysis using ultracentrifugation for Photolysis Experiments

For photolysis experiments, 40 μ M of thawed Hb was studied alone or when mixed with 2–3 mg/ml thawed Hp 1-1 or thawed Hp 2-2 in phosphate buffered saline. The absence of cell-free Hb in the mixtures with Hp, indicating complete binding of Hb to Hp, was confirmed using an analytical centrifuge (Beckman Optima XL-A with UV/vis optics). Hb mixed with Hp 1-1 or Hp 2-2, and in the absence of Hp were each loaded into one of three centrifuge cells and spun at 45000 rpm ($163,000 \times g$). Figure 1A shows an example of the measured absorption at 415 nm as a function of position for each of the three tubes. The Hp bound Hb species sediment faster due to their higher molecular weight. In Figure 1A the Hb bound to Hp 2-2 has already mostly sedimented to the bottom of the tube. We quantified the maximum amount of unbound Hb in the samples in which there was excess Hp using two methods. Firstly, we compared the absorbance from the Hp-Hb mixtures measured in the analytical centrifuge to that of the Hb alone at positions where the Hb alone sample had significant absorbance and the Hp-Hb mixtures did not (such as at 6.6 cm in Figure 1A). The ratio of absorbance at such positions yields a maximum percentage of Hp-free Hb in the Hp-Hb mixtures. Such analysis showed that there is less than 1% free Hb in the samples mixed with Hp (as expected based on the affinity of Hp for Hb). We also analyzed the percentage of cellfree Hb in the Hp-Hb mixtures by obtaining sedimentation coefficient distribution data such as that shown in Figure 1B. These profiles of the weight fraction of sedimenting species, $g(S)$ vs sedimentation coefficient, S were obtained by analyzing the sedimentation velocity profiles with DCDT+ (version 6.31) software (J. Philo, Thousand Oaks, CA) [21].

Photolysis experiments

Photolysis experiments were conducted using similar instrumentation as that described previously [22]. The Hb or Hb-Hp complexes were mixed 1:1 with 2 mM caged nitric oxide (Potassium pentachloronitrosyl-ruthenate(II)) using a Harvard Apparatus syringe pump (PHD 2000, Holliston, MA) set to a flow rate of 48 ml/min followed by a homemade mixer. The mixed sample was then passed through a 0.2 cm path-length quartz flow cell (Starna Cells, Inc., Atascadero, CA) wherein it was exposed to 355 nm, 30 mJ laser pulse from the 3rd harmonic of a Q-switched Nd:YAG laser (Spectra-Physics GCR-11, Mountain View, CA) operated at 10 Hz and focused to a 5 mm diameter spot. The absorbance was probed by also shining light from a 75W continuous wave Xenon lamp (Oriel Optics Corp.) onto the photolysis spot with the probe light spot being smaller (~ 1 mm) than the pulse one. The transmitted light was focused into a Streak camera as previously described [22]. Two Stanford Research System (Sunnyvale, CA) DG535 delay generators supplied the necessary triggers for the laser and the streak camera. Data collected over 50 laser pulses (5 sec) were averaged for each data set and divided by the corresponding images taken in the absence of photolysis to obtain the time-resolved absorbance. These data were analyzed by singular value decomposition and global

analysis, fitting to a single exponential process, using Specfit (Boston, MA). Each day, data was collected on the Hp-Hb mixtures as well as a control sample containing Hb alone using the same caged NO solution, laser power, etc. Any variations in the quantum yield of the caged NO solution (about 10%) were thus accounted for by comparing to kinetics obtained for the control sample which was assumed to have an NO dioxygenation rate constant of $6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.

Competition Experiments

Competition experiments were performed by mixing 239 μM Hb and 60 μM Hp under aerobic conditions. Thus, as opposed to the situation in photolysis experiments where mixtures were intended to have only Hp-bound species, these mixtures contained Hb both bound to and free of Hp. PROLI NONOate (Cayman Chemical Company, Ann Arbor, MI) was added to a final concentration of 60 μM . The relative rate of the reaction of NO with cell-free Hb (k_f) to that with Hb bound to Hp (k_b) was determined in similar manner as that described when competition experiments were employed for examining red blood cell vs cell-free Hb NO scavenging [23,24]. The fraction of k_f to k_b is given by

$$\text{Ln} \left(\frac{[\text{HbO}_2]_{f\text{-final}}}{[\text{HbO}_2]_{f\text{-initial}}} \right) = \frac{k_f}{k_b} \text{Ln} \left(\frac{[\text{HbO}_2]_{b\text{-final}}}{[\text{HbO}_2]_{b\text{-initial}}} \right), \quad (2)$$

where the subscripts b and f refer to the Hp bound and cell-free Hb respectively and *initial* refers to the value at the beginning of the reaction and *final* refers to that at the end (where some of the OxyHb has been converted to MetHb). We have $[\text{HbO}_2]_{\text{final}} = [\text{HbO}_2]_{\text{initial}} - [\text{MetHb}]_{\text{final}}$. Equation 2 states that the degree to which each fraction (bound Hb vs free Hb) will react depends on the initial amount of Hb in each fraction (in the form of OxyHb) and the intrinsic bimolecular rate constants k_f and k_b . The natural log term accounts for the fact that the concentrations are changing as a function of time.

In order to solve for the relative reactivity k_f/k_b we needed to determine $[\text{HbO}_2]_f$ and $[\text{HbO}_2]_b$ at the beginning and end of the reaction (initial and final). The total amount of Hb was determined using absorption spectroscopy. Absorption spectroscopy could also be used to determine the total concentration (free plus bound) of HbO₂ and MetHb. Analytical centrifugation was used to separate the Hp bound and free Hb and determine their relative concentrations. By collecting absorbance at two wavelengths in the analytical centrifuge, the amount of NO product (MetHb) and unreacted Hb could be determined for each fraction. Since the Hp bound Hb sedimented faster than free Hb, we were able to select positions along the centrifuge cell where only free Hb remained and thereby determine $[\text{HbO}_2]_{f\text{-final}}/[\text{HbO}_2]_{f\text{-initial}}$. Using this together with the knowledge of total Hb, free and bound, (obtained from analytical centrifugation) and the total concentration (free plus bound) of HbO₂ and MetHb, we could obtain $[\text{HbO}_2]_{b\text{-final}}/[\text{HbO}_2]_{b\text{-initial}}$.

The mathematical details for obtaining k_f/k_b are described in Equation 3–Equation 10. The two equations that follow relate the absorbance values at 589 and 632 nm in a region of the centrifuge cell with only free Hb present.

$$A_f^{589} = \left(\frac{[\text{HbO}_2]_{f\text{-final}}^p \times \epsilon_{\text{oxy}}^{589} + [\text{MetHb}]_{f\text{-final}}^p \times \epsilon_{\text{met}}^{589}}{[\text{HbO}_2]_{f\text{-initial}}^p \times \epsilon_{\text{oxy}}^{589} + [\text{MetHb}]_{f\text{-initial}}^p \times \epsilon_{\text{met}}^{589}} \right) \times 1 \quad (3)$$

$$A_f^{632} = \left(\frac{[\text{HbO}_2]_{f\text{-final}}^p \times \epsilon_{\text{oxy}}^{632} + [\text{MetHb}]_{f\text{-final}}^p \times \epsilon_{\text{met}}^{632}}{[\text{HbO}_2]_{f\text{-initial}}^p \times \epsilon_{\text{oxy}}^{632} + [\text{MetHb}]_{f\text{-initial}}^p \times \epsilon_{\text{met}}^{632}} \right) \times 1, \quad (4)$$

where ε is an extinction coefficient and l is the pathlength. Since the absorbance along the sedimenting free Hb border is used, it does not represent the total free Hb amount, but only part of it (indicated by the superscript p). In other words some free Hb will be both above and below the place where the absorbance is measured along the centrifuge cell. Equation 3 through Equation 4 can be rewritten as:

$$[\text{MetHb}]_{f\text{-final}}^p = \frac{A_f^{589} \times \varepsilon_{\text{oxy}}^{632} - A_f^{632} \times \varepsilon_{\text{oxy}}^{589}}{(\varepsilon_{\text{met}}^{589} \times \varepsilon_{\text{oxy}}^{632} - \varepsilon_{\text{met}}^{632} \times \varepsilon_{\text{oxy}}^{589}) * l} \quad (5)$$

$$[\text{HbO}_2]_{f\text{-final}}^p = \frac{A_f^{589} - [\text{MetHb}]_f^p \times \varepsilon_{\text{met}}^{589} \times l}{\varepsilon_{\text{oxy}}^{589} \times l} \quad (6)$$

We calculate the total amount from the partial amounts in Equation 7 and Equation 8 using the fraction of free Hb (*fractionHb_f*) determined by fitting sedimentation data with DCDT+ and SVEDBERG.

$$[\text{HbO}_2]_{f\text{-final}} = \frac{\text{fractionHb}_f \times \text{totalHb}}{[\text{HbO}_2]_{f\text{-final}}^p + [\text{MetHb}]_{f\text{-final}}^p} \times [\text{HbO}_2]_{f\text{-final}}^p \quad (7)$$

$$[\text{MetHb}]_{f\text{-final}} = \frac{\text{fractionHb}_f \times \text{totalHb}}{[\text{HbO}_2]_{f\text{-final}}^p + [\text{MetHb}]_{f\text{-f}}^p} \times [\text{MetHb}]_{f\text{-final}}^p \quad (8)$$

$$[\text{HbO}_2]_{b\text{-final}} = [\text{HbO}_2]_{f+b\text{-final}} - [\text{HbO}_2]_{f\text{-final}} \quad (9)$$

$$[\text{MetHb}]_{b\text{-final}} = [\text{MetHb}]_{f+b\text{-f}} - [\text{MetHb}]_{f\text{-final}} \quad (10)$$

In Equation 9 and Equation 10, $[\text{HbO}_2]_{f+b\text{-final}}$ and $[\text{MetHb}]_{f+b\text{-final}}$ are determined using equations similar to 5 and 6 but, at each wavelength an absorbance plateau average of the first scan is used - where both free and bound Hb are present (indicated as f+b). The *totalHb* appearing in Equation 7 and Equation 8 is the sum of $[\text{HbO}_2]_{f+b\text{-final}}$ and $[\text{MetHb}]_{f+b\text{-final}}$.

Analytical centrifugation for all experiments was performed at 40,000 to 45,000 rpm and absorbance was measured at a MetHb and HbO₂ isosbestic point (589 nm) and MetHb peak (632 nm). The fraction of cell-free Hb vs Hb-Hp was determined by fitting the data with DCDT + (ver 2.0.7.26810, John S. Philo) and SVEDBERG (ver 6.39, John S. Philo) software. These fits thus gave $([\text{MetHb}]_b + [\text{OxyHb}]_b)/([\text{MetHb}]_f + [\text{OxyHb}]_f)$. Two species diffusion coefficient fits with and without an offset parameter were performed on all samples; the concentration, diffusion coefficient, and Svedberg value parameters were all varied. On samples containing free Hb and Hb bound to Hp1-1 two species molecular mass (s/D) fits were also performed with and without the offset parameter, while the mass parameter was held constant at 64 kDa for free Hb and at 162 kDa for Hb-Hp1-1 and the remaining parameters were varied. For DCDT analysis all of the possible scans were used for two or three different number of scans per fit resulting in 20 to 50 different fits per experiment. For each experiment the ratios of the binding rates were calculated at 5 different fractions of free vs bound Hb: the maximum and the minimum fractions from all of the DCDT and Svedberg fits for that experiment and three values in between. Visual Basic in Microsoft Excel was used to calculate the average absorbances over a given range in order to determine a concentration of cell-free HbO₂ and cell-free MetHb. This analysis was applied to a region where all the Hp bound Hb was assumed to have sedimented out. The ratio k_f/k_b was calculated from about 100 to 200 absorbance pairs. From the resulting ratios the negative and those above 1000 (one instance) were thrown out and then everything outside of ± 2 standard deviations was also discarded.

Computational modeling

Using a two-dimensional model to evaluate nitric oxide (NO) production and consumption within blood vessels and the surrounding tissue, we examined the effect of Hp-Hb complex uptake rate on NO bioavailability. Computational modeling was performed similarly to that reported previously [6,25]. The governing equation for the model taken at steady state ($dC/dt = 0$) was as follows:

$$\frac{dC_{NO}}{dt} = D_{NO} \nabla^2 C_{NO} - R + S, \quad (11)$$

where R is the rate of consumption of NO ($R = k_{Hb}[Hb][NO]$), S is the production rate of NO (taken as $42.4 \mu M/s$), D_{NO} is the diffusion coefficient ($3300 \mu m^2/s$), and C_{NO} is the concentration of NO. The change in cell-free Hb with respect to time is given by the following equation

$$\frac{dHb_{CF}}{dt} = P_{Hb} - U_{Hb}, \quad (12)$$

where P_{Hb} is the production rate of cell-free Hb_{CF} ($P_{Hb} = k_p[Hb_{RBC}]$), where RBC refers to the red blood cell), and U_{Hb} is the clearance rate of Hb by Hp ($U_{Hb} = k_{1,2}[Hb][Hp]$). In this computational model, the rate of NO scavenging by cell-free Hb is assumed to be the same whether the Hb is bound to Hp or not. Thus, Hb_{CF} represents the sum of cell-free Hb that is bound and unbound to Hp and the clearance rate depends on the rate of Hp-Hb complex uptake. Assuming quasi-steady state, $dHb_{CF}/dt = 0$, and solving for $[Hb]$ in Equation 12:

$$[Hb] = \frac{k_p [Hb_{RBC}]}{k_{1,2} [Hp]}, \quad (13)$$

The change in the clearance rate of Hb by Hp thereby reduces the concentration of Hb, thus decreasing the rate of consumption of NO by Hb (R in Eq. 11).

Results

Photolysis Studies

Typical time-resolved absorption spectra obtained following photo-induced release of caged NO in the presence of Hb alone, Hb-Hp 1-1, and Hb-Hp 2-2 are shown in Figure 2A–C. The absorbance peak of the initial spectrum is near 415 nm corresponding to that of oxygenated Hb. The absorbance shifts to a 405 nm peak, characteristic of MetHb. The nature of the species involved is clearly shown in Figures 2D–F that contain the initial and final spectra resulting from global fitting to a single exponential process, $A \rightarrow B$. Also, shown are the absorbance vs time at 406 nm from the data and the global fit (insets, Figs. 2D–F). No significant differences are seen upon comparison of the spectra and kinetics between Hb alone and the Hp bound species shown in Figure 2. The lack of an effect of Hp binding on the rate of the NO dioxygenation reaction is clearly shown in Table 1, where the average bimolecular rate constants are given for each species.

Competition Experiments

To confirm our results using time-resolved absorption spectroscopy, we developed a competition assay using analytical ultracentrifugation to determine the relative rate that NO reacts with Hp bound Hb compared to unbound Hb. A mixture of Hb and Hb-Hp was incubated with an NO donor for 1 hr. The amount of MetHb formed was determined by absorption in the analytical centrifuge and the relative rate of reactions k_f/k_b was determined using Equation 2. Figure 3A shows raw data from the ultracentrifuge, one scan at 598 nm and one at 632 nm taken immediately afterwards from a total of 90 scans over 7.3 hrs. Around 6.55 cm there is a

visible inflexion point in both of the absorbance graphs from the boundary of the sedimenting free Hb and at approximately 6.8 cm the absorbance reaches a maximum plateau due to both free and bound Hb being present at that radius. To calculate the concentration of both free and bound MetHb and HbO₂ we recorded the absorbance of free Hb alone at the two wavelengths from scans such as those shown in Figure 3A where the two sedimenting species are visibly separated. For example for the scan at 589 nm, the absorbance values between 6.35 cm and 6.55 cm were assumed to be that of free Hb alone. Several absorbance values in that range were recorded, averaging over several values to reduce noise. The absorbances at 632 nm are determined in the same manner, but each radial value corresponding to 589 nm is shifted 0.005 to 0.006 cm which is roughly how far the free Hb border will have moved in the time between the start of the 589 nm scan and the following 632 nm scan. Roughly 100 to 200 different absorbance pairs result from this analysis. From these absorbance values all pairs where both absorbance values are greater than or equal to 0.2 are used to calculate k_f/k_b at 5 different amounts of free vs. bound Hb as determined by Svedberg and DCDT+ fitting (see Experimental Procedures). Figure 3B shows a summary of calculated relative rate with Hb compared to Hb bound to Hp 1-1 and Hp 2-2 (from a total of about 5000 calculations from 4 separate experiments on each Hp). The rate ratios shown were calculated based on the fraction of free Hb determined from fits that did not employ the offset parameter, when the offset was used we obtained a k_f/k_b of 1.4 ± 0.5 for free Hb vs. Hb-Hp 1-1 and 1.5 ± 1.5 for free Hb vs. Hb-Hp 2-2. These results confirm those using time-resolved absorption spectroscopy.

Computational Modeling

Since the scavenging rate of the Hb-Hp complexes are the same as for cell-free Hb, we examined the effect of uptake rate of the Hp-Hb complex on NO bioavailability. A two-fold difference in Hb-Hp uptake rate would reduce the steady-state concentration of cell-free Hb by a factor of two. We have recently modeled the effect of cell-free Hb on NO bioavailability in sickle cell disease [6]. The degree of intravascular hemolysis in sickle cell disease is less than that found in other conditions of pathological hemolysis such as paroxysmal nocturnal hemoglobinuria (PNH), thalassemia intermedia, malaria, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome and cardiopulmonary bypass.

We examined the effect of a reduced concentration of cell-free Hb on NO bioavailability under conditions that correlate to those found in normal physiology and sickle cell (hemolytic disease) during steady state and vaso-occlusive crisis. The cell-free Hb concentration in the lumen and interstitial space, Hb_{CF}, was varied by a factor of two from the previously shown mean concentration during steady state sickle cell ($4 \mu\text{M}$). The computer calculations were performed in the presence of 0.5 and $1 \mu\text{M}$ extravasated cell-free Hb and without extravasation. Figure 4 shows the steady NO concentrations plotted as a function of distance from the center of a vessel with a $50 \mu\text{m}$ radius. Each curve shows that there is virtually no NO in the lumen of the vessel and that the concentration of NO peaks at the endothelium (where it is made) and then decreases as the distance from the center of the vessel increases. Calculations for conditions mimicking steady-state in sickle cell disease, in the absence of extravasation, give a peak NO concentration of $0.14 \mu\text{M}$ (Figure 4) which is two to three times less than what is calculated in the absence of hemolysis [6]. Importantly, when the cell-free Hb concentration is lowered by a factor of two, the peak NO concentration goes up to $0.18 \mu\text{M}$ (Figure 4). Similarly, NO availability decreased for the case with $4 \mu\text{M}$ cell-free Hb in the presence of $1 \mu\text{M}$ extravasated cell-free Hb compared with the reduced concentrations of $2 \mu\text{M}$ cell-free Hb with $0.5 \mu\text{M}$ extravasated cell-free Hb. The calculated peak NO concentration for $4 \mu\text{M}$ cell-free Hb and the extravasation of $1 \mu\text{M}$ cell-free Hb out of the lumen was $0.11 \mu\text{M}$, while that measured for the reduced cell-free Hb concentrations ($2 \mu\text{M}$ cell-free Hb in the lumen and $0.5 \mu\text{M}$ extravasated cell-free Hb) was $0.16 \mu\text{M}$. These results suggest that a factor of two in Hp mediated cell-free Hb uptake rate could significantly affect NO bioavailability in disease.

Discussion

We have shown that Hb bound to either Hp 1-1 or Hp 2-2 scavenges NO via the dioxygenation reaction at the same rate as unbound Hb. This implies that oxygen remains on the heme groups when Hb is bound to Hp and that the heme groups are therefore ferrous. NO binds to R-state Hb at the same rate as it does T-state Hb where the binding rate is most likely rate-limited by diffusion through the protein to heme pocket; once inside the heme pocket the probability that NO will bind the heme is close to one [4,20,22,26–30]. As the dioxygenation rate is slightly faster than the rate of NO binding to the heme [2], it is probably also rate-limited by how fast NO diffuses through the protein to the heme pocket. Our results imply that binding to Hp does not affect the rate of diffusion of the ligand to the pocket. These results are consistent with the fact that Hb bound to Hp has been found to bind carbon monoxide at similar rates as R-state Hb [31,32]. In addition, it has previously been hypothesized that a rapid NO scavenging reaction detected in plasma was due to the dioxygenation reaction involving Hp-Hb [33].

Since complex formation of Hp with Hb itself will not reduce NO scavenging, the rate of uptake by monocyte/macrophages of the complex may be important. Once inside the monocyte/macrophage, the Hb protein is degraded and further NO scavenging is unlikely. Thus, the rate of uptake of Hb and its dependence on Hp type could have important consequences in controlling NO bioavailability. One may, however, wonder if just a two-fold difference in uptake rate could affect NO bioavailability. We explored this question using computational modeling.

In sickle cell disease, Hp is depleted. However, as this disease represents one with relatively small hemolysis, it serves as a good example to study the effects of Hp-Hb uptake on NO bioavailability. As previously shown, the effect of hematocrit (Hct) or the permeability of the RBC, no longer exists when cell-free Hb concentrations of 4 μ M and above are present in the lumen of the blood vessel [6]. Therefore, in the calculations performed with 4 μ M cell-free Hb, the results found at a Hct of 50% can be applied to the range of Hct found in sickle cell (18 to 25%). The calculations with 2 μ M cell-free Hb were performed with 50% Hct. The effects of Hb-Hp uptake rates on NO bioavailability found under these conditions would be magnified at lower Hcts found in sickle cell disease (18% to 25%) as it has been shown that lower Hcts result in a larger sensitivity to the presence of cell-free Hb [6]. In any case, the major finding of our calculations is that a two-fold difference in Hp-Hb uptake rate would significantly affect NO bioavailability. This is shown in Figure 4 where steady-state levels of NO near the smooth muscles are reduced by about an additional 25% at slower uptake rates compared to faster ones. (Note that smooth muscle would be roughly at the same place at the endothelium on the scale of Figure 4 and the position of the endothelium is marked on the figure). Given that levels of NO necessary for sGC activation and other biological processes are likely to be under tight control, it is reasonable to hypothesize that this contributes to pathology.

An additional factor that might influence how Hp-Hb affects NO bioavailability is the presence of the glycocalyx, a layer about 0.5 μ m thick of proteoglycans and glycoproteins above the endothelium [34–37]. The fact that cell-free Hb can extravasate into the interstitial space beyond the endothelium indicates it can permeate the glycocalyx. However, it is possible that Hb bound to Hp would be less likely to enter the glycocalyx, thereby reducing NO scavenging by Hp bound Hb compared to unbound cell-free Hb. In addition it is possible that the presence of Hp-Hb complexes may stimulate NO production through mechanotransduction although such an effect would have to be weighed against increased NO scavenging by cell-free Hb and Hp-Hb complexes. Another potential mediator of the effect of Hp-Hb complexes on NO bioavailability involves the extent to which these complexes effectively reduce nitrite to NO [38]. It is known that deoxygenated hemes in R-state Hb tetramers reduce Hb more effectively

than those in T-state tetramers [39,40]. Since the Hp-Hb complex appears to have R-state character [31,32], one would expect that it would be quite effective in reducing nitrite to NO as long as some of the hemes were deoxygenated. The fact that, like R-state Hb, Hb bound to Hp auto-oxidizes relatively fast would tend to decrease its ability to scavenge NO, but we did not see evidence for auto-oxidation in the conditions of our experiments. Our finding that Hp-Hb complexes scavenge NO as effectively as cell-free Hb may also have implications for the development of hemoglobin based oxygen carriers as blood substitutes. These effects require further study.

Our findings may be of profound significance in explaining the association of the Hp genotype with both vasospasm and atherothrombosis. We propose that the increased vasospasm observed in Hp 2 individuals after subarachnoid hemorrhage is the result of increased NO consumption by the dioxygenation reaction due to the slower rate of clearance of Hp-Hb in Hp 2 individuals. In individuals with the Hp 2 allele and Diabetes Mellitus the incidence of atherothrombosis is markedly increased [13]. We have shown that the expression of CD163 is markedly depressed in Hp 2-2 diabetic individuals [41]. We propose that the impaired clearance of the Hp-Hb complex within the atherosclerotic plaque of Hp 2-2-diabetic individuals, due both to an impaired efficiency of the Hp 2-2 protein to mediate clearance as well as the decreased expression of the Hp-Hb receptor CD163 in Hp 2-2 individuals, will result in decreased NO availability within the plaque leading to greater atherothrombosis in Hp 2-2 diabetic individuals.

Acknowledgments

This work was supported by NIH grants HL58091 (DK-S). DBK-S gratefully acknowledges further support from NIH grant K02 HL078706.

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Figure 1.

Sedimentation studies showing no unbound Hb in photolysis studies. (A) Raw absorption data from the analytical centrifuge taken at 415 nm, 2.5 hours after beginning the spin at 45,000 rpm. Each curve shows the absorbance due to Hb either free or bound to Hp plotted against position in a separate centrifuge tube. The radial positions of free Hb and Hb-Hp 2-2 have been shifted to line up their menisci with that of Hb-Hp 1-1. The Hb bound to the Hp 2-2 has completely sedimented at the time these data were collected and there is no evidence of free Hb in that sample. The sample containing Hp 1-1 has not completely sedimented but comparison to the free Hb sample indicates that there is little to no free Hb in the Hb-Hp 1-1

sample. (B) Species distributions were calculated using DCDDT+ (version 6.31) software (J. Philo, Thousand Oaks, CA) [21].

Figure 2.

Photolysis studies of Hb free and bound to either Hp 1-1 or Hp 2-2. Caged NO (2 mM) was mixed with Hb or Hb-Hp and released using a 355 nm wavelength laser pulse. The absorption was followed using a continuous probe beam focused onto a Streak camera. (A–C) Absorbance (after noise reduction using singular value decomposition) is plotted against wavelength following NO release for (A) unbound Hb, (B), Hb bound to Hp 1-1, and (C) Hb bound to Hp 2-2. (D–F) Initial and final species obtained by global analysis of data shown in the left hand column. The plots contain the initial species obtained from global analysis (OxyHb) and the final species (MetHb). The insets show the absorbance over time at 406 nm. (D) unbound Hb, (E), Hb bound to Hp 1-1, and (F) Hb bound to Hp 2-2.

Figure 3. Competition Experiments. (A) Absorption data collected about 141 minutes after the beginning of sedimentation. The absorbance from two consecutive scans along a centrifuge cell are shown. (B) The average relative bimolecular rate constants for the NO dioxygenation reaction. The average value of the relative rate constant of the reaction for free Hb (k_f) divided by that bound to Hp (k_b) is shown $\pm$ one standard deviation for Hp 1-1 and Hp 2-2 (from a total of about 5000 calculations from 4 separate experiments on each Hp).

Figure 4.

Effect of cell-free Hb in the lumen and interstitial space on [NO]. Concentrations of NO in the presence of cell-free Hb with and without extravasation are plotted versus the distance along the vessel axis. The simulations were performed with 50% Hct (total RBC encapsulated Hb in the lumen was 10 mM) and red blood cell permeability of $450 \mu\text{m s}^{-1}$. The position of the endothelium is indicated by the black arrow at 0.05 mm (peak NO availability). Cell-free Hb concentrations ([Hbcf]) were set to 2 and 4 μM and the concentrations of extravasated cell-free Hb into the interstitial space ([Hbextra]) were varied from 0, 0.5, and 1 μM . The figure shows that the concentration of NO at the endothelium was between about 0.11 μM and 0.18 μM for the different simulations shown. NO concentration increases when the cell-free Hb and extravasated cell-free Hb concentrations are lowered by a factor of two corresponding to the difference in Hb uptake rates by different Hp types.

Table 1

Bimolecular rate constants of NO dioxygenation. The rate of NO dioxygenation for normal adult hemoglobin (HbA) when bound to haptoglobin types (Hp) were obtained from photolysis of aged NO followed by time resolved absorption spectroscopy.

	Rate Constants ($M^{-1}s^{-1}$)
HbA	6×10^7
HbA-Hp1-1	$(6.1 \pm 0.1) \times 10^7$
HbA-Hp2-2	$(6.0 \pm 0.2) \times 10^7$