Published in final edited form as:

Nitric Oxide. 2008 March ; 18(2): 122–135.

Interactions of Nitrosylhemoglobin and Carboxyhemoglobin with Erythrocyte

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Abstract

Nitrosylhemoglobin (HbFe^{II}NO) has been detected in vivo, and its role in NO transport and preservation has been discussed. To gain insight into the potential role of HbFe^{II}NO, we performed *in vitro* experiments to determine the effect of oxygenated red blood cells (RBCs) on the dissociation of cell-free HbFe^{II}NO, using carboxyhemoglobin (HbFe^{II}CO) as a comparison. Results show that the apparent half-life of the cell-free HbFe^{II}CO was reduced significantly in the presence of RBCs at 1 % hematocrit. In contrast, RBC did not change the apparent half-life of extracellular HbFe^{II}NO, but caused a shift in the HbFe^{II}NO dissociation product from methemoglobin (metHbFe^{III}) to oxyhemoglobin (HbFe^{II}O₂). Extracellular hemoglobin was able to extract CO from HbFe^{II}CO-containing RBC, but not NO from HbFe^{II}NO-containing RBC. Although these results appear to suggest some unusual interactions between HbFe^{II}NO and RBC, the data are explainable by simple HbFe^{II}NO dissociation and hemoglobin oxidation with known rate constants. A kinetic model consisting of these reactions shows that i) deoxyhemoglobin is an intermediate in the reaction of HbFe^{II}NO oxidation to metHbFe^{III}, ii) the rate-limiting step of HbFe^{II}NO decay is the dissociation of NO from HbFe^{II}NO, iii) the magnitude of NO diffusion rate constant into RBC is estimated to be $\sim 10^4 \text{ M}^{-1}\text{s}^{-1}$, consistent with previous results determined from a competition assay, and iv) no additional chemical reactions are required to explain these data.

Keywords

nitrosylhemoglobin; carboxyhemoglobin; erythrocytes; diffusion; kinetic simulation

INTRODUCTION

Endothelium-derived nitric oxide (NO) regulates vascular tone by activating soluble guanylyl cyclase (sGC) in smooth muscle [1,2,3,4]. As a first approximation, NO that diffuses into the lumen of the blood vessel is expected to react rapidly ($\sim 10^7 \text{ M}^{-1}\text{sec}^{-1}$) with oxyhemoglobin (HbFe^{II}O₂) [5,6,7] to form methemoglobin (metHbFe^{III}) and nitrate. It is therefore necessary to preserve NO bioactivity in the proximity of a pool of hemoglobin ($\sim 10 \text{ mM}$) in the lumen. Several solutions to this paradox have been proposed, including i) the diffusion and transport limitation arises from the encapsulation of hemoglobin (Hb) in erythrocyte [8,9,10,11,12,13,14,15,16,17,18], ii) the formation of S-nitroso-hemoglobin (SNO-Hb) from the intramolecular transfer of nitrosylhemoglobin (HbFe^{II}NO) [19,20,21,22,23,24,5,25], and iii)

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the NO bioactivity transduced from nitrite [26,27,28,29]. In the latter two cases, the formation of HbFe^{II}NO can be argued to play a role for preserving NO bioactivity.

It is believed that HbFe^{II}NO is fairly stable, because the dissociation rate of NO from ferrous Hb is extremely slow ($\sim 10^{-3}$ - 10^{-5} s⁻¹) [30,31,32,33]. Therefore, preservation of NO bioactivity in HbFe^{II}NO demands a viable mechanism for NO release and re-utilization [34]. However, the metabolic fate of HbFe^{II}NO has been a subject of debate for over a decade [35]. Early studies showed that the degradation of HbFe^{II}NO under aerobic conditions led to the formation of metHbFe^{III} and nitrate, but it was uncertain whether molecular oxygen reacted directly with the NO bound to the iron-ligand, or if it required the ligand-bound NO to be released from HbFe^{II}NO first.

For over a decade the rate of oxidation of HbFe^{II}NO to metHbFe^{III} in air has been recognized as nearly identical to the rate of NO dissociation from HbFe^{II}NO under anaerobic conditions in the presence of excess sodium dithionite and carbon monoxide [30]. In early works, it has been suggested that the formation of metHbFe^{III} from HbFe^{II}NO is a direct reaction of O₂ with the bound NO. Stamler and coworkers utilized this idea of autooxidation reaction to describe denitrosylation [21]. However, later works began to consider that the autooxidation reaction may occur via three steps [36,37]: (1) the slow dissociation of NO from the iron center of hemoglobin or myoglobin, (2) the binding of oxygen to the newly formed deoxy-Fe^{II} globin, and (3) the irreversible oxidation of HbFe^{II}O₂ by NO referred to as NO dioxygenation (NOD). Olson *et al.* [38] described the NOD reaction in detail and noted that there is a 1:1 correlation between the rates of autooxidation and their rates of thermal NO dissociation over 30 different Mb and Hb mutants. When examining the rates of autooxidation of HbFe^{II}NO and the rate of thermal dissociation of NO, Herold *et al.* showed the intermediacy of deoxyHbFe^{II} during HbFe^{II}NO autooxidation reaction [39]. To expand the understanding of the HbFe^{II}NO autooxidation in heterogeneous mixture, we designed our system so that the cell-free HbFe^{II}NO autooxidation mechanism is studied and analyzed quantitatively in the presence of RBCs.

The formation of extracellular HbFe^{II}NO has been observed in patients of hemolytic diseases such as sickle-cell disease [40,41]. However, the metabolic fate of HbFe^{II}NO in the presence of RBCs has not been studied. We have previously demonstrated that there exists a diffusion barrier intrinsic to RBCs, and such barrier reduces ~ 1000 fold the NO consumption rate of RBCs [14,15]. The resulting effective reaction rate constant (k_{RBC}) for NO and intra-erythrocytic Hb becomes $\sim 10^4$ M⁻¹sec⁻¹. Examining the interaction of HbFe^{II}NO and RBCs may provide an alternative way to gain insight into the diffusion barrier to RBCs and any additional reactions that might occur between HbFe^{II}NO and RBCs.

Besides NO, another endogenously produced diatomic signaling molecule, carbon dioxide (CO), has gained increasing amounts of attention [42]. It has been found that CO has exerts similar vasoregulatory effects to NO, including inhibition of smooth muscle proliferation [43] and platelet aggregation [44]. Like NO, CO has high affinity to hemoproteins and can bind reversibly to the ferrous iron in the heme group to form carboxyhemoglobin (HbFe^{II}CO) [45]. However, unlike NO which is vulnerable to various reactions, CO is non-oxidizable and has little biochemical reactivity with non-iron compounds [46]. Therefore, differentiating the mechanism of HbFe^{II}CO and HbFe^{II}NO degradation in the presence of RBCs allows us to understand the unique chemistry that might be associated with HbFe^{II}NO.

This study focuses on elucidating the degradation pathways of HbFe^{II}CO and HbFe^{II}NO in the presence of RBCs under aerobic conditions *in vitro*. A mathematical model based on existing kinetic parameters valid for the experimental conditions is developed to explain the data.

EXPERIMENTAL PROCEDURES

Chemicals

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Sephadex G-25, and Ferricyanide ($K_3Fe(CN)_6$) was purchased from Sigma-Aldrich (St. Louis, MO). Diethylamine NONOate (DEA/NO) was purchased from Cayman Chemicals (Ann Arbor, MI). Ultrahigh purity CO gas ($> 99.999\%$) was purchased from Air Liquide (Houston, TX), and NO gas was purchased from Matheson (Montgomeryville, PA). Purified RBCs and bovine hemoglobin species were prepared as described below.

Preparation of Purified Red Blood Cells

Bovine blood was collected in a heparinized container (10 IU/mL). Plasma and buffy coat were removed from the supernatant following centrifugation of the whole blood at $800 \times g$ for 10 minutes. The packed RBCs were diluted to 50 % hematocrit (Hct) with HEPES buffer containing 40 mM HEPES, 120 mM NaCl, and 5 mM glucose at pH 7.40, 285 mOsm/kg. The cells were filtered through a column of a mixture containing 50 % α -cellulose and 50 % microcrystalline cellulose by weight to eliminate white cells. The RBCs were ready for use once all cells were resuspended and washed three times to ensure their purity. Without further specification, HEPES buffer was used for all washings of RBCs.

Preparation of Purified Hb Species

To prepare for $HbFe^{II}O_2$, purified packed RBCs were first diluted to 50 % Hct with HEPES buffer, then freeze-lysed at $-80^\circ C$ overnight. To eliminate the membrane debris, cell lysate was centrifuged at $22,000 \times g$ in $4^\circ C$ for 12 min and the top half of the supernatant was collected. Repeat the centrifugation again to ensure the removal of the cell debris. $HbFe^{II}O_2$ solution free from membrane debris was collected and purified by passage through a Sephadex G-25 column to separate small molecules such as thiols. To prevent the proteins from degrading, all hemoglobin solutions were maintained at $4^\circ C$ during the purification process.

Carboxyhemoglobin ($HbFe^{II}CO$) was prepared by passing over a stream of ultrahigh purity CO gas $HbFe^{II}O_2$ solution for about an hour under a safety hood. This method generated a CO-saturated $HbFe^{II}CO$ stock solution (100 μM) with both the liquid phase and Hb saturated with CO.

Methemoglobin ($metHbFe^{III}$) was prepared by adding a 2:1 molar ratio of potassium ferricyanide ($K_3Fe(CN)_6$) to the purified $HbFe^{II}O_2$ to oxidizes $HbFe^{II}O_2$ to $metHbFe^{III}$. The sample was then filtered through a Sephadex G-25 column to separate ferricyanide from $metHbFe^{III}$. The characteristic maximum absorbance of $metHbFe^{III}$ at 405 nm for the collected samples was verified with a spectrophotometer.

Deoxygenated hemoglobin ($deoxyHbFe^{II}$) was prepared by blowing a stream of ultrahigh-purity argon ($> 99.999\%$ Ar) over a $HbFe^{II}O_2$ solution for ~two to four hours in a deoxygenated chamber ($pO_2 < 0.5\%$). The Hb sample was chilled on ice to prevent Hb degradation and placed on a rotator to ensure homogeneous mixing. The deoxygenation was continued until the spectrum of the solution validated the pure $deoxyHbFe^{II}$ spectrum, which has maximum absorbance at 429-430 nm.

NO gas was prepared by bubbling it through deoxygenated 5 M NaOH solution for ~40 min and entrapped in a sealed-glass gas collection tube (Ace Glass). Iron-nitrosylated hemoglobin ($HbFe^{II}NO$) was prepared by transferring NO gas with a Hamilton air-tight syringe, then gently squeeze a few bubbles of NO gas from the syringe directly into the $deoxyHbFe^{II}$ prepared in the anaerobic chamber. The solution was mixed, and the purity of $HbFe^{II}NO$ was verified with

a spectrophotometer. The stock $\text{HbFe}^{\text{II}}\text{NO}$ solution (1-3 mM) was sealed in a Hamilton air-tight syringe until immediately before the start of the experiment.

Deconvolution Method and Basis Spectra

A set of “standard spectra” for pure hemoglobin species was measured for wavelength between 380 to 700 nm with a Beckman Coulter spectrophotometer DU640 (Fullerton, CA). The spectra of all Hb species mixtures were scanned between either 380-700 nm or 500-700 nm. The concentration of each Hb species in a Hb mixture was determined by linearly fitting the spectra of the mixture to the standard spectra by means of linear least-square regression [14,15].

Measurement of $\text{HbFe}^{\text{II}}\text{CO}$ Dissociation In the Presence of oxygenated RBCs

The saturated $\text{HbFe}^{\text{II}}\text{CO}$ stock solution (100 μM) was diluted 10-fold to 10 μM , and was added to the purified RBCs to make the final Hct at 0 % (absence of RBC, $[\text{HbFe}^{\text{II}}\text{CO}]$ diluted with aerobic buffer), 0.33 %, 0.66 %, and 1 %, respectively. The interaction of $\text{HbFe}^{\text{II}}\text{CO}$ and oxygenated RBCs started after mixing the $\text{HbFe}^{\text{II}}\text{CO}$ -RBC mixtures gently and swiftly. To take a sample, RBCs were pelleted by centrifugation at $14,000 \times g$ for 10 seconds. The spectrum (380 to 700 nm) of the supernatant was measured with a spectrophotometer, and the measurements were taken at several time intervals for 20 minutes. In addition, the intracellular $\text{HbFe}^{\text{II}}\text{CO}$ concentration was measured at the end of each experiment by lysing the RBCs. Briefly, cells were washed by resuspension and centrifugation three times to get rid of extracellular $\text{CO}/\text{HbFe}^{\text{II}}\text{CO}$ before RBCs were lysed. Hb species concentration was determined with the linear deconvolution method discussed above.

In order to measure $\text{HbFe}^{\text{II}}\text{CO}$ dissociation in the presence of CO -pretreated RBCs, purified oxygenated RBCs (1 % Hct) were pre-incubated with CO solutions of 0, 25, and 100 μM CO , respectively, for 20 minutes. The RBCs were washed three times to remove the extracellular CO molecules. The measurements of $\text{HbFe}^{\text{II}}\text{CO}$ degradation in the presence of CO pre-treated RBCs is performed in the same fashion described for that of $\text{HbFe}^{\text{II}}\text{CO}$ in the presence of untreated RBCs.

Determination of CO Release out of RBCs

RBC samples were pre-incubated in CO solutions of various concentrations (0, 25, and 100 μM) for ten minutes. Once treated with CO solution, the generation of $\text{HbFe}^{\text{II}}\text{CO}$ inside of the RBCs was validated with a spectra reading of the RBC lysate. RBCs were washed by resuspension and centrifugation three times to remove extracellular hemoglobin and CO molecules. CO -treated RBCs at 15 % Hct was then incubated with cell-free $\text{HbFe}^{\text{II}}\text{O}_2$ (10 μM) for 10 minutes. Finally, the extracellular spectra were recorded and the resulting percentage of $\text{HbFe}^{\text{II}}\text{CO}$ in the solution was determined.

Measurement of $\text{HbFe}^{\text{II}}\text{NO}$ Degradation In the Presence of oxygenated RBCs

Immediately before mixing $\text{HbFe}^{\text{II}}\text{NO}$ and oxygenated RBCs, a solution of $\sim 50 \mu\text{M}$ $\text{HbFe}^{\text{II}}\text{NO}$ was prepared by diluting the stock $\text{HbFe}^{\text{II}}\text{NO}$ solution prepared in the same day. The reaction started once $\sim 40 \mu\text{M}$ of $\text{HbFe}^{\text{II}}\text{NO}$ solution was added to the purified and packed RBCs adjusted for 0 % (absence of RBC, $[\text{HbFe}^{\text{II}}\text{NO}]$ diluted with aerobic buffer), 11 %, and 20 % Hcts, respectively. The total volume of all the reaction mixture was kept at 10 mL. The samples containing $\text{HbFe}^{\text{II}}\text{NO}$ and oxygenated RBCs were mixed gently to avoid lysis, poured into 10 ml syringes, and left to spin on a rotator over three hours. At each sampling time, 1 ml of solution was ejected from each syringe into a microcentrifuge tube, and the tube was spun at $14,000 \times g$ speed for 25 seconds. The spectra between 500 and 700 nm of the supernatant was recorded at 5, 20, 40, 80, 120, 180 min. Simultaneously, a sample controlling for the oxidation of $\text{HbFe}^{\text{II}}\text{O}_2$ or reduction of $\text{metHbFe}^{\text{III}}$ in the presence of oxygenated RBCs were

included throughout the course of the experiment. Specifically, the control sample contained either 10 μM of $\text{HbFe}^{\text{II}}\text{O}_2$ or 10 μM $\text{metHbFe}^{\text{III}}$ solution mixed with RBCs.

Determination of NO Diffusion Out of RBCs

To make $\text{HbFe}^{\text{II}}\text{NO}$ within RBCs, purified RBCs were first deoxygenated by passing a stream of Ar gas over a RBC/HEPES mixture of 1:1 ratio in an anaerobic chamber ($< 0.5\%$ pO_2). The RBC/HEPES mixture was placed on a rotator during the deoxygenation process to ensure homogeneous mixing. Typically, the deoxygenation took ~ 3 hrs and the deoxygenation was continued until the presence of cellular deoxy HbFe^{II} ($< 1\%$ $\text{HbFe}^{\text{II}}\text{O}_2$) was validated with spectra measurement. The deoxygenated RBCs were then treated with diethylamine-NONOate (DEA/NO) to make the intracellular HbNO concentration range between 40 to 400 μM . Once pretreated with NO, the RBCs were washed three times in HEPES buffer to clean any lysate and remove NO in the solution. Finally, 10 μM $\text{HbFe}^{\text{II}}\text{O}_2$ and 10 μM $\text{metHbFe}^{\text{III}}$ solution was added to the $\text{HbFe}^{\text{II}}\text{NO}$ -enclosed RBC at 15 % Hct, respectively, and the extracellular Hb species concentrations were monitored up to three hours.

Kinetic Simulation of $\text{HbFe}^{\text{II}}\text{CO}/\text{HbFe}^{\text{II}}\text{NO}$ Reaction with RBCs

The interactions of $\text{HbFe}^{\text{II}}\text{CO}$ and $\text{HbFe}^{\text{II}}\text{NO}$ with RBCs are simulated, respectively, using the kinetic models described below. Briefly, each interaction system ($\text{HbFe}^{\text{II}}\text{CO}$ or $\text{HbFe}^{\text{II}}\text{NO}$ with RBCs) was simplified to a homogeneous model. Ordinary differential equations (Eqns. 1-18) describe the mass actions of chemical species. For the interaction of $\text{HbFe}^{\text{II}}\text{CO}$ and RBCs (Box 1), reactions in both the phase inside of RBCs and the extracellular solution were considered. In each phase, the model incorporates two sets of reversible reactions: the association and dissociation of CO and molecular oxygen, O_2 , with Hb (Fig. 4A). The association refers to the binding of a CO molecule to a heme iron center, and the dissociation refers to the release of CO molecule from the iron center. Similarly for the interaction of $\text{HbFe}^{\text{II}}\text{NO}$ and RBCs, five reactions were included in each phase (Box 2). Four of the five reactions are the association and dissociation of NO with Hb and that of O_2 with Hb. The fifth equation is the irreversible oxidation of $\text{HbFe}^{\text{II}}\text{O}_2$ to $\text{metHbFe}^{\text{III}}$ and nitrate referred to as the NO dioxygenation reaction (Fig 4B). The detailed simulation setup, initial conditions, and reaction rate constants (k 's) are in Tables 1&2. In both models, a permeability, k_p (m/s), was introduced to account for the diffusion of either CO or NO molecules in and out of RBCs. k_p is defined in such a way that greater k_p corresponds to more CO or NO molecules permeating through the RBC membranes per unit time. In addition, Hct refers to the initial intracellular Hb concentration for both $\text{HbFe}^{\text{II}}\text{CO}$ and $\text{HbFe}^{\text{II}}\text{NO}$ interaction models in each experiment. In particular, effects of model parameters such as Hct, k_p , and reaction rate constants on either the $\text{HbFe}^{\text{II}}\text{CO}$ or $\text{HbFe}^{\text{II}}\text{NO}$ degradation, were examined closely for each model.

RESULTS

Interaction of $\text{HbFe}^{\text{II}}\text{CO}$ and RBCs

To determine the effects of RBCs on $\text{HbFe}^{\text{II}}\text{CO}$ degradation, $\text{HbFe}^{\text{II}}\text{CO}$ solutions and oxygenated RBCs (0.33, 0.66, and 1 % Hct) were mixed and the hemoglobin species in the extra-erythrocytic region were monitored with spectroscopy. The concentrations of $\text{HbFe}^{\text{II}}\text{CO}$ and $\text{HbFe}^{\text{II}}\text{O}_2$ at various Hcts (Figure 1A and B) are determined with a linear least-square deconvolution method based on pure species spectra. The concentration of each species was normalized by the initial $\text{HbFe}^{\text{II}}\text{CO}$ concentration. In the absence of RBCs (0 % Hct), $\text{HbFe}^{\text{II}}\text{CO}$ concentration remained stable during the experiment (Fig. 1A) and no other Hb species were detected (Fig. 1B). However in the presence of RBCs, the extracellular $\text{HbFe}^{\text{II}}\text{CO}$ concentration decreased. The final $[\text{HbFe}^{\text{II}}\text{CO}]_{\text{extracellular}}$ (normalized) at equilibrium decreased from 0.98 ± 0.01 to 0.57 ± 0.03 , 0.32 ± 0.02 , and 0.208 ± 0.04 as Hct increased from 0, to 0.33, 0.66, and 1% (Fig. 1A), respectively, and the initial drop in

$[\text{HbFe}^{\text{II}}\text{CO}]_{\text{extracellular}}$ occurred more rapidly at higher Hcts. On the other hand, generation of extracellular $\text{HbFe}^{\text{II}}\text{O}_2$ was observed in the presence of RBCs, and the equilibrium $[\text{HbFe}^{\text{II}}\text{O}_2]_{\text{extracellular}}$ (normalized) increased from 0.02 ± 0.003 , to 0.44 ± 0.09 , 0.62 ± 0.02 , and 0.73 ± 0.04 as the Hct increased from 0 % to 0.33, 0.66, and 1 % (Fig. 1B) respectively. In addition, no $\text{metHbFe}^{\text{III}}$ was detected at any Hcts tested (data not shown). These data suggest that oxygenated RBCs facilitated the conversion of $\text{HbFe}^{\text{II}}\text{CO}$ to $\text{HbFe}^{\text{II}}\text{O}_2$ in the extracellular region. Presumably, the CO dissociated from $\text{HbFe}^{\text{II}}\text{CO}$ is scavenged by RBCs, and thus $\text{HbFe}^{\text{II}}\text{O}_2$ was formed from free HbFe^{II} associating with oxygen in the solution.

Because the amount of Hb enclosed in RBCs (0.2 mM for 1% Hct) was much greater than the total extracellular Hb ($< 10 \mu\text{M}$), $([\text{RBC-Hb}] \gg [\text{HbFe}^{\text{II}}\text{CO}]_{\text{extracel}})$, a pseudo first-order kinetic constant, k' was introduced to represent $k[\text{RBC-Hb}]$, and was determined with Equations 19 based on the rapid change in $\text{HbFe}^{\text{II}}\text{CO}$ concentration during the first 5-6 minutes.

$$\ln \frac{[\text{HbCO}]_t}{[\text{HbCO}]_0} = -k't \quad \text{Equation 19}$$

Here we have shown that k' increased as Hct increased (Fig. 1D) and thus the corresponding apparent half-life of $\text{HbFe}^{\text{II}}\text{CO}$, as determined from k' , decreased as Hct increased. Although the half-life of $\text{HbFe}^{\text{II}}\text{CO}$ in the absence of RBCs was known to be $\sim 5 \text{ hr}$ [42], the addition of only 1 % RBCs significantly reduced the apparent half-life of $\text{HbFe}^{\text{II}}\text{CO}$ to $2.6 \pm 0.5 \text{ min}$. The intracellular Hb species was monitored and $\text{HbFe}^{\text{II}}\text{CO}$ was detected inside of RBCs at the end of the experiment. It was found that intracellular $[\text{HbFe}^{\text{II}}\text{CO}]$ increased from 0 % ($t = 0$) to up to ~ 4 % at $t = 20 \text{ min}$. For all trials, the concentration of intracellular $\text{metHbFe}^{\text{III}}$ remained low ($\sim 0 \mu\text{M}$) and unchanged.

The effect of oxygenated RBCs in destabilizing $\text{HbFe}^{\text{II}}\text{CO}$ was reduced when RBCs had been pretreated with CO solutions of 25 and 100 μM . Figure 1C shows that with Hct fixed at 1%, the effect on final $[\text{HbFe}^{\text{II}}\text{CO}]_{\text{ext}}$ concentration is diminished when in the presence of CO-pretreated RBCs as compared to untreated RBCs. Furthermore, increasing the pre-treatment concentration of CO from 25 to 100 μM diminishes the decrease in $[\text{HbFe}^{\text{II}}\text{CO}]_{\text{ext}}$ concentrations and the final normalized concentration changed from 0.45 ± 0.05 to 0.75 ± 0.04 . These results suggest that the diffusion of CO from the extracellular region into the RBCs can be attributed to the change in $[\text{HbFe}^{\text{II}}\text{CO}]$ and $[\text{HbFe}^{\text{II}}\text{O}_2]$ in the solution.

Several other experiments were performed to eliminate potential factors that may affect the dissociation of $\text{HbFe}^{\text{II}}\text{CO}$. To test whether the conversion of $\text{HbFe}^{\text{II}}\text{CO}$ to $\text{HbFe}^{\text{II}}\text{O}_2$ involves a change in Hb conformation induced by an allosteric effector, inositol hexaphosphate (IHP) was added to the $\text{HbFe}^{\text{II}}\text{CO}$ solution. Results showed that $\text{HbFe}^{\text{II}}\text{CO}$ concentration remained unchanged in the presence of IHP at 40, 100, and 500 μM (data not shown). In addition, it was determined that RBCs do not release any molecule to the supernatant to activate the conversion of $\text{HbFe}^{\text{II}}\text{CO}$ to $\text{HbFe}^{\text{II}}\text{O}_2$ in solution, since $\text{HbFe}^{\text{II}}\text{CO}$ was stable when mixed with the supernatant in which RBCs had been incubated for 2 hrs ($n \geq 3$). Moreover, the stability of $\text{HbFe}^{\text{II}}\text{CO}$ was also shown to be unaffected in the absence of glucose. Therefore, the involvement of energy consumption was also eliminated.

Interaction of $\text{HbFe}^{\text{II}}\text{NO}$ and RBCs

Analogous to the above experiments, the composition of the extracellular Hb species ($[\text{HbFe}^{\text{II}}\text{NO}]$, $[\text{HbFe}^{\text{II}}\text{O}_2]$, and $[\text{metHbFe}^{\text{III}}]$) were monitored spectrophotometrically at various time intervals ($t = 0, 5, 20, 40, 80, 120$, and 180 min) for the solutions containing $\text{HbFe}^{\text{II}}\text{NO}$ and RBCs (11 and 20% Hcts) under aerobic condition *in vitro*. Likewise, the concentration of each species was determined with a linear least-square deconvolution method from the spectra. The pseudo first-order kinetic constant, k' , was determined using equation 20.

$$\ln \frac{[HbNO]_t}{[HbNO]_o} = -k't$$

Equation 20

In contrast to the case of $HbFe^{II}CO$, the stability of $[HbFe^{II}NO]_{ext}$ was not significantly affected with or without the presence of oxygenated RBCs (Figs. 2A & D). The change of $[HbFe^{II}NO]_{ext}$ at $t = 180$ min are -26.6 ± 5.4 and -21.9 ± 2.8 μM for 11 and 20 % Hct, respectively.

Extracellular $HbFe^{II}O_2$ and $metHbFe^{III}$ were also determined from the same sets of spectra. In the absence of RBCs, $[HbFe^{II}O_2]_{ext}$ remained low (~ 0 μM) and unchanged whereas $metHbFe^{III}$ increased as a function of time (change of $[metHbFe^{III}]_{ext}$ at $t = 180$ min is 30.9 ± 4.7 μM) (Figs. 2BCE), indicating that $metHbFe^{III}$ is the only product under this condition. However, when oxygenated RBCs are present, extracellular $HbFe^{II}O_2$ formation increased with Hct (Figs. 2B&E), whereas extracellular $metHbFe^{III}$ formation is reduced at higher Hcts (Figs. 2C&E). The change in $[HbFe^{II}O_2]_{ext}$ at $t = 180$ min increased to 8.1 ± 1.5 and 11.7 ± 0.8 μM as Hct increased to 11 and 20 %, respectively, whereas the change in $[metHbFe^{III}]_{ext}$ at $t = 180$ min reduced to 20.3 ± 4.0 and 12.3 ± 3.1 μM at 11 and 20 %, respectively. Apparently, RBCs changed the product formation from $metHbFe^{III}$ to $HbFe^{II}O_2$ but not the stability of $HbFe^{II}NO$. Note that the total Hb concentration stayed reasonably constant (< 4 % change of the total Hb concentration) throughout all experiments, which showed little or no RBC lysis during the experiment.

In order to control for auto-oxidation or reduction of the cell-free Hb species ($HbFe^{II}O_2$ and $metHbFe^{III}$) in the presence of RBCs, extracellular $HbFe^{II}O_2$ or $metHbFe^{III}$ were mixed with oxygenated RBCs and were found to remain stable in the presence of RBCs over three hours. These results showed that the activity of $metHbFe^{III}$ reductase and the auto-oxidation of $HbFe^{II}O_2$ to $metHbFe^{III}$ were insignificant in the extracellular space of RBCs. Moreover, it was verified that the initial presence of $metHbFe^{III}$ does not affect the rate of $HbFe^{II}NO$ degradation, and the oxidation state of $metHbFe^{III}$ is not reduced (from Fe^{III} to Fe^{II}) nor affected by the presence of $HbFe^{II}NO$ (data not shown). Lastly, we also verified that superoxide anion ($O_2^{\cdot -}$) was not induced-released by oxygenated RBCs upon the addition of $HbFe^{II}NO$ to RBCs to reduced $metHbFe^{III}$ to $HbFe^{II}O_2$ when $HbFe^{II}NO$ was added to the cells. It was observed that the attenuated production of $metHbFe^{III}$ or the generation of $HbFe^{II}O_2$ remained statistically unchanged when superoxide dismutase (SOD) was added to the interaction of $HbFe^{II}NO$ and RBCs (data not shown).

Induced-Release of CO or NO from RBCs

To determine whether RBC-enclosed $HbFe^{II}CO$ can release CO out of RBCs upon the addition of an extracellular Hb species, 10 μM of cell-free $HbFe^{II}O_2$ was added to a batch of purified RBC-enclosed $HbFe^{II}CO$ (15 % Hct). Extracellular Hb species concentration was measured from mixtures with RBCs containing 0 to 20 mole % of intracellular $HbFe^{II}CO$. Samples were taken from the extracellular region 10 minutes after the addition of $HbFe^{II}O_2$. Fig. 3A shows that cell-free $HbFe^{II}CO$ was detected and there is a linear correlation between the molar percentage of the intracellular $HbFe^{II}CO$ and the percent extracellular $HbFe^{II}CO$ detected. These results demonstrated that CO can be released to the extracellular space upon the addition of cell-free $HbFe^{II}O_2$.

In contrast, the release of NO from RBC-enclosed $HbFe^{II}NO$ upon the addition of cell-free Hb species was not observed. Since NO can either oxidize $HbFe^{II}O_2$ to form $metHbFe^{III}$ or reduce $metHbFe^{III}$ to form $HbFe^{II}$, the release of NO out of RBCs would be detected by the change in cell-free $[HbFe^{II}O_2]$ and $[metHbFe^{III}]$. However, it was shown that the extracellular

[HbFe^{II}O₂] and [metHbFe^{III}] remained unchanged over 180 min (Fig 3B&C) in the presence of RBCs containing up to ~10 % of HbFe^{II}NO inside the cells.

Kinetic Simulation

To understand the chemistry underlying the above results, the interactions of HbFe^{II}CO and HbFe^{II}NO with RBCs were modeled. The interaction scheme between HbFe^{II}CO and RBCs is illustrated in Fig. 4A, and the kinetic constants are listed in Table 1. When the association and dissociation of CO and O₂ with Hb, are considered simultaneously in a system of ordinary differential equations, the predicted kinetics agrees well with the experimental data (Fig. 5). This result shows that the scheme shown in Fig. 4A is sufficient to explain the data for HbFe^{II}CO interaction with RBC. In this case the results are insensitive to the permeability, k_p , of RBC to CO as long as it is greater than 7×10^{-5} m sec⁻¹. Thus the permeability of RBC to CO cannot be determined from these data.

The kinetic model for the interaction of HbFe^{II}NO and RBCs follows the three-step reaction mechanism depicted in Fig 4B with kinetic parameters listed in Table 1. Simulation results for all three Hb species, HbFe^{II}NO, HbFe^{II}O₂, and metHbFe^{III}, at different Hcts are also in good agreement with the experimental data. Specifically, the simulation is consistent with the three experimental observations: (1) The degradation of HbFe^{II}NO was unaffected by Hct when RBCs are present (Fig 6A); (2) the formation of HbFe^{II}O₂ increased as Hct increased (Fig 6B), and (3) metHbFe^{III} formation reduced as Hct increased from 0 to 20 % (Fig 6C). The permeability, k_p , that would allow the fitting of simulated data to the experimental results within the experimental error range is determined to be between $8 \sim 30 \times 10^{-4}$ m sec⁻¹, which is slightly greater but comparable to the permeability estimated in Vaughn *et al.* [15]. Furthermore, the simulation of HbFe^{II}NO degradation using the kinetic model also demonstrates the independence of HbFe^{II}NO half-life from changes in Hct and k_p . Again, these results demonstrate that the reaction scheme in Fig. 4B explains the chemistry underlying the experimental data very well. The effect of various parameters on the interaction of HbFe^{II}NO and RBCs were closely examined using the kinetic model. Simulation shows that while the Hct is fixed, an increase in k_p increases the formation of HbFe^{II}O₂ (Fig. 7A) and reduces the formation of metHbFe^{III} (Fig. 7B). The only parameters that could affect the half-life of HbFe^{II}NO are the association (k_{assoc}) and dissociation (k_{dis}) rate constants of HbFe^{II}NO. As shown in Fig 7C, an increase in k_{dis} from 10^{-5} to 10^{-2} sec⁻¹ accelerates the degradation of HbFe^{II}NO dramatically. In addition, a hypothetical increase in k_{assoc} from 10^7 to 10^{10} M⁻¹sec⁻¹ decreases the degradation rate of HbFe^{II}NO (data not shown). In summary, simulation results strongly suggest that the rate-limiting step of the degradation of the HbFe^{II}NO in the interaction with RBCs is the dissociation of NO from Hb, and the ratio of product formation for HbFe^{II}O₂ and metHbFe^{III} are dependent on both Hct and k_p .

DISCUSSION

Since HbFe^{II}NO has been proposed to be an important intermediate in preserving the NO bioavailability *in vivo*, the chemistry involving HbFe^{II}NO metabolism has gained significant interest. Although the effect of RBCs on the apparent half-lives of the HbFe^{II}CO and HbFe^{II}NO appear different, with the apparent half-life of HbFe^{II}CO shortened at Hct as low as 1% while that of the HbFe^{II}NO remained statistically unchanged at Hct up to 20 %, the mechanisms of the interactions are explainable with known chemistries and kinetic parameters.

RBCs facilitate the conversion of HbFe^{II}CO to HbFe^{II}O₂

The interaction of HbFe^{II}CO and oxygenated RBCs suggests that RBCs facilitate the conversion of HbFe^{II}CO to HbFe^{II}O₂ in the solution, since the apparent rates of extracellular HbFe^{II}CO dissociation and HbFe^{II}O₂ generation increased in the presence of RBCs. Both the

kinetic model and the experimental results suggest that such conversion involves the transport of CO molecules into the cells. In the absence of RBCs, the long half-life of $\text{HbFe}^{\text{II}}\text{CO}$ in the solution is due to the slow interfacial diffusion of dissolved CO from solution to gas phase. In the presence of RBCs, CO molecules “see” a concentrated pool of Hb introduced by the RBCs and associate with the internal Hb. Therefore, it appeared that the extracellular $\text{HbFe}^{\text{II}}\text{CO}$ concentration decreased as the CO molecules redistribute/re-equilibrate themselves among the extra- and intracellular Hb's. Immediately after CO dissociates from the cell-free Hb, surrounding O_2 binds with the newly formed deoxy HbFe^{II} and forms $\text{HbFe}^{\text{II}}\text{O}_2$. For those RBCs that were pretreated with CO, fewer ligand-binding sites of the cellular Hb are available for further binding. The phenomenon is confirmed by the fact that pre-treating RBCs with CO results in a diminished effect that RBCs have on $\text{HbFe}^{\text{II}}\text{CO}$ dissociation. These results also demonstrated that, indeed, RBCs act as a “sink” for ligands.

$\text{HbFe}^{\text{II}}\text{NO}$ degradation is independent of RBCs scavenging of NO

Unlike $\text{HbFe}^{\text{II}}\text{CO}$ interaction with RBCs, in which CO is a non-oxidizable ligand, the interaction of $\text{HbFe}^{\text{II}}\text{NO}$ with oxygenated RBCs involved the oxidation of NO to nitrate through the irreversible dioxygenation (NOD) reaction in addition to the reversible binding of O_2 and NO to Hb. $\text{HbFe}^{\text{II}}\text{NO}$ in solution is known to degrade under aerobic conditions [35] and eventually form $\text{metHbFe}^{\text{III}}$. The overall autooxidation reaction is summarized in Reaction 1.

Immediately, two questions are raised by this. First, is the autooxidation reaction an elementary reaction through which the molecular oxygen irreversibly reacts with NO directly? This question has been debated for over a decade. Secondly, do RBCs introduce novel chemistry other than the association and dissociation of NO and O_2 with Hb, respectively, and the NOD reaction?

If autooxidation involves direct NO binding to the molecular oxygen forming nitrate, the subsequent formation of $\text{HbFe}^{\text{II}}\text{O}_2$ must come from the reduction of $\text{metHbFe}^{\text{III}}$ by RBCs. However this reaction does not occur, because the extracellular $\text{metHbFe}^{\text{III}}$ concentration remains constant in the presence of either $\text{HbFe}^{\text{II}}\text{NO}$ or RBCs ($n > 3$, data not shown). In

addition, the possibility that superoxide anion (O_2^-) may reduce $\text{metHbFe}^{\text{III}}$ to $\text{HbFe}^{\text{II}}\text{O}_2$ was also eliminated. These findings strongly indicate that deoxy HbFe^{II} is an intermediate for $\text{HbFe}^{\text{II}}\text{O}_2$ formation in the presence of RBCs. Confirmed by the simulation results, our findings demonstrate that the oxidation of $\text{HbFe}^{\text{II}}\text{NO}$ to $\text{metHbFe}^{\text{III}}$ in the presence of RBCs involves three elementary steps (Fig. 4C): the dissociation of $\text{HbFe}^{\text{II}}\text{NO}$ resulting in deoxy HbFe^{II} and NO, ii) the association of O_2 with deoxy HbFe^{II} to form $\text{HbFe}^{\text{II}}\text{O}_2$ ($\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$), and iii) the oxidation of $\text{HbFe}^{\text{II}}\text{O}_2$ by NO (also $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$) to form $\text{metHbFe}^{\text{III}}$ and nitrate. Therefore, no unusual chemistry is introduced by RBCs. Recent publications studying the oxidation of $\text{HbFe}^{\text{II}}\text{NO}$ in the absence of RBCs also suggested the three-step mechanism to form $\text{metHbFe}^{\text{III}}$ in aerobic conditions [38,39].

The Rate-limiting step is the Dissociation of NO from $\text{HbFe}^{\text{II}}\text{NO}$

Using the kinetic model, we analyzed the interaction of $\text{HbFe}^{\text{II}}\text{NO}$ and RBCs by examining factors that could affect $\text{HbFe}^{\text{II}}\text{NO}$ degradation and product generation. Unlike the interaction of RBCs and $\text{HbFe}^{\text{II}}\text{CO}$, RBCs do not accelerate the dissociation of $\text{HbFe}^{\text{II}}\text{NO}$ in the solution. Our results demonstrate that the apparent rate of $\text{HbFe}^{\text{II}}\text{NO}$ dissociation is independent of Hcts and permeability. Simulation also shows that the association and dissociation rate constants are the only parameters that affect the degradation of $\text{HbFe}^{\text{II}}\text{NO}$. Because $k_{\text{assoc, NO}}$ at $\sim 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ is extremely rapid and near the diffusion-limited regime, further increasing the rate

constant does not provide much physical meaning. We have also found that $\text{HbFe}^{\text{II}}\text{NO}$ degradation is independent of Hct level at $k_{\text{dis, NO}}$ ranging between 10^{-2} to 10^{-5} sec^{-1} . Since the dissociation rate constant differs from the α - ($< 10^{-5} \text{ s}^{-1}$) to β - ($< 10^{-3} \text{ s}^{-1}$) subunit of hemoglobin, the subunit to which NO is bound to can change the distribution of product formation. In summary, our results demonstrate that just as it is the case in the absence of RBCs, k_{dis} is the rate-determining parameter for $\text{HbFe}^{\text{II}}\text{NO}$ degradation in the presence of RBCs, and it is the bottleneck preceding the NO-diffusion into RBCs. Therefore, the degradation of $\text{HbFe}^{\text{II}}\text{NO}$ is independent of RBC-scavenging of NO.

Effects of k_p and Hct

Our results demonstrate that RBCs act as a ligand “sink” for NO as well as for CO. When $\text{HbFe}^{\text{II}}\text{NO}$ is mixed aerobically with suspensions of oxygenated RBCs, the formation of cell-free $\text{HbFe}^{\text{II}}\text{O}_2$ may seem counter-intuitive initially. However, simulation results show that the formation of $\text{HbFe}^{\text{II}}\text{O}_2$ is a manifestation of the NO dissociation from Hb and the competition of intracellular $\text{HbFe}^{\text{II}}\text{O}_2$ with extracellular $\text{HbFe}^{\text{II}}\text{O}_2$. Despite the diffusional resistance due to the unstirred layers, cellular $\text{HbFe}^{\text{II}}\text{O}_2$, which is much higher in concentration than the cell-free Hb and NO, can compete with external Hb for NO. Consequently, the external Hb remains reduced and oxygenated at the end of the reaction, and the $\text{HbFe}^{\text{II}}\text{O}_2$ generated is still capable of reacting further with other species.

Using the kinetic model, we found that any factor that may influence the rate of NO uptake by RBCs determines the fate of NO metabolism. We showed that both Hct and the permeability can affect the product distribution between $\text{HbFe}^{\text{II}}\text{O}_2$ and $\text{metHbFe}^{\text{III}}$ (Fig 2E). On the other hand, the permeability, k_p (m/s), indicates the amount of NO diffusion across RBCs at a given RBCs surface area. Therefore, higher k_p allows less NO available to oxidize extracellular $\text{HbFe}^{\text{II}}\text{O}_2$ to form $\text{metHbFe}^{\text{III}}$. Comparing the mechanism for the interaction of $\text{HbFe}^{\text{II}}\text{NO}$ and RBC to that of $\text{HbFe}^{\text{II}}\text{CO}$ and RBCs, we found that the mechanism for $\text{HbFe}^{\text{II}}\text{CO}$ and RBCs interaction is a subset of that for $\text{HbFe}^{\text{II}}\text{NO}$ and RBCs interaction.

Role of Diffusional Barrier for NO uptake

This study also provides an alternative approach to quantify the diffusion resistance for NO uptake by RBCs. It was previously shown that there exists a diffusion barrier intrinsic to RBCs [14,15], and it reduces the rate of NO reaction with intracellular Hb by ~ 1000 -fold from the reaction rate between NO and cell free Hb ($\sim 10^7 \text{ M}^{-1}\text{sec}^{-1}$). Thus the effective reaction rate constant for NO and intracellular Hb, also known as the NO uptake rate by RBCs (k_{RBC}), is $\sim 10^4 \text{ M}^{-1}\text{sec}^{-1}$. Based on our simulation model, the consumption term of NO in the species mass balance, $-k_p \times \text{Area} \times \text{Hct} \times ([\text{NO}]_{\text{ext}} - [\text{NO}]_{\text{int}})/V_{\text{total}}$, is equivalent to the NO consumption as defined previously, $-k_{\text{RBC}} \times [\text{Hb}]_{\text{internal}} \times \text{Hct} \times [\text{NO}]$. Therefore, the NO uptake rate by RBCs (k_{RBC}) can be calculated from the estimated k_p . The k_{RBC} calculated from k_p ranges between 5.2×10^4 and $1.9 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$, consistent with previous results [14,15]. This finding corroborates the existence and the magnitude of the diffusion barrier between NO and RBC-Hb. Increasing the diffusional barrier between NO and RBCs would result in decreased extracellular $\text{HbFe}^{\text{II}}\text{O}_2$ generation and increased $\text{metHbFe}^{\text{III}}$ formation under the same condition. Hence, our results indicate that the diffusion resistance plays a role in product formation from $\text{HbFe}^{\text{II}}\text{NO}$ degradation, and the distribution of product formation reflects the existence of a diffusion barrier to reach internal Hb (k_{RBC}).

Conservation of CO but not NO by RBCs

Once CO or NO is in the RBCs, it is important to know whether the molecule can be released from RBCs. The release of the molecules from RBCs shows non-consumption of the molecule by RBCs. In short, CO diffuses freely out of RBCs into the solution upon the addition of extracellular oxyHb, whereas no NO-export out of the RBCs-enclosed $\text{HbFe}^{\text{II}}\text{NO}$ was observed

for three hours. These results confirm that: (1) the bioavailability of NO is not conserved under this condition, which is consistent with previous findings [47]; and (2) that the autooxidation of HbFe^{II}NO to metHbFe^{III} involves the intermediate deoxyHbFe^{II}.

In summary, while the preservation of NO in the presence of RBCs and the biological functions of CO *in vivo* remain unclear, our findings demonstrate how RBCs affect HbFe^{II}CO and HbFe^{II}NO degradation. Complete understanding of the biochemistry and the diffusion-reaction mechanism between NO, CO, Hb, and RBCs is fundamental to the design of a red blood cell substitute and the extent and limitations of NO and CO drug delivery. Although NO release from HbFe^{II}NO appears to be a slow and rate-limiting step for subsequent reactions in our system, much more rapid NO release from HbFe^{II}NO has been observed while the HbFe^{II}O₂-nitrite reaction occurred in parallel [47]. Such a reaction suggests novel HbFe^{II}NO metabolism mechanisms in the presence of nitrite, and perhaps it would provide further details to the HbFe^{II}NO chemistry by studying the interaction of HbFe^{II}NO and RBCs in the presence of nitrite under conditions of different oxygenation.

Box 1

Extracellular Region For the Interaction of HbCO/RBCs

$$\frac{d[HbCO]_{ext}}{dt} = -k_{dis,CO}[HbCO]_{ext} + k_{assoc,CO}[Hb]_{ext}[CO]_{ext} \quad (\text{Eqn. 1})$$

$$\frac{d[Hb]_{ext}}{dt} = k_{dis,CO}[HbCO]_{ext} - k_{assoc,CO}[Hb]_{ext}[CO]_{ext} + k_{dis,O_2}[HbO_2]_{ext} - k_{assoc,O_2}[Hb]_{ext}[O_2]_{ext} \quad (\text{Eqn. 2})$$

$$\frac{d[CO]_{ext}}{dt} = k_{dis,CO}[HbCO]_{ext} - k_{assoc,CO}[Hb]_{ext}[CO]_{ext} - k_{p,CO} * Hct, CO * Area * ([CO]_{ext} - [CO]_{in}) / Vt \quad (\text{Eqn. 3})$$

$$\frac{d[HbO_2]_{ext}}{dt} = -k_{dis,O_2}[HbO_2]_{ext} + k_{assoc,O_2}[Hb]_{ext}[O_2]_{ext} \quad (\text{Eqn. 4})$$

Intracellular Region For the Interaction of HbCO/RBCs

$$\frac{d[HbCO]_{in}}{dt} = -k_{dis,CO}[HbCO]_{in} + k_{assoc,CO}[Hb]_{in}[CO]_{in} \quad (\text{Eqn. 5})$$

$$\frac{d[Hb]_{in}}{dt} = k_{dis,CO}[HbCO]_{in} - k_{assoc,CO}[Hb]_{in}[CO]_{in} + k_{dis,O_2}[HbO_2]_{in} - k_{assoc,O_2}[Hb]_{in}[O_2]_{in} \quad (\text{Eqn. 6})$$

$$\frac{d[CO]_{in}}{dt} = k_{dis,CO}[HbCO]_{in} - k_{assoc,CO}[Hb]_{in}[CO]_{in} + k_{p,CO} * HctCO * Area * ([CO]_{ext} - [CO]_{in}) / Vt \quad (\text{Eqn. 7})$$

$$\frac{d[HbO_2]_{in}}{dt} = -k_{dis,O_2}[HbO_2]_{in} + k_{assoc,O_2}[Hb]_{in}[O_2]_{in} \quad (\text{Eqn. 8})$$

Box 2

Extracellular Region For the Interaction of HbNO/RBCs

$$\frac{d[HbNO]_{ext}}{dt} = k_{dis,NO}[HbNO]_{ext} + k_{assoc,NO}[Hb]_{ext}[NO]_{ext} \quad (\text{Eqn. 9})$$

$$\frac{d[Hb]_{ext}}{dt} = k_{dis,NO}[HbNO]_{ext} - k_{assoc,NO}[Hb]_{ext}[NO]_{ext} + k_{dis,O_2}[HbO_2]_{ext} - k_{assoc,O_2}[Hb]_{ext}[O_2]_{ext} \quad (\text{Eqn. 10})$$

$$\frac{d[NO]_{ext}}{dt} = k_{dis,NO}[HbNO]_{ext} - k_{assoc,NO}[Hb]_{ext}[NO]_{ext} - k_{p,NO} * Area * HctNO * ([NO]_{ext} - [NO]_{int}) / Vt - k_{NOD}[HbO_2]_{ext}[NO]_{ext} \quad (\text{Eqn. 11})$$

$$\frac{d[HbO_2]_{ext}}{dt} = -k_{dis,O_2}[HbO_2]_{ext} + k_{assoc,O_2}[Hb]_{ext}[O_2]_{ext} - k_{NOD}[HbO_2]_{ext}[NO]_{ext} \quad (\text{Eqn. 12})$$

$$\frac{d[metHb]_{ext}}{dt} = k_{NOD}[HbO_2]_{ext}[NO]_{ext} \quad (\text{Eqn. 13})$$

Intracellular Region For the Interaction of HbNO/RBCs

$$\frac{d[HbNO]_{in}}{dt} = -k_{dis,NO}[HbNO]_{in} + k_{assoc,NO}[Hb]_{in}[NO]_{in} \quad (\text{Eqn. 14})$$

$$\frac{d[Hb]_{in}}{dt} = k_{dis,NO}[HbNO]_{in} - k_{assoc,NO}[Hb]_{in}[NO]_{in} + k_{dis,O_2}[HbO_2]_{in} - k_{assoc,O_2}[Hb]_{in}[O_2]_{in} \quad (\text{Eqn. 15})$$

$$\frac{d[NO]_{in}}{dt} = k_{dis,NO}[HbNO]_{in} - k_{assoc,NO}[Hb]_{in}[NO]_{in} + k_{p,NO} * Area * HctNO * ([NO]_{ext} - [NO]_{in}) / Vt - k_{NOD}[HbO_2]_{in}[NO]_{in} \quad (\text{Eqn. 16})$$

$$\frac{d[HbO_2]_{in}}{dt} = k_{dis,O_2}[HbO_2]_{in} - k_{assoc,O_2}[Hb]_{in}[O_2]_{in} \quad (\text{Eqn. 17})$$

$$\frac{d[metHb]_{in}}{dt} = k_{NOD}[HbO_2]_{in}[NO]_{in} \quad (\text{Eqn. 18})$$

ACKNOWLEDGEMENT

This work was partially supported by grant R01HL065741 from National Heart Lung and Blood Institutes.

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(A)

(B)

Fig. 1. Interaction of extracellular $\text{HbFe}^{\text{II}}\text{CO}$ with oxygenated RBCs
 (A) In the absence of RBCs, the $\text{HbFe}^{\text{II}}\text{CO}$ was stable. As Hct increased, the concentration of $\text{HbFe}^{\text{II}}\text{CO}$ decreased more rapidly. The normalized $[\text{HbFe}^{\text{II}}\text{CO}]_{\text{ext}}$ at $t = 20$ min are 0.98 ± 0.01 , 0.57 ± 0.03 , 0.32 ± 0.02 , and 0.21 ± 0.04 for 0, 0.33, 0.66, and 1 % Hct, respectively. (B) Minimal $\text{HbFe}^{\text{II}}\text{O}_2$ was generated in the absence of RBCs. However, the presence of RBCs resulted in the generation of $\text{HbFe}^{\text{II}}\text{O}_2$ and the normalized $[\text{HbFe}^{\text{II}}\text{O}_2]_{\text{ext}}$ at $t = 20$ min are 0.02 ± 0.003 , 0.44 ± 0.09 , 0.62 ± 0.02 , and 0.73 ± 0.04 for 0, 0.33, 0.66, and 1 % Hct, respectively.

For (A)&(B), the symbols for Hct's were 0 % (■), 0.33 % (○), 0.66 % (◆), and 1 % (Δ). (C) The stability of $\text{HbFe}^{\text{II}}\text{CO}$ increased when 1% of RBCs were pre-treated with 100 μM CO solution (○), 25 μM CO solution (◆), and without CO pretreatment (Δ). The normalized $[\text{HbFe}^{\text{II}}\text{CO}]_{\text{ext}}$ at $t = 20$ min are 0.75 ± 0.04 and 0.45 ± 0.05 for 100 and 25 μM of CO pretreatment, respectively, and 0.23 ± 0.08 for no CO-pretreatment. The $\text{HbFe}^{\text{II}}\text{CO}$ solution without RBC was controlled (■). D) The $\text{HbFe}^{\text{II}}\text{CO}$ pseudo-first order dissociation rate constant, k' ($k' = k [\text{RBC}]$), increased as the Hct increased. (* $P \leq 0.005$, $n \geq 4$).

(A)

(B)

(C)

(D)

Fig. 2. Interaction of extracellular $\text{HbFe}^{\text{II}}\text{NO}$ with oxygenated RBCs

(A) In contrast to $\text{HbFe}^{\text{II}}\text{CO}$, $\text{HbFe}^{\text{II}}\text{NO}$ degradation was not affected by RBCs up to 20 % Hct. The change in $[\text{HbFe}^{\text{II}}\text{NO}]$ over three hours shown at various Hct's was not statistically different. The change in $[\text{HbFe}^{\text{II}}\text{NO}]_{\text{ext}}$ for 0, 11, 20 % Hcts are -27.2 ± 3.6 , -26.7 ± 5.3 , and -21.9 ± 2.8 μM for 0, 11, and 20 % Hct, respectively. (B) In the absence of RBCs, $[\text{HbFe}^{\text{II}}\text{O}_2]$ remained unchanged. However, $\text{HbFe}^{\text{II}}\text{O}_2$ was generated in the presence of RBCs, and the generation of $\text{HbFe}^{\text{II}}\text{O}_2$ are -1.4 ± 0.5 , 8.1 ± 1.5 , to 11.7 ± 0.9 μM at 0, 11, and 20 % Hcts, respectively. (C) The presence of RBCs reduced the amount of $\text{metHbFe}^{\text{III}}$ generated. The change in $[\text{metHbFe}^{\text{III}}]_{\text{ext}}$ at $t = 180$ min are 30.9 ± 4.7 , 20.3 ± 4.0 , and 12.3 ± 3.1 μM for 0, 11, and 20 %, respectively. (D) The pseudo-first order degradation rate constant of $\text{HbFe}^{\text{II}}\text{NO}$ was independent of Hct. (E) The formation of $\text{HbFe}^{\text{II}}\text{O}_2$ and $\text{metHbFe}^{\text{III}}$ depended on Hct. As the Hct increased, the amount of $\text{HbFe}^{\text{II}}\text{O}_2$ generated increased and the formation of $\text{metHbFe}^{\text{III}}$ reduced. For (A), (B), & (C), the Hct's were 0 % ($\blacklozenge$), 11 % ($\blacksquare$), and 20 % ($\blacktriangle$). (* $p < 0.05$, data shown as mean values $\pm$ S.D. and $n \geq 5$)

(A)

(B)

Fig. 3. Induced export of CO or NO from RBCs

(A) Addition of 10 μM $\text{HbFe}^{\text{II}}\text{O}_2$ solution to 15 % Hct of RBC-enclosed $\text{HbFe}^{\text{II}}\text{CO}$ (■) induced the release of CO from cells. After the system equilibrates, extracellular $\text{HbFe}^{\text{II}}\text{CO}$ was detected, and the percent extracellular $\text{HbFe}^{\text{II}}\text{CO}$ detected correlated linearly with the percent $\text{HbFe}^{\text{II}}\text{CO}$ enclosed in RBCs. (B)&(C) Addition of either 10 μM $\text{HbFe}^{\text{II}}\text{O}_2$ or 10 μM $\text{metHbFe}^{\text{III}}$ solution to 15 % Hct of $\text{HbFe}^{\text{II}}\text{NO}$ -enclosed in RBCs (◆) did not induce the release of NO from cells to extracellular region. Extracellular $[\text{HbFe}^{\text{II}}\text{O}_2]$ and $[\text{metHbFe}^{\text{III}}]$ from either solution ($\text{HbFe}^{\text{II}}\text{O}_2$ or $\text{metHbFe}^{\text{III}}$) in the cell-free region remained unchanged. Normal RBCs without the enclosure of $\text{HbFe}^{\text{II}}\text{NO}$ (□) were controlled. (data shown as mean values $\pm$ S.D., $n \geq 4$)

(A)

(B)

(C)

Fig. 4. The reaction schemes used in kinetic simulations

(A) The presence of RBCs drives the cell-free CO to diffuse into cells and bind to the intracellular deoxygenated Hb. The surrounding molecular oxygen associates rapidly with HbFe^{II} to form HbFe^{II}O₂. (B) The HbFe^{II}NO and RBC system involves the irreversible oxidation of NO to NO₃⁻ by HbFe^{II}O₂ in addition to the reversible dissociation of NO and O₂ from HbFe^{II}NO and HbFe^{II}O₂, respectively. (C) The competition between extracellular and intracellular HbFe^{II}O₂ to scavenge NO results in shifts in product formation in the presence of RBCs. The dissociation of NO is the rate-limiting step.

(A)

(B)

(C)

Fig. 5. Kinetic simulation of HbFe^{II}CO and oxygenated RBC interactions

Dotted lines (---) are the simulated results for (A) HbFe^{II}CO dissociation, and (B) HbFe^{II}O₂ generation. For both (A) & (B) symbols are representative experimental results for 0 % RBC (■), 0.33 % Hct (○), 0.66 % Hct (◆), and 1 % Hct (Δ). (C) The effect of permeability (k_p) on HbFe^{II}CO dissociation at a fixed Hct, 0.66 % (◆). Increasing k_p increases the rate of HbFe^{II}CO dissociation.

(A)

(B)

(C)

Fig. 6. Kinetic simulation (solid curves) of HbFe^{II}NO interaction with oxygenated RBCs
 Representative experimental data shown are 0 % (□), 10 % (◆), and 20 % (Δ) Hct. (A) Stability of [HbFe^{II}NO] does not differ much in the absence (0 % Hct) or presence (10, 20 % Hct) of RBCs. Simulation results are insensitive to k_p . (B)&(C): The generation of HbFe^{II}O₂ and metHbFe^{III}, respectively, as a function of Hct from both the simulated results ($k_p = 16 \times 10^{-4}$ m/s) and experimental data. The range of k_p that would fit the experimental data is between $8\text{--}30 \times 10^{-4}$ m/s.

(A)

(B)

(C)

Fig. 7. Effect of k_p and k_{diss}

(A) Increasing k_p increases the generation of $\text{HbFe}^{\text{II}}\text{O}_2$. The simulation shown is for Hct fixed at 10 %. (B) Increase in k_p decreases the metHbFe^{III} formation (Hct = 10 %). (C) Increase in k_{dissoc} from 10^{-5} to 10^{-2} increases the rate of $\text{HbFe}^{\text{II}}\text{NO}$ degradation. Representative experimental data for 10 % Hct (◆) are shown.

Table 1

Simulation Parameters

Constants	Values	Unit	Ref
$k_{\text{dis,CO}}$	0.01	sec^{-1}	[48,31]
$k_{\text{assoc,CO}}$	5×10^6	$\text{sec}^{-1} \text{M}^{-1}$	[48,31]
$k_{\text{dis,O2}}$	50	sec^{-1}	[49,50]
$k_{\text{assoc,O2}}$	6×10^7	$\text{sec}^{-1} \text{M}^{-1}$	[49,50]
$k_{\text{p,CO}}$	-	m sec^{-1}	-
Hct_{CO}	0, 0.33, 0.66, 1	% vol/vol	-
$k_{\text{dis, NO}}$	1.5×10^{-4}	sec^{-1}	[30,31,32,33]
$k_{\text{assoc, NO}}$	6×10^7	$\text{sec}^{-1} \text{M}^{-1}$	[48,51,52,53]
$k_{\text{dis, O2}}$	50	sec^{-1}	[49,50]
$k_{\text{assoc, O2}}$	6×10^7	$\text{sec}^{-1} \text{M}^{-1}$	[49,50]
k_{NOD}	8.9×10^7	$\text{sec}^{-1} \text{M}^{-1}$	[6,7,5]
$k_{\text{p,NO}}$	$(8-30) \times 10^{-4}$	m sec^{-1}	-
Hct_{NO}	0, 10, 20	% vol/vol	-
Area (RBC surface area)	0.143	$\text{m}^2 (\% \text{ Hct})^{-1}$	[15]
RBC volume	95	$\mu\text{m}^3 (\text{cell})^{-1}$	[15]
RBC Surface Area	144	$\mu\text{m}^2 (\text{cell})^{-1}$	[15]
Total System Volume (Vt)	10^{-5}	m^3	-

Table 2

Initial Conditions

Reactants	Initial Conditions	Concentration (M)
HbCO _{ext}		8.9×10^{-6}
Hb _{ext}		0
CO _{ext}		3.5×10^{-5}
HbO ₂ _{ext}		0
HbCO _{in}		0
Hb _{in}		2×10^{-6}
HbO ₂ _{in}		$(0, 67, 134, 200) \times 10^{-6}$
CO _{in}		0
HbNO _{ext}		40×10^{-6}
Hb _{ext}		0
NO _{ext}		49.8×10^{-6}
HbO ₂ _{ext}		3×10^{-6}
metHb _{ext}		0
HbNO _{in}		0
Hb _{in}		0
NO _{in}		0
HbO ₂ _{in}		$(0, 2, 4) \times 10^{-3}$