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Contribution of membrane permeability and unstirred layer diffusion to nitric oxide-red blood cell interaction

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Abstract

Nitric oxide (NO) consumption by red blood cell (RBC) hemoglobin (Hb) in vasculature is critical in regulating the vascular tone. The paradox of NO production at endothelium in close proximity of an effective NO scavenger Hb in RBCs is mitigated by lower NO consumption by RBCs compared to that of free Hb due to transport resistances including membrane resistance, extra- and intra- cellular resistances for NO biotransport to the RBC. Relative contribution of each transport resistance on NO-RBC interactions is still not clear. We developed a mathematical model of NO transport to a single RBC to quantify the contributions from individual transport barriers by analyzing the effect of RBC membrane permeability (P_m), hematocrit (Hct) and NO-Hb reaction rate constants on NO-RBC interactions. Our results indicated that intracellular diffusion of NO was not a rate limiting step for NO-RBC interactions. The extracellular diffusion contributed 70–90% of total transport resistance for $P_m > 1$ cm/s whereas membrane resistance accounts for 50–75% of total transport resistance for $P_m < 0.1$ cm/s. We propose a narrow P_m range of 0.21–0.44 cm/s for 10–45% Hct, respectively, below which membrane resistance is more significant and above which extracellular diffusion is a dominating transport resistance for NO-RBC interactions.

Keywords

Nitric oxide; red blood cells; biotransport; permeability; extracellular diffusional resistance; internal diffusion

Introduction

Nitric oxide (NO) acts as a potent vasodilator in vasculature (Furchgott and Zawadzki, 1980; Ignarro et al., 1987) and is a key player in physiological and pathophysiological processes (Moncada et al., 1991). In vasculature, NO homeostasis is maintained with its production by endothelial cells and consumption by smooth muscle cells and red blood cell (RBC) hemoglobin (Hb). It is established that Hb is an effective scavenger of NO ($k_{Hb-NO} \sim 10^7$ M⁻¹ s⁻¹) (Cassoly and Gibson, 1975; Doyle and Hoekstra, 1981; Eich et al., 1996; Moncada et al., 1991). This has raised an important question about how NO is able to escape its potent scavenger Hb to maintain NO bioavailability in the vasculature. The paradox of vasoactive nitric oxide (NO) production in the close proximity of an effective NO scavenger Hb encapsulated in red blood cells has resulted in multitude of theoretical and experimental

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studies to examine NO interaction with RBCs (Deonikar and Kavdia, 2010a; Han and Liao, 2005; Huang et al., 2007; Liao et al., 1999; Vaughn et al., 2000).

The NO consumption by RBCs is much lower than that of NO consumption by equivalent amount of free Hb (Carlsen and Comroe, 1958; Deonikar and Kavdia, 2010b; Vaughn et al., 2001). A series of transport resistances are reported to slow the NO-RBC interactions. In blood vessels, a red blood cell-free layer near the vessel wall provides diffusional resistance for NO transport (Liao et al., 1999). Furthermore, an unstirred layer surrounding an RBC can provide additional diffusional resistance (Liu et al., 2002; Liu et al., 1998b). The membrane of the RBC can also act as a resistance for NO transport into the RBC. Sakai et al. (Sakai et al., 2008) recently reported that a diffusional transport resistance is also present inside the RBC. Although it is established that RBCs consume NO at a lower rate than free Hb, the mechanisms that lead to the lower consumption are still under intense investigation.

Several experimental studies have reported that RBC membrane permeability plays a critical role in the NO-RBC interactions. In the literature, RBC membrane permeability for NO varies four orders of magnitude from 0.0415–40 cm/s (Liu et al., 2007; Tsoukias and Popel, 2002; Vaughn et al., 2001). Han et al. (Han and Liao, 2005) observed an increase in NO diffusion into the RBCs when they reduced the submembrane cytoskeleton compared to that with an intact submembrane cytoskeleton. Additionally, a study by Huang et al. (Huang et al., 2001) reported that the NO-RBC interaction increased and decreased when RBC membrane was chemically modified with azide and bis(sulfosuccinimidul)-suberate, respectively. Recently, we reported that a RBC membrane permeability of 0.0415–0.4 cm/s is required to maintain sufficient NO bioactivity (Deonikar and Kavdia, 2010a). In a computational analysis of an earlier experimental study by Azarov et al. (Azarov et al., 2005), Huang et al. (Huang et al., 2007) suggested that the binding of deoxygenated hemoglobin at the band-3 protein in submembrane cytoskeleton increased the membrane permeability NO by approximately ~10 fold than that under oxygenated conditions, thereby allowing more NO under deoxygenated conditions and less NO under oxygenated conditions through the RBC membrane for NO-RBC interactions.

Experimental and theoretical analyses have proposed that the extracellular diffusional resistance plays a major role in slowing NO-RBCs interactions (Liu et al., 2002; Liu et al., 1998b). Given that the extracellular diffusional resistance lowers the oxygen uptake by RBCs (Huxley and Kutchai, 1981), it is extrapolated that the transport of similar sized gaseous molecule NO is also limited to the RBCs by the extracellular diffusional resistance (Huxley and Kutchai, 1981; Liu et al., 2002). By comparing the measured rate of NO disappearance in presence of RBCs and oxyHb, Liu et al. (Liu et al., 2002) concluded that the lower consumption of NO by RBCs is due to extracellular diffusional resistance.

The understanding of NO-RBC interactions is also critical in developing and optimizing the Hb based oxygen carriers (HBOC) because the NO bioavailability at smooth muscle cell layer for vasodilatation is dependent on NO consumption in the vascular lumen by the HBOC. Diffusional resistance and permeability are important design parameters for HBOC. Sakai et al. (Sakai et al., 2008) reported that the internal diffusional resistance and not the lipid membrane barrier is responsible for lowering the consumption of NO by the Hb vesicles designed to carry oxygen, and they extrapolated this to NO-RBC interactions. A recent study by Azarov et al. (Azarov et al., 2011) concluded that extracellular diffusion was the rate limiting step for NO-RBC and NO-Hb vesicle interactions while NO membrane permeability was the rate limiting step for NO-RBC microparticle interactions.

The understanding of NO-RBC interactions will also provide insight into the mechanisms of NO export out of the RBCs. RBCs have been reported to preserve NO bioactivity in the

form of S-nitrosohemoglobin (SNOHb) (Jia et al., 1996; Stamler et al., 1997). In addition, deoxyHb can act as a nitrite reductase to form NO under deoxygenated conditions (Crawford et al., 2006; Gladwin et al., 2006). Export of NO from either SNOHb or nitrite reductase pathway would strongly depend on the transport resistances in the RBC and vascular lumen.

Thus, the quantification of individual transport resistance can provide useful insights in NO-RBC interactions. To understand the individual contributions of transport resistances including membrane permeability, extra-, and intra- cellular resistances on NO-RBC interactions, we developed a computational model of NO transport to a single RBC. We predicted the NO concentration profile across the unstirred plasma layer, the RBC membrane and the RBC core for different values of NO concentration outside the unstirred plasma layer, Hct and RBC membrane permeability. We also estimated the NO concentrations inside and outside the RBC membrane as a function of extracellular diffusional resistance and RBC membrane permeability.

Mathematical model

Model Geometry

The NO biotransport to a single RBC at steady state was modeled in spherical geometry as shown in Fig. 1. The spherical model consisted of three concentric spheres representing the RBC core, the RBC membrane, and an unstirred plasma layer (PL). The inner sphere represents the RBC-hemoglobin and the outer sphere represents the unstirred plasma layer. The RBC membrane separates the inner sphere to the outer sphere and has a thickness of δ . NO diffuses from the unstirred plasma layer into the RBC membrane and reaches the RBC core to react rapidly with Hb. We simulated how NO-RBC interactions are affected from various hemodynamic, biophysical and biochemical parameters.

Model Equation

The steady state NO biotransport in the spherical geometry is represented as follows:

$$D_{NO}^i \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C_{NO}}{\partial r} \right) - R_i = 0 \quad (1)$$

where, C_{NO} represents the NO concentration, D_{NO}^i represents the diffusivity of NO in the respective region. R_i represents the net reaction rate to describe the NO consumption in each region. In the RBC core NO reacts with Hb. The reaction rate is given as $R_{cyt} = k_{NO-Hb} C_{NO} C_{Hb}$. k_{NO-Hb} is the reaction rate constant for NO-Hb reaction in the RBC core and C_{Hb} represents the hemoglobin concentration inside the RBC. No reaction is considered in the RBC membrane, hence $R_m = 0$. In the unstirred plasma layer surrounding the RBC, NO autooxidation reaction is considered and the reaction rate is given as $R_{pl} = k_{NO-O_2} C_{NO}^2 C_{O_2}$, where k_{NO-O_2} represents the reaction rate constant for auto-oxidation reaction of NO in the unstirred plasma layer and C_{O_2} represents the oxygen concentration in the unstirred plasma layer.

Boundary Conditions

A zero flux boundary condition was assumed at the center of the RBC to represent the symmetry of NO concentration. Thus,

At the center of the RBC ($r = 0$):

$$\frac{\partial C_{NO}}{\partial r} = 0 \quad (2a)$$

Continuous flux boundary conditions were assumed at the membrane- unstirred plasma layer and the membrane-RBC core interfaces.

At the unstirred plasma layer –membrane interface:

$$D_{NO}^{pl} \frac{\partial C_{NO}}{\partial r} \Big|_{r=r_{rbc}^+} = D_{NO}^m \frac{\partial C_{NO}}{\partial r} \Big|_{r=r_{rbc}^-} \quad (2b)$$

D_{NO}^{pl} and D_{NO}^m are the NO diffusivities in unstirred plasma layer and the membrane, respectively. r_{rbc} is the radius of the RBC.

At the membrane–RBC core interface:

$$D_{NO}^m \frac{\partial C_{NO}}{\partial r} \Big|_{r=(r_{rbc}-\delta)^+} = D_{NO}^{cyt} \frac{\partial C_{NO}}{\partial r} \Big|_{r=(r_{rbc}-\delta)^-} \quad (2c)$$

D_{NO}^{cyt} is the NO diffusivity in the RBC core, δ is the thickness of the RBC membrane. NO concentration at the outer edge of unstirred plasma layer was assumed constant.

At the outer surface of unstirred plasma layer ($r = r_{pl}$)

$$C_{NO} = \text{constant} = C_{NO}^{out} \quad (3)$$

Model Parameters

Parameter values used to solve the model equation are listed in Table 1. The radius of the RBC, r_{rbc} , is $2.8 \mu\text{m}$ and was based on a RBC volume of $\sim 90 \mu\text{m}^3$ (Fung, 1997). The thickness of the RBC membrane (δ) is $7 \times 10^{-3} \mu\text{m}$ (Malinski et al., 1993). To understand the effect of Hct and consequently the diffusional resistance provided by the unstirred plasma layer surrounding the RBC on the NO transport to RBC, we used Hct values ranging from 1 to 45%. The thickness of the unstirred plasma layer surrounding the RBCs is dependent on the local Hct. Hct is the volumetric ratio of RBC to the blood. For a single RBC, the radius of the unstirred plasma layer surrounding it is obtained by taking the ratio of RBC volume to blood volume. Since the RBC in this study is approximated to a spherical shape, the Hct can be rewritten as $\text{Hct} = r_{rbc}^3 / r_{pl}^3$ where r_{rbc} is the RBC radius and r_{pl} is the unstirred layer (outer sphere) radius. Thus,

$$r_{pl} = r_{rbc} \times \text{Hct}^{-\frac{1}{3}} \quad (4)$$

D_{NO}^{pl} is $3.3 \times 10^{-5} \text{ cm}^2/\text{s}$ (Malinski et al., 1993; Vaughn et al., 1998). D_{NO}^m was obtained from the permeability (P_m) values of NO in the RBC membrane by using following equation.

$$D_{NO}^m = P_m \times \delta \quad (5)$$

In the cytosol of RBC, D_{NO}^{cyt} was considered to be $1.6 \times 10^{-5} \text{ cm}^2/\text{s}$ (Goldstick et al., 1976; Malinski et al., 1993). To study the effect of RBC membrane permeability on steady state

NO concentrations inside and outside the RBC membrane, we used P_m values ranging from 0.0415 – 40 cm/s (Liu et al., 2007; Tsoukias and Popel, 2002; Vaughn et al., 2001).

The NO concentration outside the unstirred plasma layer was kept constant and was varied from 1, 10, 100 and 1000 nM for the simulations conducted in this study. To analyze the effect of NO-Hb reaction rate on NO-RBC interactions, simulations were performed for NO-Hb reaction rate constants of 2.5×10^7 and $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Cassoly and Gibson, 1975; Eich et al., 1996; Herold et al., 2001). In the unstirred plasma layer surrounding the RBC, the NO transport was assumed to be due to diffusion and convective NO transport was neglected.

Estimation of diffusional resistances for NO transport to RBC

As shown in Fig. 1, NO encounters three transport resistances extracellular diffusion in the plasma, RBC membrane, and intracellular diffusion as it approaches the Hb encapsulated in the RBC. We evaluated the significance of each of the resistances as follows. To estimate the significance of intracellular diffusional resistance, we evaluated the internal effectiveness factor (η) for NO-RBC diffusion/reaction inside the RBC cytoplasm. η is an indicator of relative significance of diffusion and reaction for the overall reaction rate. Internal effectiveness is defined as the ratio of actual reaction rate to the reaction rate obtained if the entire interior surface of the spherical body was exposed to the outside surface concentration. The value of η ranges from 0 to 1. If η is close to 0, it indicates that the reaction rate is diffusion limited and a value close to 1 indicates reaction rate to be limited by surface-reaction.

The RBC core was considered a homogenous medium. For a spherical particle, η is calculated as follows (Fogler, 1986):

$$\eta = \frac{3}{\phi^2} (\phi \coth \phi - 1)$$

ϕ is the Thiele modulus and is calculated as follows:

$$\phi = \sqrt{\frac{(r_{\text{rbc}} - \delta)^2 / D_{\text{NO}}^{\text{cyt}}}{1 / (k_{\text{Hb-NO}} C_{\text{NO, core-m}})}}$$

where, $(r_{\text{rbc}} - \delta)$ is the radius of the RBC core and $C_{\text{NO, core-m}}$ is the NO concentration at the RBC core – membrane interface.

In order to determine the relative contributions of transport resistance from RBC membrane and extracellular diffusion in unstirred plasma layer on NO transport to RBCs, we evaluated the mass transfer coefficients in the unstirred layer by equating the mass transfer of NO from outside the unstirred plasma layer to the RBC membrane- unstirred plasma layer interface and unstirred plasma layer-membrane interface to membrane-RBC core interface. A mass balance of NO in the unstirred plasma layer and RBC membrane is given as follows.

$$\begin{aligned}
 \text{Flux}_{\text{unstirred plasma layer}} \times \text{Area of RBC} &= \text{Flux}_{\text{membrane}} \times \text{Area of RBC core} \quad k_{ec} \left(C_{NO}^{\text{out}} - C_{NO,pl-m} \right) \\
 &\times 4\pi r_{rbc}^2 \\
 &= P_m \times \left(C_{NO,pl-m} - C_{NO,core-m} \right) \\
 &\times 4\pi (r_{rbc} - \delta)^2
 \end{aligned} \quad (6)$$

where, k_{ec} is the mass transfer coefficient in the unstirred plasma layer, C_{NO}^{out} is the NO concentration outside the unstirred layer, $C_{NO, core-pl}$ is the NO concentration at the RBC membrane–unstirred plasma layer interface, P_m is the RBC membrane permeability. k_{ec} was evaluated using Eq. 6 for all the Hct and P_m values used in this study. The total transport resistance for NO-RBC interactions was calculated by adding the extracellular diffusion, RBC membrane and internal diffusion resistances in series as follows equation.

$$\text{Total NO transport resistance} = \frac{1}{k_{ec}} + \frac{1}{P_m} + \frac{1}{k_{cyt}} \quad (7)$$

where k_{cyt} is the mass transfer coefficient in the RBC cytosol. Percent contributions from individual resistances were calculated as follows.

$$\begin{aligned}
 \% \text{Transport resistance from RBC membrane} &= \left(\frac{1}{P_m} \times 100 \right) / \text{Total NO transport resistance} \\
 \% \text{Transport resistance from extracellular diffusion} &= \left(\frac{1}{k_{ec}} \times 100 \right) / \text{Total NO transport resistance}
 \end{aligned}$$

Numerical Solution

Equation 1 was solved numerically using appropriate boundary conditions to estimate the NO concentration profiles across the model geometry using FlexPDE5 software (PDE Solutions Inc., Antioch, CA). The adaptive mesh finite element algorithm used in this software allows mesh generation in proportion to the concentration gradients in respective regions. The relative accuracy used in this study was 0.0005 for all simulations.

Results

Effect of RBC membrane permeability on NO biotransport to the RBC

The role of resistance from RBC membrane in reducing the NO consumption by RBCs is under intense investigation (Deonikar and Kavdia, 2010a; Han and Liao, 2005; Huang et al., 2007; Liu et al., 2002; Liu et al., 1998b; Tsoukias and Popel, 2002). We studied the NO-RBC interactions for RBC membrane NO permeability of 0.0415, 0.4, 1 and 40 cm/s at physiological Hct of 45%. Fig. 2 shows the steady state NO concentration profiles as a function of distance from the RBC center (center at zero) for C_{NO}^{out} values of 1, 10, 100 and 1000 nM (panel A–D, respectively).

Steady NO concentrations across the model geometry were predicted by solving Eq (1) numerically using appropriate boundary conditions given in Eq (2a, 2b, 2c) and (3). The concentration gradient arise from NO consumption by RBC encapsulated Hb or oxygen and the NO diffusion across the model geometry. Before NO reaches the RBC core and reacts with RBC bound Hb, NO has to cross the unstirred layer and the membrane. As expected, the steady state NO concentrations decreased as NO diffused towards the center of the RBC for all the C_{NO}^{out} and P_m values used in the simulations. The steady state NO concentrations across the model geometry decreased with increased in P_m . This is due to the fact that at low P_m values, less amount of NO would pass through the membrane and as the P_m increases,

more NO can pass through the membrane. Hence, the steady state NO concentrations will be higher at lower P_m and the steady state NO decrease with increase in P_m .

Three gradients of NO concentrations were observed across the model geometry. The NO concentrations gradients include between 1) the edge of the unstirred plasma layer and the outside RBC membrane; 2) the out and in- side RBC membrane; and 3) the inside RBC membrane and the RBC core. As the RBC membrane permeability increased, NO concentration gradients across the unstirred plasma layer and from inside membrane to the RBC core increased whereas NO concentration gradients across the membrane decreased (Fig. 2). For all the simulations, NO concentrations reached zero close to the membrane inside the RBC core. The RBC membrane permeability of 40 cm/s did not have significant transport resistance as demonstrated by almost linear NO profile across the whole geometry.

Relationship between RBC membrane NO concentration, RBC membrane permeability

In order to understand the effect of membrane permeability on NO availability across RBC membrane, NO concentrations as a function of RBC membrane permeability on the either side of the RBC membrane are shown in the Figure 3. $C_{NO, core-m}$ increased with permeability. The highest $C_{NO, core-m}$ were observed at P_m of 40 cm/s and the lowest $C_{NO, core-m}$ were observed at P_m of 0.0415 cm/s. A logarithmic relationship was observed between $C_{NO, core-m}$ and P_m which is given by $C_{NO, core-m} = C_{NO}^{out}(0.01033 \ln(P_m) + 0.047)$, where concentrations are in nM and P_m is in cm/s. The steady state $C_{NO, pl-m}$ (Fig. 3B) decreased with RBC membrane permeability. For a given C_{NO}^{out} , $C_{NO, pl-m}$ were highest for $P_m = 0.0415$ cm/s and lowest for $P_m = 40$ cm/s. The logarithmic relationship between $C_{NO, pl-m}$ and P_m is given by $C_{NO, pl-m} = C_{NO}^{out}(-0.117 \ln(P_m) + 0.54)$, where concentrations are in nM and P_m is in cm/s.

NO concentrations at the RBC membrane increased with Hct in a membrane permeability dependent manner

Unstirred plasma layer thickness surrounding the RBC offers diffusional resistance for NO transport to the RBC and is a function of Hct (Liu et al., 2002; Liu et al., 1998b). We performed simulations for Hct ranging from 1 to 45% to examine the contribution of extracellular diffusional resistance on NO transport to RBC. C_{NO}^{out} was kept constant at 100 nM. Steady state $C_{NO, core-m}$ and $C_{NO, pl-m}$ were estimated and plotted in Fig. 4A and B, respectively.

Increasing Hct increased both $C_{NO, core-m}$ and $C_{NO, pl-m}$ for all the RBC membrane permeability values. The increase in membrane NO concentration was linear for Hct = 10%. The linear relationships between membrane NO concentrations and Hct are provided in Table 2. For Hct = 10%, the membrane NO concentrations increased non-linearly with Hct. At higher P_m values of 1 and 40 cm/s, the steady state NO concentrations inside and outside the RBC membrane increased linearly with Hct. For a given Hct, we observed that $C_{NO, pl-m}$ decreased whereas $C_{NO, core-m}$ increased with increase in membrane permeability.

Effect of NO-Hb reaction rate constant on membrane NO concentrations

The reaction rate constant of NO-Hb reaction has been reported in the range of $2.5-8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Cassoly and Gibson, 1975; Eich et al., 1996; Herold et al., 2001; Vaughn et al., 1998). To assess the effect of NO consumption rate by Hb on NO transport to the RBCs, we estimated $C_{NO, core-m}$ and $C_{NO, pl-m}$ for NO-Hb reaction rate constants of $2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Ratios of $C_{NO, core-m}$ and $C_{NO, pl-m}$ estimated for $2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ w.r.t. $C_{NO, core-m}$ and $C_{NO, pl-m}$ estimated for $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ are plotted as a function of Hct in Fig. 5A and B, respectively. $C_{NO, core-m}$ were ~1.8 fold higher for NO-Hb reaction

rate constant of $2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ than those for $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and remained similar for all Hct and P_m . For membrane permeability of 0.0415 cm/s, $C_{\text{NO, pl-m}}$ for NO-Hb reaction rate constants 2.5 and $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ were similar to each other and did not change with Hct. However, for membrane permeability of 0.4, 1 and 40 cm/s, $C_{\text{NO, pl-m}}$ for NO-Hb reaction rate constant $2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ were ~ 1.05 , 1.1 and 1.7 -fold higher than those at NO-Hb reaction rate constant of $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively.

Relative contribution of intracellular diffusion, extracellular diffusion and RBC membrane permeability on NO transport to the RBC

To determine the relative importance of internal diffusional transport resistance on overall NO reaction rate with RBCs, we estimated the internal effectiveness factor. The internal effectiveness factor is the ratio of actual reaction rate and the rate of reaction if the entire interior of the RBC were to be exposed to the surface NO concentration, which will indicate the rate limiting step for NO reaction with RBCs. The internal effectiveness factor along with the Thiele modulus (a ratio of characteristic diffusion time to characteristic reaction time (Fogler, 1986)) was also evaluated for different NO-Hb reaction rate constants and membrane permeability values for physiological Hct of 45 and listed in Table 3. As the P_m increased from 0.0415 to 40 cm/s, the Thiele modulus changed from ~ 0.01 to 0.05 and 0.01 to 0.03 for NO-Hb reaction rate constants of 8.9 and $2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The internal effectiveness factor approached ~ 1 for all the RBC membrane permeability values for both the NO-Hb reaction rate constants at 45% Hct. Based on the internal effectiveness (~ 1) and Thiele modulus values ($\ll 1$) estimates, the NO reaction with Hb and not the internal diffusion is the rate limiting step in the RBC core (Fogler, 1986). We observed similar Thiele modulus and internal effectiveness factor values for 1, 5, 10, 15, 20, 30 and 40% Hct values (data not shown).

Individual contributions from membrane permeability and extracellular diffusion in the unstirred layer towards the overall transport resistance for NO transport to RBCs were calculated for all the Hct and membrane permeability values. The percentage of each individual transport resistance with respect to the overall NO transport resistances were calculated using Eq 8a and 8b and plotted as a function of RBC membrane permeability for all the Hct values in Fig 6. Our results showed that contributions from extracellular diffusion and RBC membrane resistance toward total transport resistance increased and decreased, respectively, with RBC membrane permeability for all Hct. At a Hct of 1%, the extracellular diffusion and RBC membrane resistance contributions were $\sim 84\%$ and $\sim 16\%$, respectively, of the total transport resistance for NO consumption by RBCs for RBC membrane permeability of 0.4 cm/s. On the other hand, for the same membrane permeability value of 0.4 cm/s at higher Hct of 45%, we observed that the transport resistance from the membrane accounted for $\sim 52\%$ whereas the extracellular diffusion resistance accounted for 47% of the total transport resistance. For all the Hct values used in this study, the contribution from membrane resistance was more for P_m values in the 0.0415–0.4 cm/s range whereas the extracellular diffusion resistance contribution was higher for the higher P_m in the 1–40 cm/s. Our results indicate that under physiological Hct, the membrane resistance is more significant if the P_m were in the range of 0.0415–0.4 cm/s whereas extracellular resistance was the major transport resistance for P_m in the range of 1–40 cm/s. Note that the most significant aspect of these results is that the extracellular diffusion contribution to overall transport resistance overtakes the membrane resistance contribution at P_m value over a narrow range from 0.21–0.44 cm/s for hematocrit range of 10–45%, respectively.

Validation of modeling approach

To validate the effects of unstirred plasma layer diffusional resistances on the modeling approach used in this study, we estimated NO concentrations for extreme values of diffusion

coefficient for NO. We used 2 orders of magnitude higher diffusion coefficient of $3.3 \times 10^{-3} \text{ cm}^2 \cdot \text{s}^{-1}$ and a membrane permeability of $40 \text{ cm} \cdot \text{s}^{-1}$ to estimate NO concentrations across the model geometry. In addition, As shown in Fig. 7, we observed that for the high diffusion coefficient of $3.3 \times 10^{-3} \text{ cm}^2 \cdot \text{s}^{-1}$, the gradient in steady state NO concentrations in the unstirred plasma layer was much lower as compared to that of low diffusion coefficient of $3.3 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ for the same RBC membrane permeability of $40 \text{ cm} \cdot \text{s}^{-1}$. Additionally, the steady state concentrations were almost identical to those obtained at the low RBC membrane permeability value of $0.0415 \text{ cm} \cdot \text{s}^{-1}$. When we increased the diffusion coefficient to $3.3 \times 10^{-2} \text{ cm}^2 \cdot \text{s}^{-1}$, we observed that the effect of unstirred plasma layer on NO transport was negligible as evidenced by a nearly flat NO concentration profile. This indicates that the unstirred plasma layer does not offer significant diffusional resistance for NO transport and is not a major limitation for NO-RBC interactions when the NO diffusivity is very high.

Discussion

It is established that NO consumption by Hb in RBCs is much lower than that of free Hb and this lower rate of consumption is attributed to transport resistances from cell free layer (Liao et al., 1999), extracellular diffusion in unstirred layer surrounding the RBCs (Liu et al., 2002), RBC membrane (Vaughn et al., 2000) and intracellular diffusion inside the RBCs (Sakai et al., 2008). However, the relative contribution of these transport resistances is still not clear. The analysis of the relative contribution from individual transport resistance has proven more difficult due to lack of direct physical measurements of parameters like RBC membrane permeability. Computational modeling of NO biotransport can provide useful insights into quantifying the relative contributions of the individual transport resistances. Towards this end, we present a computation analysis of NO biotransport to a single RBC to analyze the role of intracellular diffusion resistance in the RBC core, RBC membrane permeability, extracellular diffusion resistance from unstirred layer surrounding the RBC and NO consumption rates by Hb in lowering the NO-RBC interactions.

Role of intracellular diffusional resistance in NO-RBC interactions

A number of recent studies have analyzed the contribution of intracellular diffusional resistance in the RBC cytosol. A recent study involving NO interactions with polyethylene glycol conjugated with liposome encapsulated Hb (PEG-LEH) by Rameez et al. (Rameez and Palmer, 2011) attributed the lower NO consumption by PEG-LEH to the intracellular diffusional resistance. In an experimental analysis of NO transport to Hb vesicles (HbV), Sakai et al. (Sakai et al., 2008) reported that as the concentration of Hb inside the HbV and the size of the HbV increased, the NO binding to Hb was reduced. They concluded that the NO binding to Hb in the HbV is dependent on the Hb concentration indicating a role for intracellular diffusion in reducing the NO binding to Hb. They further postulated that the intracellular diffusion may also play a role reducing the NO consumption by Hb in the RBC cytosol.

To understand the significance intracellular diffusional resistance, we estimated the Thiele modulus and internal effectiveness factor, indicators of rate limiting mechanisms (Fogler, 1986) in a spherical particle (RBC). Based on our results of Thiele modulus and internal effectiveness factor, the rate limiting step for NO transport to the RBCs is the reaction of NO with the Hb in RBCs. In other words, the NO consumption rate by RBC is determined by how fast the RBC encapsulated Hb consumes NO rather than how fast NO can reach Hb. It is worth noting that the internal effectiveness factor remained ~ 1 and the Thiele modulus was $\ll 1$ for all the RBC membrane permeability and NO-Hb reaction rate constant values used in this study. These results suggest that internal diffusional resistance in the RBC cytosol may not play a significant role in the NO consumption by RBCs.

Role of membrane resistance in NO-RBC interactions

Resistance from the RBC membrane for the transport of small gaseous molecules such as O_2 was shown to be much lesser than that from the unstirred layer surrounding the RBCs (Coin and Olson, 1979; Huxley and Kutchai, 1981). Since NO is similar in size to O_2 , RBC membrane was shown to provide insignificant resistance for NO transport into the RBCs (Liu et al., 2002; Liu et al., 1998b). However, some studies have estimated a low P_m value for NO in the RBC membrane indicating a significant role for RBC membrane in NO-RBC interactions (Han and Liao, 2005; Vaughn et al., 2000). Additionally, oxygen tension in the RBCs was also shown to affect the P_m , and hence the NO-RBC interactions (Huang et al., 2007).

The value of P_m is a critical factor in determining the impact of membrane resistance in NO-RBC interactions. Lack of direct experimental measurement for P_m value adds to the complexity of determining the role of RBC membrane resistance in NO-RBC interactions. In this model, we estimated steady state NO concentrations at the inside and outside membrane using P_m range of 0.0415–40 cm/s (Liu et al., 2002; Tsoukias and Popel, 2002; Vaughn et al., 2000). For physiological Hct of 45% (Fig.2), as the P_m increased, the steady state NO concentrations inside the membrane increased. This indicates that the transport resistance for NO from the RBC membrane decreased thereby allowing more NO inside the RBC. The decrease in steady state NO concentrations outside the membrane with an increase in P_m further corroborates that the RBC membrane transport resistance decreased as the P_m values increased. An increase in NO auto-oxidation rate was reported by Liu et al. in the biological membranes (Liu et al., 1998a). To understand the effect of increased NO auto-oxidation reaction rate, we incorporated the NO auto-oxidation reaction in the RBC membrane. We observed that the estimated NO concentrations across the model geometry remained the same as those without NO auto-oxidation reaction in the membrane (data not shown).

For lower P_m of 0.0415 cm/s, the membrane NO concentrations for Hct >10% remained similar indicating that NO consumption by RBCs remained similar despite an increase in the Hct. In an experimental and theoretical analysis of NO-RBC interactions using a novel flow system, we also previously observed that the NO consumption by RBCs remained similar for Hct values >10% (Deonikar and Kavdia, 2010b) which indicates a significant role for transport resistance from the RBC membrane at higher Hct.

Role of extracellular diffusion in unstirred layer on NO-RBC interactions

The unstirred layer surrounding the RBC has been reported to be a major barrier for NO transport to the RBC (Liu et al., 2002; Liu et al., 1998b; Tsoukias and Popel, 2002). The unstirred layer surrounding the RBCs is reported to exist even in rapid mixing devices (Coin and Olson, 1979; Rice, 1980). The thickness of the unstirred layer around the RBC indicates the diffusional distance NO has to overcome before it reaches RBCs. The thickness of the unstirred layer is dependent on the Hct (Deonikar and Kavdia, 2010a; Liu et al., 2002; Tsoukias and Popel, 2002) as shown in Eq. (4). To determine the influence of the thickness of unstirred layer, we estimated the steady state NO concentrations at the inside and outside RBC membrane for Hct values ranging from 1–45%. As shown in the Fig. 3, the inside and outside steady state NO concentrations increased with the Hct in a P_m dependent manner. For Hct>10%, we observed a linear increase in membrane concentrations w.r.t. Hct (Table 2). For a given P_m , the diffusional distances for NO should decrease with an increase in Hct, thereby increasing NO availability at the inside and outside RBC membrane at steady state. It is worth noting that the inside membrane concentrations at steady state were higher for high P_m and vice versa, as NO receives little membrane resistance at high P_m values. On the

other hand, the outside membrane NO concentrations were higher for lower P_m and vice versa.

Decrease in NO consumption by changing the NO-Hb reaction rate constant from 8.9 to $2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ increased the inside as well as outside membrane steady NO concentrations. However, the fold increase in the membrane NO concentrations remained unaffected by Hct as well as membrane permeability. These results indicate that the steady state NO concentrations are less sensitive to the change in the NO consumption inside the RBC.

Relative contributions of individual transport resistances on NO-RBC interactions

We estimated the contributions from membrane transport resistance and the resistance from unstirred layer by equating the fluxes in the unstirred layer and the RBC membrane. The transport resistance from the internal diffusion inside the RBC was deemed negligible based on the internal effectiveness factor and Thiele modulus. As shown in Fig. 6, the transport resistance from the extracellular resistance increased while the transport resistance from membrane decreased with membrane permeability for all the Hct.

Our results indicate that the resistance from the RBC membrane plays a dominant role if the P_m was very low. The threshold value of P_m below which RBC membrane resistance is dominant over extracellular resistance is dependent on Hct. For a low Hct of 1%, the fraction of transport resistance was ~16% for RBC membrane and 84% from extracellular resistance at a low membrane permeability value was 0.4 cm/s. Liu et al. (Liu et al., 2002) also found that at lower Hct, the extracellular resistance was a significant resistance for NO transport to RBCs. We recently reported that the P_m should be in the range of 0.0415–0.4 cm/s so that sufficient NO bioactivity is maintained at the smooth muscle cell layer for vasodilation (Deonikar and Kavdia, 2010a). In that case, at physiological Hct of 45%, the RBC membrane resistance would account for ~50–90% of the total transport resistance for NO-RBC interactions. If the P_m was in the range of 1–40 cm/s, the contribution of membrane resistance towards the overall transport resistance would be in the range of ~1–30%.

NO transport models proposed by Liu et al. (Liu et al., 2002; Liu et al., 1998b) and Tsoukias et al. (Tsoukias and Popel, 2002) reported that extracellular diffusion resistance was a major transport resistance for NO-RBC reaction indicating no resistance from the RBC membrane and a high P_m value. On the other hand, modeling and experimental studies from Liao group reporting a significant role for membrane resistance have indicated very low values of P_m (0.0415–0.4 cm/s) (Han and Liao, 2005; Vaughn et al., 2000; Vaughn et al., 2001). One of the key findings of our study is the narrow range of RBC membrane permeability values of 0.21–0.44 cm/s for Hct range of 10–45% which provides a conclusive criteria in determining the significance of extracellular and membrane transport resistances in NO-RBC interaction. We predict that RBC membrane is significant transport resistance if the P_m value is lower than P_m range of 0.21–0.44 cm/s range, consistent with findings of Liao group (Han and Liao, 2005; Vaughn et al., 2000; Vaughn et al., 2001). The extracellular diffusion and membrane resistance both play significant role if the P_m is ~ 0.21–0.44 cm/s. Extracellular diffusion is the dominating transport resistance if the P_m is higher than the 0.21–0.44 cm/s range consistent with findings of Liu et al. (Liu et al., 2002; Liu et al., 1998b) and Tsoukias et al. (Tsoukias and Popel, 2002).

Although not analyzed in this study, along with extracellular diffusion from unstirred layer RBC membrane, cell free layer near the vessel wall also provides a significant source of resistance for NO diffusion in the vascular lumen and can lower the NO-RBC interactions (Deonikar and Kavdia, 2012; Kavdia and Popel, 2003; Kavdia et al., 2002; Kim et al., 2007). RBCs under flow tend to migrate towards the center of the vessel leaving an almost

RBC free layer near the vessel wall consisting mainly of plasma. The width of cell free layer plays a significant role in modulating the effective blood viscosity thereby modulating the wall shear stress dependent NO production from the endothelium, in small arterioles (Ong et al., 2011).

Physiological Significance

The computational model developed in this study provides a unique tool for quantification of individual transport resistances involved in lowering NO-RBC interactions. We report a logarithmic relationship between steady state membrane NO concentrations and P_m , which is dependent on the NO concentration outside the unstirred layer. This relationship, in conjunction with experimental data, can be utilized to determine NO scavenging rates for HBOCs and help optimize the design parameters like the size, Hb concentration inside HBOC, thickness of HBOC membrane for developing HBOCs. RBCs have been reported to preserve NO bioactivity in the form of either S-nitrosohemoglobin (SNOHb) (Jia et al., 1996; Stamler et al., 1997). Nitrite reductase activity of deoxyHb in the RBCs is also reported to release NO and the NO bioactivity can be transported out of RBCs to cause vasodilation (Crawford et al., 2006; Gladwin et al., 2006). The relationship between P_m and NO concentrations at the membrane obtained in this study can be used to quantify the amount of NO exported out of RBCs from SNOHb/nitrite pathways.

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Highlight

- We developed a model of nitric oxide (NO) transport to a red blood cell (RBC)
- Effect of diffusion transport resistances on NO-RBC interaction was analysed
- Intracellular diffusion of NO does not affect NO-RBC interaction
- Membrane transport resistance is dominant if membrane permeability (P_m) < 0.21 cm/s
- Extracellular diffusion transport resistance is dominant if P_m > 0.44 cm/s

Figure 1. Model Geometry

NO biotransport to a single RBC is modeled in spherical geometry. The model consists of three concentric spheres. The inner sphere represents the RBC enclosed by an outer sphere representing unstirred plasma layer surrounding the RBC. NO concentration outside the unstirred plasma layer is considered constant.

Figure 2. NO concentration profiles

NO concentrations across the model geometry are plotted as a function of distance from the center of the RBC at different P_m values. Panels A, B, C and D represent the NO concentration profiles for unstirred plasma layer NO concentrations (C_{NO}^{out}) values of 1, 10, 100 and 1000 nM, respectively. The Hct used for these simulations was 45% and NO-Hb reaction rate constant was $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 3. NO concentrations at the RBC membrane

$C_{NO,core-m}$, NO concentration at the RBC core-RBC membrane interface, (panel A) and $C_{NO,pl-m}$, NO concentrations at the RBC membrane-plasma interface (panel B), are plotted as a function of P_m for C_{NO}^{out} values of 1, 10, 100 and 1000 nM. The Hct used for these simulations was 45% and NO-Hb reaction rate constant was $8.9 \times 10^7 M^{-1}s^{-1}$.

Figure 4. NO concentrations at the membrane as a function of Hct

$C_{NO,core-m}$, NO concentration at the RBC core-RBC membrane interface (panel A), and $C_{NO,pl-m}$, NO concentrations at the RBC membrane-plasma interface (panel B), are plotted as a function of Hct for $C_{NO}^{out} = 100$ nM. NO-Hb reaction rate constant used was 8.9×10^7 $M^{-1}s^{-1}$, Hct ranged from 1–45%.

Figure 5. Effect of NO-Hb reaction rate constants on NO membrane concentrations

$C_{NO,core-m}$ and $C_{NO,pl-m}$ were estimated for NO-RBC reaction rate constants of $2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. Ratios of $C_{NO,core-m}$ at $2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ to $C_{NO,core-m}$ at $8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ are plotted as a function of Hct in panel A and ratios of $C_{NO,pl-m}$ at $2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ to $C_{NO,pl-m}$ at $8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ are plotted in panel B as a function of Hct.

Figure 6. Relative contributions of transport resistances

Contribution of membrane permeability and extracellular resistance in NO-RBC interaction was plotted for Hct ranging from 1–45% and P_m values ranging from 0.0415–40 cm/s.

Figure 7. Validation of model

NO concentration profiles are plotted as a function of distance from the RBC center. NO concentrations were estimated for diffusion coefficients of 3.3×10^{-5} , 3.3×10^{-3} and $3.3 \times 10^{-2} \text{ cm}^2 \cdot \text{s}^{-1}$ at high P_m of $40 \text{ cm} \cdot \text{s}^{-1}$, and compared with NO profiles at diffusion coefficient of $3.3 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ at low P_m of $0.0415 \text{ cm} \cdot \text{s}^{-1}$.

Table 1**Model Parameters**

Parameter	Description	Value/Expression
P_m	NO permeability in RBC membrane	0.0415, 0.4, 1 and 40 cm/s (Liu et al., 2007; Tsoukias and Popel, 2002; Vaughn et al., 2001)
δ	Thickness of RBC membrane	$7 \times 10^{-3} \mu\text{m}$ (Malinski et al., 1993)
$D_{\text{NO}}^{\text{pl}}$	NO diffusivity in plasma	$3.3 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Malinski et al., 1993; Vaughn et al., 1998)
D_{NO}^{m}	NO diffusivity in RBC membrane	$P_m \times \delta$
$D_{\text{NO}}^{\text{cyt}}$	NO diffusivity in RBC core	$1.6 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Goldstick et al., 1976; Malinski et al., 1993)
$k_{\text{Hb-NO}}$	NO-Hb reaction rate constant	2.5×10^7 and $8.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Cassoly and Gibson, 1975; Eich et al., 1996; Herold et al., 2001)
$k_{\text{NO-O}_2}$	NO autooxidation rate constant	$9.6 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$
r_{rbc}	Radius of RBC	$2.8 \mu\text{m}$ (Fung, 1997)
C_{Hb}	Hemoglobin concentration in RBC	20.3 mM (Altman and Katz, 1971)
C_{O_2}	Oxygen concentration	27 μM (Kavdia and Popel, 2003)
$C_{\text{NO}}^{\text{out}}$	NO concentration outside unstirred plasma layer	1, 10, 100 and 1000 nM

Table 2

Membrane NO concentration and Hct relationship for Hct>10%

P_m , cm/s	RBC core-membrane NO conc., nM	R^2	Plasma-membrane NO conc., nM	R^2
0.0415	$C_{NO, core-m} = 0.36 \text{ Hct} + 75.75$	0.98	$C_{NO, pl-m} = 0.0028 \text{ Hct} + 0.58$	0.98
0.4	$C_{NO, core-m} = 0.71 \text{ Hct} + 22.62$	0.99	$C_{NO, pl-m} = 0.049 \text{ Hct} + 1.56$	0.99
1	$C_{NO, core-m} = 0.54 \text{ Hct} + 9.84$	0.99	$C_{NO, pl-m} = 0.085 \text{ Hct} + 1.57$	0.99
40	$C_{NO, core-m} = 0.15 \text{ Hct} + 1.46$	0.99	$C_{NO, pl-m} = 0.14 \text{ Hct} + 1.29$	0.99

$C_{NO, core-m}$ is the NO concentration at the RBC core-RBC membrane interface; $C_{NO, pl-m}$ is the NO concentration at the RBC membrane-unstirred plasma layer interface; Hct units are in %

Table 3

Internal Effectiveness factors and Thiele modulus for 45% Hct

P_m , cm/s	Thiele Modulus		Internal Effectiveness	
	$k_{Hb-NO}=8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$	$k_{Hb-NO}=2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$	$k_{Hb-NO}=8.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$	$k_{Hb-NO}=2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$
0.0415	0.0177	0.0129	0.999979	0.999989
0.4	0.0410	0.0295	0.999888	0.999942
1	0.0488	0.0352	0.999841	0.999917
40	0.0581	0.0308	0.999775	0.999937

P_m is the NO permeability of the RBC membrane; k_{Hb-NO} is the reaction rate constant for NO-Hb reaction.