Published in final edited form as:

Microvasc Res. 2009 June ; 78(1): 107–118. doi:10.1016/j.mvr.2009.02.009.

Hemorrhagic shock and nitric oxide release from erythrocytic nitric oxide synthase: A quantitative analysis

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Abstract

A large loss of blood during hemorrhage can result in profound shock, a state of hypotension associated with hemodynamic abnormalities. One of the hypotheses to account for this collapse of homeostasis is that the production of nitric oxide (NO), a gas molecule that dilates blood vessels, is significantly impaired during hemorrhage, resulting in a mismatch between O₂ delivery and the metabolic activity in the tissues. NO can be released from multiple sources in the vasculature. Recent studies have shown that erythrocytes express functional endothelial nitric oxide synthase (NOS3), which potentially serves as an intraluminal NO source. NO delivery from this source is complex: Erythrocytes are not only NO producers but also act as potent sinks because of the high affinity of NO for hemoglobin. To test our hypothesis that the loss of erythrocytic NOS3 during hemorrhage contributes to NO deficiency-related shock, we have constructed a multicellular computational model that simulates NO production and transport to allow us to quantify the loss of NO under different hemorrhagic conditions. Our model shows that: (1) during mild hemorrhage and subsequent hemodilution (hematocrit >30%), NO from this intraluminal source is only slightly decreased in the vascular smooth muscle, but the NO level is significantly reduced under severe hemorrhagic conditions (hematocrit <30%); (2) whether a significant amount of NO from this source can be delivered to vascular smooth muscle is strongly dependent on the existence of a protective mechanism for NO delivery; (3) if the expression level of NOS3 on erythrocytes is similar to that on endothelial cells, we estimate ~13 pM NO at the vascular smooth muscle from this source when such a protective mechanism is involved. This study provides a basis for detailed studies to characterize the impairment of NO release pathways during hemorrhage and yield important insights for the development of resuscitation methods.

Keywords

computational model; nitric oxide; hemorrhage; endothelial nitric oxide synthase; erythrocyte

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INTRODUCTION

Loss of a large intravascular volume of blood results in hemorrhagic shock, a state of hypotension associated with hemodynamic abnormalities that lead to the collapse of homeostasis (Gutierrez *et al.*, 2004). In the microcirculation, the functional capillary density decreases significantly during hemorrhage, reducing the oxygen supply to the surrounding tissues (Salazar Vazquez *et al.*, 2008; Torres *et al.*, 2008). In the absence of timely and proper treatment, severe hemorrhagic shock is associated with high morbidity and mortality.

The primary goal in treating hemorrhagic shock is to stop the bleeding and restore the volume of the circulating blood. Blood transfusion is widely considered the best treatment for patients who have lost a large volume of blood, although some studies have suggested that blood transfusion itself may sometimes be problematic (Sugerman *et al.*, 1970; Rao *et al.*, 2004). The whole blood transfusion is often impractical, particularly in the case of accidents or on the battlefield, when the injured are at high risk of losing blood but have no immediate access to red blood cells.

Current fluid resuscitation methods include the administration of colloid or crystalloid solutions (Falk *et al.*, 1992), followed by the transfusion of oxygen-carrying blood. These resuscitation methods are aimed at restoring circulating blood volume, even at the cost of a lower-than-normal hematocrit or hemoglobin concentration (Figure 1). Alternative resuscitation methods have been developed, including the use of blood substitutes, which are usually hemoglobin-based oxygen carriers. Unlike whole red blood cell suspensions, hemoglobin-based blood substitutes can be carried in emergency trauma bags and injected into any recipient without the need for the time-consuming process of blood type matching. However, significant side effects have been reported for the blood substitutes currently under development (Alayash, 2004; Buehler & Alayash, 2008; Natanson *et al.*, 2008). Commonly used resuscitation fluids, such as crystalloids or colloids, are designed to restore intravascular volume. Administration of a resuscitation fluid restores the circulation and maintenance of systemic blood pressure and metabolic balance, but the circulating blood now has a lower hematocrit after treatment. This hemodilution can cause problems, one of which is vasoconstriction, which further decreases functional capillary density.

One of the hypotheses to explain the hemorrhage-induced collapse of homeostasis is that the production of nitric oxide (NO) is significantly impaired because of the reduced blood viscosity and flow (Salazar Vazquez *et al.*, 2008). NO is a gas molecule that dilates blood vessels and is necessary for matching O₂ delivery to the metabolic activity of the local tissue. Once formed, NO can diffuse into the vascular smooth muscle, where it reacts with soluble guanylate cyclase (sGC), which catalyzes the formation of 3',5'-cyclic guanosine monophosphate (cGMP) to induce vasodilation.

NO synthesized by endothelial NOS (eNOS or NOS3) in endothelial cells is widely considered to be the major source of this signaling molecule in the vasculature (Lancaster, 1994; Buerk, 2007; Tsoukias, 2008). However, experimental evidence and theoretical analyses have suggested that NO from non-endothelial cells, including those from intraluminal sources, can play a significant role in a variety of physiological functions in the vasculature (reviewed in (Chen *et al.*, 2008b)). In addition to its presence in endothelial cells, NOS3 has been shown to be expressed in erythrocytes as a potential NO source. Chen and Mehta (Chen & Mehta, 1998) have reported the presence of NO-producing enzymes in erythrocytes, but later studies have suggested that the identified NOS enzymes were inactive proteins (Kang *et al.*, 2000). Recently, Kleinbongard *et al.* (Kleinbongard *et al.*, 2006), using multiple experimental approaches, found that erythrocytes express a functional NOS3 that potentially can serve as an intraluminal NO source. This NOS3 is located both on the plasma membrane and in the

cytoplasm of the erythrocytes. However, although some subsequent studies have confirmed the presence or function of NOS3 in erythrocytes (Webb *et al.*, 2007; Ulker *et al.*, 2008), others have questioned its existence (Hilarius *et al.*, 2007). NO from this source has been thought to regulate the function of erythrocytes and platelets (Gladwin, 2006; Kleinbongard *et al.*, 2006; Yalcin *et al.*, 2008). Thus, it is still unclear whether a significant amount of NO from this intraluminal source can reach the vascular wall and cause blood vessel relaxation.

Certain intraluminal sources of NO (e.g. *S*-nitrosohemoglobin, intraluminal nitrite) have been proposed to exert a hypoxic vasodilation effect (Singel & Stamler, 2005; Kim-Shapiro *et al.*, 2006; Chen *et al.*, 2008b). If the NO synthesized by intraerythrocytic NOS3 can escape scavenging by hemoglobin as other blood-born NO, it could be another important source regulating vascular tone. During hemorrhagic shock, the loss of blood must result in a concomitant loss of erythrocytic NOS3. If this source is indeed important for NO-mediated vasodilation, hemorrhage would not only result in the loss of erythrocytes that carry oxygen, but also of the vasodilating NO that they “carry”.

We have therefore hypothesized that the loss of erythrocyte-based NOS3 during hemorrhage contributes to NO deficiency observed in shock. Because of the technical difficulties involved in attempting to obtain quantitative measurements of intraluminal NO, there are no published data concerning how much NO from this source is available to smooth muscle. In the present study, we have constructed a multicellular computational model that simulates NO production from NOS3 in erythrocytes and its transport through an arteriole. Our simulations indicate that whether a significant amount of NO from this source can be delivered to vascular smooth muscle is strongly dependent on the existence of a protected mechanism for NO delivery.

MODEL FORMULATION

Model Geometry and Assumptions

We have previously formulated a computational model that simulates NO release and transport in and around microvessels, as a means of predicting the NO concentration in vascular smooth muscle when NO is liberated from intraluminal *S*-nitrosohemoglobin or through nitrite reduction (Chen *et al.*, 2007a; Chen *et al.*, 2008a). Here, we have modified the model to predict the smooth muscle NO exposure and approximate NO distribution in the vessel when NOS3 in the erythrocytes is the sole source of NO; the other previously considered sources have additive effects within a linear range of NO dependency. We considered (1) a two-dimensional cross-section of an arteriole with its surrounding tissue and (2) a discrete distribution of erythrocytes in the lumen. The model consists of five layers (Figure 1): Layer 1 is the intraluminal layer containing plasma and discrete erythrocytes; erythrocytes are modeled as circles. The erythrocyte membrane has been reported to have a low permeability (high resistance) to NO diffusion (El-Farra *et al.*, 2003). Thus, the cell is divided into two sub-regions: a thin membrane and the homogeneous intraerythrocytic hemoglobin. In Layer 1, NO can be formed as a result of NOS3 synthesis, which is distributed on the cell membrane and in the cytoplasm (Kleinbongard *et al.*, 2006). Layer 2 consists of the endothelium and associated interstitial space; Layer 3 is composed of smooth muscle cells containing sGC, which catalyzes the formation of cGMP; Layer 4 consists of non-perfused tissue containing nerve fibers and stromal and parenchymal cells; and Layer 5 consists of tissue perfused by capillaries.

We have used different hematocrit values to mimic the conditions of hemorrhage and resuscitation, from 8% to 45%. A hematocrit of 45% is close to the physiological value (Sharan & Popel, 2001; Chen *et al.*, 2007b), while lower values correspond to conditions in which blood cells are lost, but the total volume is replenished by the transfusion of a resuscitation fluid. We represented the hematocrit as the area occupied by the erythrocytes in the lumen divided by the total luminal cross-section; this value was the so-called vessel hematocrit. We

also considered a physiological erythrocyte-free layer near the vessel wall: No erythrocytes were placed in this erythrocyte-free region, which was 2 μm in size and was just adjacent to the vascular wall.

Governing Equations and Chemical Reactions

The governing equation that describes the diffusion and reactions of NO in the vasculature is:

$$\frac{\partial C_{NO}}{\partial t} = D_{NO} \nabla^2 C_{NO} + R_{NO} \quad (1)$$

where C_{NO} is the NO concentration, D_{NO} is the diffusivity of NO, and R_{NO} is the sum of total production and consumption rates of NO. In our model, we ignored the effect of convective transport because of the large Damkohler number (~ 173 , (Chen *et al.*, 2007a)), which represents the ratio of reaction to convection. In addition, because of the short half-life of NO in the bloodstream (Butler *et al.*, 1998; Thomas *et al.*, 2001; Jeffers *et al.*, 2005), a steady state of NO profiles in the blood vessel can be achieved within milliseconds (Tsoukias *et al.*,

2004) and we considered a steady state ($\frac{\partial C_{NO}}{\partial t} = 0$) in our model. The governing equation was applied to all the layers described above.

In the vasculature, NO can be consumed through reactions with a variety of chemical species. In the intraluminal layer (Layer 1), oxygen acts as a sink through its reactions with NO. Inside erythrocytes, NO is synthesized as the result of NOS3 activity, and a portion of the generated NO is scavenged by the intraerythrocytic hemoglobin. In the endothelium and the interstitial space (Layer 2), NO is consumed through reactions with oxygen; the endothelial production of NO catalyzed by NOS3 was ignored here as well, since we excluded all other sources in order to isolate the effect by NOS3 expressed on the erythrocytes. In Layer 3, NO reacts with the sGC located in the smooth muscle cells, resulting in vasodilation. In the non-perfused tissue layer (Layer 4), there is no NO production, since the NOS activity in this region was necessarily ignored when we focused on the NO availability from our specific intraluminal source. Also, NO is consumed in this layer through its reactions with oxygen. Because of the relative hypoxic conditions associated with hemorrhage, we also ignored the NO consumption by parenchymal tissue that occurs through an oxygen-dependent mechanism (Thomas *et al.*, 2001). In the perfused tissue layer (Layer 5), NO is consumed by oxygen and hemoglobin contained in erythrocytes flowing through the capillaries.

The means by which the synthesized NO is transported out of erythrocytes is as yet unclear, but it determines the eventual NO availability from this source. In the case of NO derived from intraerythrocytic sources, there are three likely delivery mechanisms (Kim-Shapiro *et al.*, 2006; Chen *et al.*, 2008a): (1) NO freely diffuses out of the erythrocyte; (2) erythrocyte membrane-bound proteins provide a mechanism that facilitates NO transport without its being rapidly scavenged by intracellular hemoglobin; (3) another unknown species, other than NO itself, is the immediate product in the reaction chain. This unknown molecule is not reactive with hemoglobin and is converted to NO in the extracellular region. Case 3 seems unlikely in scenario of erythrocytic NOS3, since studies have shown that the final product leaving NOS3 catalytic heme is NO itself, rather than another intermediate species (Xia & Zweier, 1997; Alderton *et al.*, 2001). Thus, we have only modeled the effects of Cases 1 and 2: Case 1 was modeled as a free diffusion process, during which NO also rapidly reacts with hemoglobin; Case 2 was modeled as a surface release determined by the NOS3 activity, ignoring the intermediate processes. We assumed that all of the NO molecules that were formed were able to leave the erythrocyte. This release was unidirectional: The NO molecules that were released encountered transport resistance when they attempted to diffuse back into the erythrocytes.

This resistance can be specified by a high diffusivity in the thin membrane sublayer that is related to the membrane permeability (El-Farra *et al.*, 2003; Chen *et al.*, 2007a; Chen *et al.*, 2008a). When NO diffuses back into the erythrocytes, it rapidly reacts with the intraerythrocytic hemoglobin.

As previously mentioned, Layer 1 contains two sub-regions: the plasma and the erythrocytes. In each erythrocyte, NO is released through NOS3 catalytic activity and, meanwhile, is consumed through hemoglobin scavenging:

$$R_{NO} = Q_{NO} - k_{Hb} C_{Hb} C_{NO} \quad (2)$$

where k_{Hb} is the kinetic reaction rate between hemoglobin and NO, C_{Hb} is the hemoglobin concentration, C_{NO} is the NO concentration, and Q_{NO} is the NO release rate inside the cell when NO transport outside the cell occurs through free diffusion. A thin sub-layer along an erythrocyte was created to represent the erythrocyte membrane, which possesses an intrinsic resistance to NO diffusion. When NO transport occurs via free diffusion, the NO production is modeled as a uniform reaction inside erythrocytes; when the formed NO is exported through facilitated mechanisms, the release rate of this process (Q_{NO}) is modeled as a surface reaction and expressed as a boundary flux, as discussed below. In the plasma sub-region, NO can freely diffuse in any direction. Also, the chemical reaction between NO and oxygen is expressed as:

$$R_{NO} = -k_{O_2} C_{O_2} C_{NO}^2 \quad (3)$$

where k_{O_2} is the kinetic reaction rate between oxygen and NO and C_{O_2} is the oxygen concentration.

In Layer 2, the NO production catalyzed by NOS3 in the endothelium is not considered, in accordance with our goal of assessing the NO production from the erythrocytic NOS3 as the sole source of NO; NO is consumed through reaction with oxygen:

$$R_{NO} = -k_{O_2} C_{O_2} C_{NO}^2 \quad (4)$$

In Layer 3, NO is consumed through reactions with sGC:

$$R_{NO} = -k_{sGC} C_{NO}^2 \quad (5)$$

where k_{sGC} is the kinetic reaction rate between sGC and NO.

In Layer 4, we also ignore the possible NO production by NOS1 located in nerve fibers and mast cells; NO is consumed through the reaction with oxygen:

$$R_{NO} = -k_{O_2} C_{O_2} C_{NO}^2 \quad (6)$$

In Layer 5, NO is consumed through reactions with oxygen and intracellular hemoglobin flowing through the capillaries in the capillary-perfused region. Thus, the net reaction in this layer is:

$$R_{NO} = -k_{O_2} C_{O_2} C_{NO}^2 - k_{cap} C_{NO} \quad (7)$$

where k_{cap} is the effective reaction rate between NO and cellular hemoglobin in the capillaries.

Parameter values

In our calculations, the geometric information was similar to that in our previous studies of S-nitrosohemoglobin and nitrite reduction modeling (Chen *et al.*, 2007a; Chen *et al.*, 2008a): We chose 4 μm as the effective radius of an erythrocyte (r_1), which was modeled as a circle. The erythrocyte membrane is resistant to NO transport, and its thickness (r_{mem}) was chosen to be 0.0078 μm (Vaughn *et al.*, 2001). The radius of the pre-capillary arteriolar lumen (r_3) was chosen as 17 μm . We also simulated the effect of the erythrocyte-free zone adjacent to the vascular wall with a thickness of 2 μm , resulting in a region with a radius of 15 μm (r_2) that contained erythrocytes. We assumed that the thickness of the endothelium and that of the interstitial space was 0.5 μm . Thus, the total thickness of the endothelium and interstitial space layer was 1 μm , and the radius (r_4) chosen for this layer was 18 μm . The radius (r_5) of the outer edge of the smooth muscle layer was 24 μm , with the assumption that the thickness of the smooth muscle was 6 μm (Haas & Duling, 1997). The radius (r_6) of the non-perfused tissue layer was 30 μm , and we chose 6 μm as the thickness of the non-perfused tissue adjacent to the blood vessel. Finally, we chose 20 μm as the thickness of the perfused tissue, making the radius (r_7) of the entire region of our model 50 μm .

NO has a diffusivity of 3300 $\mu\text{m}^2/\text{s}$ in tissues (Vaughn *et al.*, 2001; El-Farra *et al.*, 2003). Inside erythrocytes, it diffuses less rapidly, with a diffusivity of 880 $\mu\text{m}^2/\text{s}$ (El-Farra *et al.*, 2003). The intracellular concentration of hemoglobin heme (C_{Hb}) was 20 mM. Here, we ignored the effect of cell-free hemoglobin because, under normal conditions, the cell-free hemoglobin concentration is very low (Chen *et al.*, 2008a). NO can react with hemoglobin to form relatively stable metabolites, and the reaction rate (k_{Hb}) between hemoglobin and NO has been reported in the range of 12 to 90 $\mu\text{M}^{-1}\cdot\text{s}^{-1}$ (Gibson & Roughton, 1957; Morris & Gibson, 1980; Herold *et al.*, 2001; Chen *et al.*, 2007b). Here we chose 18 $\mu\text{M}^{-1}\cdot\text{s}^{-1}$ as the kinetic rate (Chen *et al.*, 2007b). The concentration of sGC (C_{sGC}) used was 0.1 μM , and the reaction rate between sGC and NO (k_{sGC}) was 0.05 $\mu\text{M}^{-1}\cdot\text{s}^{-1}$ (Vaughn *et al.*, 2001). The effective reaction rate between NO and the capillary hemoglobin (k_{cap}) was 12.4 s^{-1} , as justified in (Kavdia & Popel, 2003). The reaction rate between oxygen and NO was $9.6 \times 10^{-6} \mu\text{M}^{-2}\cdot\text{s}^{-1}$ (Kavdia & Popel, 2003). The oxygen concentration was 52 μM , corresponding to ~ 30 mm Hg, as a constant. All the parameter values, including the size (radius) information for each layer that is based on experimental data for arterioles, are listed in Table 1.

The NO release rate depends on the expression level of NOS3 in the erythrocyte and the concentrations of the substrates and cofactors that are required for NO synthesis (Stuehr, 2004). We have previously constructed a computational model based on biochemical pathway analysis to allow us to predict the NO release rate from the endothelial cells (Chen & Popel, 2006, 2007). We could have used that model to estimate the NO release rate from the NOS3 on erythrocytes; however, the NOS3 concentration, which is a key parameter in this model, has not been reported in the literature. Since our primary goal in this study was to investigate the relative loss of NO from this specific source during hemorrhage, we selected a value for the NO release rate on the basis of the expression of NOS3 in the endothelial cells; since the resulting NO bioavailability was approximately proportional to the NOS3 expression, estimates for other values of NOS3 could be readily obtained. As discussed in our previous studies, the NO molecules that are formed could be transported either through free diffusion or transferred to membrane-associated proteins first, then further exported out of the cell. In

the former case, we used a fixed value as the NO release rate, assuming that NO production is a uniform process in the cell. In the latter case, the NO group can avoid being scavenged through rapid rebinding to the hemoglobin iron. In our model, we represented this series of biochemical reactions using an apparent surface release reaction. For comparison purposes, we used the same surface release rate for all the calculations.

Boundary conditions

In our model, the NO concentration is continuous at all the boundaries between the regions, except for the interface between the erythrocyte membrane and the plasma, where a finite transport resistance was introduced. Here we assumed that the solubility of NO in each region in the model was the same. At the outer interface of the whole region, we assumed a no-flux condition. Thus, the boundary conditions were:

1. At the outer boundary of Layer 5:

$$\frac{\partial C_{NO}}{\partial r} = 0 \quad (8)$$

2. At the interface between the plasma and the erythrocyte, the NO release from the erythrocyte was incorporated as the surface boundary condition:

$$S_{NO} = D_{NO,mem} \frac{\partial C_{NO,mem}}{\partial r} - D_{NO,plasma} \frac{\partial C_{NO,plasma}}{\partial r} \quad (9)$$

where S_{NO} is the NO release from the erythrocyte surface through the membrane protein-associated mechanism, $D_{NO,mem}$ is the diffusivity of NO in the erythrocyte membrane, $D_{NO,plasma}$ is the diffusivity of NO in the plasma, $C_{NO,mem}$ is the NO concentration in the thin erythrocyte membrane layer, and $C_{NO,plasma}$ is the NO concentration in plasma. The membrane permeability (P_{mem}) is determined by the diffusivity of NO in the erythrocyte membrane ($D_{NO,mem}$) and the membrane thickness (r_{mem}) (El-Farra *et al.*, 2003):

$$P_{mem} = \frac{D_{NO,mem}}{r_{mem}} \quad (10)$$

The membrane permeability has been reported in a large range (Vaughn *et al.*, 2001; El-Farra *et al.*, 2003; Jeffers *et al.*, 2005; Liu *et al.*, 2007) and here we chose the value of 450 $\mu\text{m/s}$ for this parameter (Vaughn *et al.*, 2001; El-Farra *et al.*, 2003). The effect of P_{mem} will be discussed below. The membrane thickness (r_{mem}) was 0.0078 μm (El-Farra *et al.*, 2003). Thus, the apparent diffusivity of NO inside the erythrocyte membrane was 3.51 $\mu\text{m}^2/\text{s}$. In our numerical calculations, we used a larger r_{mem} (0.078 μm , a value still much less than the erythrocyte size) and therefore a higher $D_{NO,mem}$ (35.1 $\mu\text{m}^2/\text{s}$), in order to make our numerical solution more efficient; the value of P_{mem} remained fixed. This numerical procedure has been justified in our previous study (Chen *et al.*, 2007a; Chen *et al.*, 2008a).

Numerical Method

The governing equation (Eq. 1), coupled with the appropriate boundary conditions, was solved numerically using the FlexPDE software package (PDESolutions, Antioch, CA). A 3-GHz processor with 2-GB SDRAM was used to implement the code.

RESULTS

Loss of NO delivery from the erythrocytic source during hemorrhage when NO transport out of the cell occurs through free diffusion

During hemorrhagic shock, a large quantity of erythrocytes as well as plasma is lost. Fluid resuscitation can restore the circulating blood volume, with a lower hematocrit. Here we calculated the percentage of erythrocytic NO that was lost with various hemodilution levels, as compared to physiological conditions.

As discussed in *Model Formulation*, two mechanisms of NO delivery from the erythrocytes are likely: free diffusion and a facilitated mechanism across the erythrocyte membrane. We calculated the NO distribution around an arteriole and compared the relative change in NO delivery for various hematocrit values in the case of free diffusion (Figure 2). Figure 2A and B show the NO distribution when the hematocrit was 45% and NO diffused freely out of the cells. Note that a value corresponding to the NOS3 expression in endothelial cells was chosen as the NO release rate from each erythrocyte for all our calculations and that our focus was on comparing the relative change in NO availability with the lower hematocrit conditions. Thus, we show the NO concentration in arbitrary units (A. U.). In this particular calculation (and all following calculations unless otherwise indicated), we used $2.53 \times 10^{-2} \mu\text{M/s}$ as the NO synthesis rate value.

Figure 2C and D show how the NO distribution changed as the hematocrit was reduced to 30%, equivalent to 33% hemodilution. The NO delivered to the smooth muscle dropped slightly, by 4%, when compared to the physiological hematocrit. The drop was more significant, by 27%, when there was a larger blood loss (and subsequent fluid resuscitation), corresponding to a hematocrit of 15% (Figure 2E, F). Note that under physiological conditions, hematocrit in the normal or non-hemorrhage state covers a range from approximately 40 – 45% (~45% for men and ~42% for women) (Purves *et al.*, 2004). Here we consider the hemorrhage situation of up to 1/3 blood volume “moderate” and hemorrhage with 50 – 60% reduction in blood volume “severe” (Martini *et al.*, 2008). Thus, moderate hemorrhage corresponds to a 30 – 40% hematocrit range and severe hemorrhage is in the hematocrit range of 15 – 30%, or even lower, after restoration of the initial blood volume with a resuscitation fluid not containing erythrocytes.

Figure 2G shows the NO concentration, normalized by the value for a hematocrit of 45%, at the vascular wall as a function of hematocrit when NOS3 in the erythrocytes was the sole source of NO. It is interesting that the NO presence in smooth muscle began to decrease as the hematocrit was reduced from 45%; this decrease was accelerated as hemorrhage and hemodilution became more significant. With an extremely low hematocrit of 7.5%, the NO from this erythrocytic source was reduced by 58%.

Thus far, our calculations have been based on a specific distribution of erythrocytes in the lumen (Figure 2). As shown in our previous studies on NO release and transport from other intraerythrocytic sources (Chen *et al.*, 2007a; Chen *et al.*, 2008a), other types of distribution of the cells had no significant effect on the results for NO delivery to the vascular wall, although the distribution inside the lumen was not the same.

Loss of NO delivery from the erythrocytic source during hemorrhage when NO is transported out of the cell via a facilitated mechanism

As previously mentioned, NO formed inside erythrocytes could potentially be shuttled out of the cells through the mediation of membrane-bound proteins, thereby avoiding being rapidly scavenged by the intracellular hemoglobin (Singel & Stamler, 2005; Kim-Shapiro *et al.*, 2006). Figure 3 shows how NO delivery, if indeed protected by this mechanism, would be

affected by the loss of blood cells during hemorrhage and resuscitation. As we observed for the free diffusion scenario, a hemodilution of 33% after hemorrhage resulted in a moderate decrease in NO concentration in the vascular wall (Figure 3C, D) by approximately 5%, when compared to physiological conditions (Figure 3A, B). A more aggressive hemodilution that resulted in an even smaller hematocrit (15%) significantly reduced the NO delivery by approximately 38% (Figure 3E, F). If the erythrocyte-derived NO were indeed an important vasodilator, such a striking reduction in NO would severely limit the vasodilatory capacity of this source during severe hemorrhage (i.e., a large amount of blood was lost and/or an aggressive fluid resuscitation protocol was applied). Whether this erythrocyte-derived NO is indeed of importance for vasodilation depends on how much NO can be delivered under physiological conditions: if too little, how much it decreased with hemorrhage would not matter in any case; if the amount delivered from this source is significant under physiological conditions, then a sharp drop in the level of this signaling molecule as a result of blood loss could cause vasoconstriction and a lower functional capillary density downstream. This question will be further discussed below.

The NO synthesis rate used to determine the surface flux rate for each erythrocyte in this calculation was the same as that used in Figure 2 for our modeling of the free diffusion mechanism. Note that the level of NO delivered to the smooth muscle through this mechanism was far greater than that achieved through free diffusion, a finding that is consistent with our previous studies of NO delivery from other intraluminal sources (Chen *et al.*, 2008a).

Amount of NO delivery from erythrocytes

How much NO from this erythrocytic source is present in the vascular wall? This question is of great importance because the answer would allow us to determine whether NO from this source can have a significant vasoregulatory effect, when compared to the EC₅₀ value for the effect of NO on sGC activity (i.e., the amount of NO required to stimulate half sGC maximal activity). However, as we discussed earlier, values for the NO production rate from erythrocytes or the expression level of NOS3 in erythrocytes are not available in the literature, presumably because of methodological constraints. Thus, we cannot use our model to directly predict the absolute value of NO present in the vasculature from this source. Instead, we have used an alternative method to estimate the NO production rate by assuming an erythrocyte-NOS3 expression level and fitting this parameter to a biochemical pathway model that we previously developed (Chen & Popel, 2006, 2007). In the previous studies, we constructed a kinetic model that describes the biochemical reactions involved in NO synthesis under different conditions and predicted an NO production rate of 17 nM/s. Moreover, Kleinbongard *et al.* determined that the ratio of NOS3 activity levels in erythrocytes and endothelial cells was 3:7 in their cell culture system (although not necessarily *in vivo*, as they pointed out) (Kleinbongard *et al.*, 2006). If we assume that the NOS3 concentration is the same as that in vascular endothelial cells, we can estimate that the NO synthetic rate is 7.3 nM/s (3/7 of the rate for an endothelial cell) from this erythrocytic source. The total amount of released NO is the product of the erythrocyte volume (V) and the NO production rate (Q_{NO} , 7.3 nM/s). Thus, we calculated the surface release rate of NO from the erythrocyte membrane per unit time per unit area to be:

$S_{NO} = \frac{Q_{NO} \cdot V}{A}$, where A is the surface area of the erythrocyte. Both V and A are determined by the radius of the erythrocyte. Given the values of Q_{NO} and the radius of the erythrocyte from Table 1, S_{NO} was $1.0 \times 10^{-17} \mu\text{mol} \cdot \mu\text{m}^{-2} \cdot \text{s}^{-1}$. It should be noted that erythrocytes actually have a biconcave shape, rather than the circle as approximated in our model. Thus, our approximation may have had a slight effect on the value of S_{NO} , but it is expected to produce a negligible difference in the NO concentration around an arteriole (Jeffers *et al.*, 2005). The production of NO by the erythrocyte was incorporated as surface NO release in the boundary

conditions at the interface between the erythrocyte membrane and the plasma (see *Boundary Conditions*).

With that estimated parameter in our model, we calculated how much NO could be delivered to the vascular wall, as well as to other regions. Figure 4A and B show ~0.01 pM NO present at the vascular wall if free diffusion is the transport mechanism. This value increased to about 13 pM if the facilitated transport mechanism indeed exists (Figure 4C and D). In the plasma within the lumen, the NO level was ~0.02 pM or ~22 pM, respectively, for the diffusion and facilitated transport mechanisms. These values would be expected to decrease if hemorrhagic shock and the subsequent fluid resuscitation resulted in a lower hematocrit. According to Figure 3G, for a hematocrit of 30%, NO delivery to the vascular smooth muscle would be reduced to 12 pM if the formed NO were protected and then released from the cell through the membrane-associated protein transport; for a hematocrit of 15%, the value would be 8 pM.

Again, it is important to note that the NO production rate from this erythrocytic source in vivo is not yet available, and the value that we used was based on an assumption that the expression level of NOS3 on erythrocytes, as well as other information about other NOS cofactors, was the same as that for vascular endothelial cells. Thus, the predicted levels given above should be regarded as estimates only until the detailed characteristics of the NOS3 activity in erythrocytes have been experimentally determined. However, our predictions provide a reasonable starting point for quantifying the NO contribution to the vasculature from this erythrocytic source, since NO bioavailability at the vascular wall is a function of NOS3 expression, which has been addressed in our model.

DISCUSSION

Hemorrhagic shock is a state of hypotension that is characterized by diminished blood flow and decreased functional capillary density in the microcirculation as well as impaired oxygen supply to the tissues. The loss of nitric oxide has been hypothesized as one of the mechanisms responsible for this impaired oxygen supply during hemorrhage (Salazar Vazquez *et al.*, 2008). NOS3 has been experimentally identified in erythrocytes and may play a role in maintaining homeostasis. We have constructed a computational model to analyze the change in NO from this erythrocytic source, which is lost along with the erythrocytes during hemorrhage and fluid resuscitation. We have also predicted the presence of NO in the vascular wall and in the lumen in a simulated situation in which NOS3 in erythrocytes was the sole source of NO. Our model also considered the effect of the alternate mechanisms by which NO may be transported out of the cell.

Model validation and comparison of model predictions with previous studies

NO is a highly reactive species and has a sub-second half-life time in the bloodstream (Butler *et al.*, 1998). As a result, direct measurement of the NO concentration distribution in vivo is very difficult to obtain. Moreover, any change in NO that occurs as a result of the loss of erythrocytic NOS3 during hemorrhage cannot be easily discriminated from NO that may come from other sources. Thus, to our knowledge, there have been no experimental values reported in the literature concerning either the amount of delivered NO or the relative changes that occur during hemorrhage in the NO derived from this erythrocytic source. Our theoretical modeling in this study has provided a “best-case scenario” in which all other sources of NO were excluded, and only NOS3 in erythrocytes was considered. Previous theoretical models, including ours, have pointed to a low value (pM level) of NO concentration for NO from intraluminal sources (*S*-nitrosohemoglobin, nitrite reduction, iron-nitrosyl-hemoglobin) (Jeffers *et al.*, 2005; Chen *et al.*, 2007a; Liu *et al.*, 2007; Chen *et al.*, 2008a). Our calculations in the present analysis were close to these predicted values (Figure 4), although all these models

will need to be validated experimentally as more sophisticated experimental approaches are developed.

Hemorrhage: Is there a loss of bioactive NO as well as blood?

During hemorrhage, erythrocytes are lost; moreover, one of the treatments, administration of volume replacement fluids, has not worked as well as anticipated. As a result of erythrocytes loss, it is certain that the oxygen-carrying hemoglobin is lower than under physiological conditions. The previous finding (Kleinbongard *et al.*, 2006) that the erythrocyte expresses functional NOS3 in the cytoplasm and cell membrane raises the question of whether this source of the potent vasodilator NO is reduced in the bloodstream. Its loss would certainly exacerbate the disruption of homeostasis associated with hemorrhagic shock. Unlike the loss of oxygen, the loss of NO produced by erythrocytic NOS3 is more complex: Erythrocytes are not only NO producers (because of their functional NOS3) but also an NO sink, since NO binds to hemoglobin with a very high affinity (Buerk, 2007; Chen *et al.*, 2008b). Thus, the delivery of NO from intraluminal sources represents a balance between production and scavenging.

Our simulations indicate that NO delivery from this erythrocytic source to the vascular wall as well as the bloodstream is decreased with lower hematocrit (Figures 2 and 3). This conclusion applies to both of the hypothesized NO transport mechanisms (Kim-Shapiro *et al.*, 2006), free diffusion and a protected transport mechanism, although with the facilitated mechanism (which is more likely to convey physiologically significant NO, Figure 4), the NO delivery to smooth muscle showed a slightly lesser dependence on the hematocrit when hemodilution was considered (Figures 2G and 3G). According to our simulations, mild hemorrhage (hematocrit of >30% after the addition of an equal volume of plasma expander) only slightly reduced the NO availability at the vascular wall; under severe conditions of hemorrhage (hematocrit of <30%), NO from this source was significantly reduced (Figure 3G). Thus, if erythrocytic NOS3 is indeed a significant source of circulating vasodilator, the loss of a small fraction of the total erythrocyte population would have only a minimal effect on the capacity for vessel regulation, presumably thanks to NO production and the scavenging effects of erythrocytes. However, the loss of a large amount of blood causes not only a loss of oxygen-carrying erythrocytes but also a significant fraction of the available erythrocyte-derived NO. A treatment that increases NO bioactivity, rather than volume replacement fluid alone, may be necessary to compensate adequately for the loss of NO produced by erythrocytic NOS3.

Is the amount of NO delivered by erythrocytic NOS3 sufficient for vasodilation?

As discussed above, a large portion of the NO derived from erythrocytic NOS3 is lost during severe hemorrhage (hematocrit of <30%), and whether this loss significantly affects vasoactivity is highly dependent on how much NO from this source is delivered to the smooth muscle under physiological conditions. We have pointed out that if only a little NO from this source, far lower than the amount required to induce vessel relaxation, eventually gets to the vascular wall when the hematocrit is normal, any further decrease in this NO delivery as the hemorrhage continues would not matter. In such a case, the enzymatic function may represent a “vestigial” activity that yields nonfunctional molecules or those that are exclusively responsible for other physiological functions. It has been hypothesized that erythrocytic NO may serve to regulate the functional characteristics of the erythrocyte deformability and/or platelet aggregation (Kleinbongard *et al.*, 2006; Yalcin *et al.*, 2008), depending on the availability of NO in the lumen. We have estimated the amount of the NO that is delivered to the vascular wall. Our simulations, based on the assumption that the erythrocytic NOS3 is expressed at the same level as the enzyme in vascular endothelial cells, show that NO is present at 0.01 pM in the smooth muscle when only free diffusion is involved (Figure 4A, B). This value, when compared to all the reported values for EC₅₀ (likely at the nanomolar level, reviewed in (Chen *et al.*, 2008b)), seems too low to be physiologically relevant or functional.

Thus, assuming the value for NOS3 expression that we have used in this study, NO from erythrocytes is likely to have no influence under either physiological or hemorrhagic conditions if the synthesized NO is transported merely by free diffusion.

Our calculations indicate that ≈ 13 pM NO can reach the vascular smooth muscle if the NO bioactivity is protected and is transported via a facilitated mechanism across the cell membrane (Figure 4C and D). Although this 13 pM level of NO is below the currently reported values for the EC₅₀ (Chen *et al.*, 2008b), it is close to the levels contributed by other endocrine NO sources, such as S-nitrosohemoglobin and nitrite reduction in the bloodstream (Jeffers *et al.*, 2005; Chen *et al.*, 2007a; Chen *et al.*, 2008a), that have been shown to play an important role in vasodilation during hypoxia (Singel & Stamler, 2005; Kim-Shapiro *et al.*, 2006). Our calculations suggest that if a facilitated transport mechanism is responsible for NO delivery from this erythrocytic source, a sharp decline in this NO source during hemorrhagic shock could reduce the intrinsic vasodilation capacity of erythrocytes as a large number of them are lost.

Another question concerning the amount of NO available from this source is related to a discrepancy that we have previously encountered: The amount of NO synthesized from endothelial cells does not account for the experimentally measured NO concentration obtained by using microelectrodes in the perivascular region; this discrepancy is on the order of 100 nM (Chen *et al.*, 2008b). We have hypothesized that NO derived from other sources combined with endothelium-derived NO is responsible for the microelectrode measurements (Zani & Bohlen, 2005; Lowe *et al.*, 2008) and have analyzed a number of biochemical pathways that lead to NO release within the vasculature (Chen *et al.*, 2008b). We now conclude that NO derived from erythrocytic NOS3 does not account for the discrepancy described above, unless the erythrocytic NOS3 expression is much higher than that assumed in the present simulations.

Membrane permeability that affects the delivery of NO generated by erythrocytic NOS3

The relative NO distribution within the vascular wall and the lumen may affect certain vascular activities. The NO distribution is also strongly dependent on the resistance that is encountered when extracellular NO molecules diffuse into erythrocytes. In our previous studies, we have demonstrated that the membrane permeability is important for NO signaling from intraluminal sources such as S-nitrosohemoglobin and nitrite. The membrane of erythrocytes was initially thought to have little resistance to NO diffusion, but subsequent studies have found that this lipid bilayer may possess an intrinsic barrier to NO transport (Vaughn *et al.*, 2001; El-Farra *et al.*, 2003). In our calculations, we first used a low value of 450 $\mu\text{m/s}$ as the membrane permeability (Table 1). However, we reasoned that it would also be useful to determine how a higher membrane permeability would affect the amount of NO that finally reaches the vascular wall, since a number of studies have shown that this membrane resistance to NO diffusion may not be that high (Tsoukias & Popel, 2002; Azarov *et al.*, 2005; Liu *et al.*, 2007). Therefore, we also calculated the NO concentration in smooth muscle under the same conditions as in Figure 4C and D, except that we used a high value of P_{mem} . Liu *et al.* (Liu *et al.*, 2007) reported 45000 $\mu\text{m/s}$ as the membrane permeability in their experiments and simulations. Using this value in our calculations, we found that the NO concentration in smooth muscle was approximately 0.57 pM, about 23-fold lower than the 13 pM obtained with the low value for P_{mem} . The nature of the membrane resistance to the uncharged NO requires further investigation if we are to gain a more complete understanding of NO signaling from this erythrocytic source.

Limitations of the model

In our model, we considered the steady-state of NO profiles to address the dynamic mass transport of NO during hemorrhage. This approximation approach has been previously justified

(Tsoukias *et al.*, 2004) based on the short half-life of NO in the blood stream (Butler *et al.*, 1998; Thomas *et al.*, 2001; Jeffers *et al.*, 2005) and the large Damkohler number (Chen *et al.*, 2007a). In addition, hemorrhage and the associated resuscitation are dynamic and complex processes and the final outcome depends on a variety of factors, e.g. species, magnitude of blood loss, bleeding rate, type of resuscitation fluids, rate of resuscitation, start and end points of resuscitation. In addition to the magnitude of hemorrhage, the activity and expression of NOS3 may depend on the bleeding rate as well as the resuscitation approaches. In the model formulation, we assumed that the expression and activity of erythrocytic NOS3 do not change. This assumption may not be accurate. It has been found that the enzymatic activity of NOS3 in vascular endothelial cells is controlled by a variety of factors, e.g. intracellular Ca^{2+} /calmodulin and shear stress (Alderton *et al.*, 2001). During hemorrhage and subsequent resuscitation, the viscosity of the blood is lower than under physiological conditions, resulting in lower shear stress imposed on the erythrocytes where NOS3 is expressed (Salazar Vazquez *et al.*, 2008). Thus, it is likely that the enzymatic activity of NOS3 decreases with bleeding. Moreover, experimental evidence has shown that higher shear stress can increase the expression level of NOS in endothelial cells (Milkiewicz *et al.*, 2005). Thus, the decrease of the NO bioavailability because of hemorrhage could be higher than the predictions in our model. In this study, we were focused on the relationship between NO availability from erythrocyte-NOS3 and the magnitude of hemorrhage, which directly determines the availability of erythrocytic NOS3. We hypothesized that the loss of erythrocyte-based NOS3 during hemorrhage contributes to NO deficiency observed in shock and our model provides an approximate prediction of NO availability around an arteriole during normal conditions and then in moderate and severe hemorrhage. Future studies are needed to address the dynamic relationship between NO bioavailability and other factors, such as bleeding rate, blood viscosity and Ca^{2+} concentration in blood, during hemorrhage.

Conclusions

One of the hypotheses to account for the hemorrhage-induced collapse of vascular homeostasis is that the production of NO is significantly impaired. We hypothesized that the loss of NOS3 in erythrocytes during hemorrhage contributes to NO deficiency-related shock, and we have constructed a multicellular computational model that simulates the complex NO delivery from this source under different hemorrhagic conditions: Erythrocytes are not only NO producers but also act as potent sinks because of the high affinity of NO for hemoglobin. Our model shows that: (1) during mild hemorrhage and subsequent hemodilution (hematocrit > 30%), the amount of NO from this intraluminal source that is present at the vascular smooth muscle falls only slightly (by approximately 5%); whereas the NO presence is significantly (by approximately 30% or more) reduced under severe hemorrhagic conditions (hematocrit of <30%); (2) whether a significant amount of NO from this source can be delivered to vascular smooth muscle is strongly dependent on the existence of a protected mechanism for NO delivery; and (3) if the expression level of NOS3 in erythrocytes is similar to that in endothelial cells, we estimate a level of ~13 pM NO in the vascular smooth muscle from this source with such a protected NO transport mechanism. This study provides a basis for detailed studies designed to expand our understanding of the impaired NO release pathway during hemorrhage, and thereby provide important information for the development of more effective resuscitation methods after severe blood loss.

Acknowledgments

This research was supported by NIH grants R01 HL079087 and R01 HL018292. We thank Dr. Nikolaos Tsoukias for the contribution to the design of Figure 1.

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LIST OF ABBREVIATIONS

NO	nitric oxide
NOS	nitric oxide synthase
NOS3	endothelial NOS
sGC	soluble guanylate cyclase
cGMP	3',5'-cyclic guanosine monophosphate

Figure 1.

Blood flow and release of NO released from NOS3 in erythrocytes. The region shown can be divided into five layers: intraluminal region, endothelium and interstitial space, smooth muscle cells, non-perfused tissue, and tissue perfused by capillaries. (A) normal physiological conditions; (B) conditions in which resuscitation fluid has been applied after a certain volume of blood is lost, resulting in a lower hematocrit.

(G)

Figure 2.

NO distribution around an arteriole for different values of hematocrit when NOS3 in erythrocyte is the sole source of NO and free diffusion is the transport mechanism. (A), (B) physiological conditions (hematocrit of 45%); (C), (D) hematocrit reduced to 30%; (E), (F): hematocrit reduced to 15%. Note that we used an arbitrary value of $2.53 \times 10^{-2} \mu\text{M/s}$ as the NO release rate from each erythrocyte for all calculations and, thus, the NO concentration is in arbitrary units (A. U.), but the relative change in NO delivery can be compared under different hematocrit conditions. Panels A, C, and E represent the cross-sections of an arteriole and its surrounding tissues. Panels B, D, and F are plots of the NO concentration from the lumen center to the perivascular region along a path whose starting and ending points are represented as 1 and 2, respectively, in the inset. RBC: red blood cell; Hct: hematocrit.

Figure 3.

The NO distribution under the same simulation conditions as in Figure 2, but considering the case in which NO leaves the erythrocytes via a facilitated transport mechanism, instead of through free diffusion. Note that the NO synthesis rate from each erythrocyte ($2.53 \times 10^{-2} \mu\text{M/s}$) in this calculation is also an arbitrary value, allowing the relative levels of NO distribution with different hematocrit to be compared, as in Figure 2.

Figure 4.

Estimate of the amount of NO delivered to the smooth muscle as a result of the synthesis by NOS3 on erythrocytes. The intraerythrocytic NOS3 concentration was assumed to be the same as that in the endothelial cells. No other NO production sources were considered in the simulation. (A), (B) NO transport out of the cells was assumed to occur through free diffusion; (C), (D) NO transport was assumed to occur via the facilitated mechanism (as discussed in *Model Formulation*). Panels B and D are plots of the NO concentration from the lumen center to the perivascular region along a path, whose starting and ending points are represented as 1 and 2, respectively, in the inset.

Table 1

Model Parameters. The radius of each layer, with the exception of r_1 , represents the distance from the center of the lumen to the outer boundary of the layer.

Parameters	Value	Unit	Reference
Radius			
Erythrocyte (r_1)	4	μm	(Vaughn <i>et al.</i> , 2001; El-Farra <i>et al.</i> , 2003)
Erythrocyte-rich layer (r_2)	15	μm	text
Erythrocyte-free layer (r_3)	17	μm	text
Endothelium and interstitial space (r_4)	18	μm	text
Smooth muscle layer (r_5)	24	μm	text
Non-perfused tissue (r_6)	30	μm	text
Perfused tissue (r_7)	50	μm	text
Physiological hematocrit	45%	N/A	(Sharan & Popel, 2001; Chen <i>et al.</i> , 2007b)
Erythrocyte membrane thickness (r_{mem})	0.0078	μm	(Vaughn <i>et al.</i> , 2001; El-Farra <i>et al.</i> , 2003)
Erythrocyte membrane permeability (P_{mem})	450	$\mu\text{m}^2/\text{s}$	(Vaughn <i>et al.</i> , 2001; El-Farra <i>et al.</i> , 2003)
NO diffusion coefficient in membr. (D_{mem})	3.51	$\mu\text{m}^2/\text{s}$	text
Intracellular NO diffusion coefficient (D_{int})	880	$\mu\text{m}^2/\text{s}$	(Vaughn <i>et al.</i> , 2001)
Extracellular NO diffusion coefficient (D_{ext})	3300	$\mu\text{m}^2/\text{s}$	(El-Farra <i>et al.</i> , 2003)
Hemoglobin-NO reaction rate (k_{Hb})	18	$\mu\text{M}^{-1}\cdot\text{s}^{-1}$	(Chen <i>et al.</i> , 2007b)
Intracellular hemoglobin concentration	20	mM	text
NO consumption rate by sGC (k_{sGC})	0.05	$\mu\text{M}^{-1}\cdot\text{s}^{-1}$	(Vaughn <i>et al.</i> , 2001)
Oxygen concentration (C_{O_2})	52	μM	text
Reaction rate with O_2 (k_{O_2})	9.6×10^{-6}	$\mu\text{M}^{-2}\cdot\text{s}^{-1}$	(Kavdia & Popel, 2003)
NO consumption rate by perfused tissue (K_{cap})	12.4	s^{-1}	(Kavdia & Popel, 2003); text