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Forum Review

The Red Blood Cell and Vascular Function in Health and Disease

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ABSTRACT

Nitric oxide (NO) is widely accepted as a central regulator of vascular tone and a vast array of other cardiovascular signaling mechanisms. An emerging player in these mechanisms is hemoglobin (Hb), an erythrocytic protein that serves as the archetypical model for an allosteric protein. Specifically, red blood cells (RBC) are suggested to be integral in matching blood flow to tissue oxygen demands. The mechanisms proposed involve the ability of Hb to sense changes in oxygen concentrations and coupling this process to modulating vascular NO levels. The molecular basis of these mechanisms remains under investigation, but is clearly diverse and discussed in this article from the basis of the blood flow responses to hypoxia. Another emerging theme in RBC biology is the role of these cells during inflammatory disease in which disease processes promote the interaction of vascular NO and the RBC. This is exemplified in hemolytic diseases, in which released Hb has drastic affects on vascular homeostasis mechanisms. Additionally, it is becoming evident that RBC express numerous molecules that mediate interactions with the extracellular matrix and cellular mediators of inflammation. The functional implications for such interactions remain unclear but highlight potential roles of the RBC in modulating inflammatory disease. *Antioxid. Redox Signal.* 6, 992–999.

INTRODUCTION

Recent insights have led to the appreciation that the red blood cell (RBC) plays an important role in the physiological mechanisms through which circulatory hemodynamics are controlled and, furthermore, that dysfunction in these mechanisms may underlie the development of vascular disease (4, 23, 45, 48, 58, 62). The primary function for the RBC is to reversibly transport oxygen and carbon dioxide, thereby sustaining aerobic respiration. This function is carried out by hemoglobin (Hb), and it is not surprising then that this protein comprises >90% of the dry weight of the total RBC protein. However, the RBC contains many other proteins, whose functions are now being appreciated in the context of both modulating oxygen transport functions mediated by Hb and, more recently, affecting inflammation and vascular cell signaling (2, 18, 57, 76). The central theme of this overview is to discuss the current concepts surrounding the question "How does the RBC control vascular function?" The most obvious answer to this question as alluded to above is delivering oxygen. This is regulated by local oxygen concentration gradients to which Hb responds by either binding to (becoming oxygenated) or releasing (becoming deoxygenated) oxygen. The fundamental principles behind these effects are discussed in most biochemistry textbooks, and it is assumed in writing this article that the reader is knowledgeable in the basic principles of oxygen transport by Hb. Specifically, in this review, we will focus on the models and mechanisms intrinsic to the red cell that impact on vascular blood flow and inflammation.

Blood flow is a physiologic parameter that determines oxygen delivery by modulating the flux of RBC through a tissue and also is an important conduit for the inflammatory re-

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sponse. Vascular inflammation is an important component of host defense, but is now also appreciated, together with endothelial dysfunction, as a primary cause of morbidity and/or mortality of a number of acute and chronic diseases, including atherosclerosis, sepsis, and sickle cell disease (21, 26, 46, 57). In the following sections, mechanisms through which the RBC may be involved in controlling blood flow will be discussed.

RBC AND VASCULAR BLOOD FLOW

The proposed mechanisms by which the RBC regulates blood flow involves modulation of factors that promote either dilation or constriction of the vessel wall. This can occur through interactions with factors produced in the vessel and released into the circulation [*e.g.*, nitric oxide (NO) or endothelin] or by direct release [*e.g.*, adenosine triphosphate (ATP) and NO] from the RBC itself.

RBC and NO metabolism

NO is an integral component of vascular homeostasis mechanisms that include regulation of blood flow, platelet and leukocyte function, and antioxidant effects (35, 53, 55, 61). It is been recognized for many years that NO reacts with deoxygenated or oxygenated ferrous Hb (10, 14, 15, 71). The rapid nature of this reaction has directed thinking toward a role of the RBC in limiting/inhibiting NO-dependent activity in the vascular compartment. In fact, consideration of the concentrations of Hb, and the kinetics of the reaction with NO, led to the conclusion that NO should not be able to exert significant control of the array of vascular functions mentioned above (44). Clearly, however, this is not the case, and several models have been proposed to explain how NO can control vascular biology despite the presence of a seemingly limitless sink, *i.e.*, the RBC (11, 22, 28, 34, 40, 49–52, 63). These models have taken essentially two forms: (a) active exclusion of NO from interacting with Hb inside the RBC or (b) active participation of the RBC in regulating NO function by generating NO in a controlled manner (Fig. 1). The following sections will address these theories in the context of the regulation of blood flow, as well as the consequences of disrupting the NO regulatory functions of the RBC during pathology.

Exclusion of NO from the RBC

This model is based on the fact that in order for NO to react and be scavenged by Hb, it must enter the RBC first. A series of elegant studies have led to the concept that, under physiological conditions, entrance of NO into the RBC is significantly slowed due to a variety of biochemical and biophysical factors that serve to create diffusion barriers limiting NO reactions with RBC-Hb. These barriers include (a) a RBC-free zone formed by laminar flow of plasma along the endothelium within the lumen (9, 49), and (b) the RBC membrane (Fig. 1). The precise mechanisms through which the RBC membrane modulates NO entrance are still under investigation, but include increased rate of reaction of NO with oxygen within the hydrophobic interior of the membrane, formation of unstirred

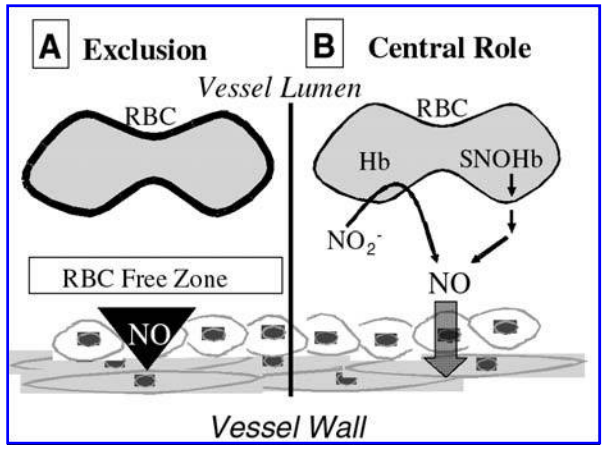

FIG. 1. Proposed models through which the RBC regulates NO flux in the vasculature. (A) Architecture of the vessel wall and the RBC result in diffusional barriers that prevent/limit NO entering the RBC. **(B)** The RBC activates NO-storage pools (*e.g.*, nitrite) to generate NO.

layers (a consequence of the friction between the bulk aqueous solution outside the red cell and the red cell membrane), and formation of barriers comprised of RBC membrane and associated protein matrix (31, 34, 50, 51).

The critical role of compartmentalizing Hb within the RBC is illustrated by the effects of cell-free Hb or pathologies associated with hemolysis (*e.g.*, sickle cell disease) on NO-dependent regulation of blood flow. In this situation, neither blood flow nor RBC membrane-dependent diffusional barriers are present and rapid scavenging and inhibition of NO function are observed (64, 65). In fact, hypertensive effects of cell-free Hb-based blood substitutes can be explained by this mechanism. Furthermore, capillary occlusion and hemolysis, followed by RBC-free Hb-dependent scavenging of endothelial-derived NO, have recently been proposed to underlie crisis events in patients with sickle cell disease. Novel therapeutics at preventing Hb-dependent NO scavenging therefore may offer clinical benefit in such circumstances and likely applies to other hemolytic diseases also, including cerebral vasospasm and malarial infection (45, 64).

Active role of the RBC in NO-dependent regulation of vascular function

The second concept involves a controlled stimulation of NO-dependent function in the vasculature by the RBC. The molecular mechanisms by which this can occur remain under investigation, and have yielded three models:

(a) S*-Nitrosohemoglobin (SNOHb) hypothesis.* The first involves storage, transport, and then delivery of NO from the RBC to the vasculature. The central mediator proposed in this process is SNOHb, a derivative of Hb in which NO is bound to a specific cysteine residue present at the 93rd position of the β-chain and that is a chemically distinct entity from NO-heme of Hb (52, 62, 63). According to this mechanism (also referred to as the SNOHb hypothesis), SNOHb is

formed from HbNO upon oxygenation and transported in the arterial circulation. As SNOHb enters regions of low oxygen tensions and becomes deoxygenated, the "SNO" (*S*-nitrosothiol) moiety is transferred to thiols on the RBC surface (specifically on the anion-exchange protein), where it can stimulate an increase in blood flow through interactions with the vessel wall. This pathway is regulated by the oxygen tension, which controls whether the Hb is oxygenated or deoxygenated (and hence controls protein conformation). In this way, oxygen is the switch that in one direction (*i.e.*, oxygenation) results in SNOHb synthesis and in the other (*i.e.*, deoxygenation) releases NO-like activity. This hypothesis is elegant in its construction because it matches the oxygen demand to blood flow. However, several studies have failed to reproduce the basic tenets of the mechanisms proposed and concluded that SNOHb does not play a role in the physiological regulation of blood flow. The basis of these arguments includes questions surrounding physiological levels of SNOHb and reaction mechanisms pertaining to SNOHb formation and vasoactivity (12, 22, 40, 60, 81, 82). It is beyond the purview of this article to discuss in detail the opposing views on the SNOHb hypothesis, which are discussed extensively in other recent review articles (17, 23, 24, 33, 48, 62, 83).

(b) Nitrite reductase activity of Hb. The second mechanism that fits into the concept of the RBC actively being involved in controlling NO function in the vascular compartment involves metabolism of nitrite to a vasodilator. Recent studies have shown that circulating concentrations of nitrite (<1 μ*M*) closely reflect endogenous activity of endothelial NO production (42). Chemically, nitrite is relatively inert, with biological functions limited to mediating nitration reactions during inflammation (70). The pioneering studies that led to the identification of NO as a biologically important regulatory product of vessel tone also showed that nitrite can vasodilate (36, 37). However, the concentrations required to elicit vasodilation *in vitro* were in the high micromolar to millimolar range (two to three orders of magnitude higher than physiologic concentrations), leading to a general dismissal of a role for this species in controlling vascular blood flow. However, more recent studies demonstrated that systemic decreases in blood pressure during NO-inhalation therapy were associated with arterial–venous gradients in nitrite (22). Furthermore, infusion of low (2 μ*M*) concentrations of nitrite into the forearm of healthy volunteers significantly increased local blood flow by ~30% (11). The mechanism of this effect was proposed to involve deoxygenated Hb-dependent reduction of nitrite to NO (11, 15). Similar to the principles outlined in the SNOHb hypothesis, in this case, deoxygenation of Hb serves as a switch to initiate reactions that lead to an increase in blood flow. In this case, nitrite is a precursor for the formation and release of the vasodilatory stimulus from the RBC, and deoxyHb acts as a nitrite reductase. It is interesting to note that nitrite has also been proposed to modulate blood flow via interactions with RBC in fish and may represent an evolutionary conserved mechanism (38).

Many questions surrounding the SNOHb and RBC-nitrite hypotheses remain. It is interesting to speculate, however, that in each case the RBC acts as a hypoxic sensor, responding to low oxygen environments to deliver NO to the vasculature and increase blood flow. Moreover, the sensor in the RBC is Hb itself, necessitating a revised thinking of the effects of Hb on NO function that include stimulation of function and not simply inhibition.

(c) RBC and ATP release. The third mechanism involves controlled release of ATP from the RBC. Specifically, in vascular beds in which rapid changes in blood flow are required, *e.g.*, lungs and skeletal muscle during exercise, the RBC may contribute to hypoxic vasodilation via release of ATP (27, 73, 74). Stimuli including hypoxia and mechanical deformation promote ATP release from the RBC via activation of G protein-dependent signaling (56). Through binding to endothelial P2Y receptors, ATP stimulates NO production from endothelial NO synthase thereby increasing blood flow. Interestingly, hypoxia also stimulates adenosine release directly from the endothelium through an NO-dependent mechanism (16). In turn, adenosine stimulates vasodilation via activation of β-adrenergic receptors. This suggests a concerted model whereby the RBC and the endothelium sense hypoxia to increase ATP or adenosine in the circulation for the purposes of increasing blood flow.

RBC AND INFLAMMATION

Over the last decade, the role of inflammatory processes in mediating acute and chronic vascular diseases has been widely documented. Inflammation is a diverse, multistep process involving leukocyte interactions with endothelial or epithelial cells. Whereas evidence for specific leukocyte populations has been demonstrated in a given inflammatory response, little attention has been paid to the RBC. A change in blood flow is a critical and early element of the inflammatory response. As discussed below, the RBC may modulate inflammation via regulation of NO bioavailability and hence blood flow. In addition, recent insights have underscored the potential role for direct interactions between normal RBC and the endothelium or circulating leukocytes. The following sections discuss the potential roles for the RBC in inflammation and highlight this emerging area of red cell biology.

Interactions of RBC with vascular and inflammatory cells

Direct interactions of RBC and vascular endothelial cells have been discussed in the context of hemolytic diseases, including sickle cell disease, hereditary spherocytosis, and malaria (19, 57, 79, 80). Studies have demonstrated an increased adhesion of RBC containing sickle Hb (HbS) to endothelial cells under hypoxia and low-flow conditions (57). The functional implications of this adhesion include increased Hb deoxygenation that results in sickling and may contribute to the mechanism of vasoocclusive crisis. Increased expression/avidity of adhesion molecules on the endothelium and the RBC appear to be the primary causes of increased adhesion (20), and understanding the molecular events that lead to increased adhesiveness therefore may yield important therapeutic targets.

In addition to endothelial cell interactions, binding to the matrix components thrombospondin, laminin, and fibronectin has been described and provides further molecular mechanisms for HbS RBC adhesion to the vessel wall (for review, see 57). Adhesion molecules expressed on the RBC (*e.g.*, CD36, VLA-4, and others) have been identified, and similar to leukocytes it appears that multiple (or redundant) ligand pairs can exist, the end point being increased adhesion. Why HbS RBC have increased levels of these adhesion molecules is not clear and may be related to the higher percentage of reticulocytes (or immature RBC that have higher levels of adhesion molecules than their adult counterparts) in blood from sickle cell patients. Additional mechanisms include stimulation of adhesion molecule expression by dehydration or dysfunctional RBC deformability.

Other hemolytic disorders associated with increased RBC adhesion molecule expression include malaria, in which sequestration of the parasite into the RBC confers an increased adhesiveness with the endothelium. It is thought this is important in sequestering infected RBC to specific organs, which underlies the pathogenesis of malaria. Interestingly, a recent study suggests that infected RBC first adhere to platelets, which in turn bind the endothelium (80). It was proposed that the functional consequences of this were to increase the vascular beds to which RBC can bind, and allow such interactions to occur under conditions of higher blood flow. In addition to adhesion molecules, the lipid composition in the outer leaflet of the RBC plasma membrane can also mediate adhesion interactions. This has been characterized with respect to phosphatidylserine, which under normal conditions is present only on the inner leaflet of the membrane, but under disease conditions (*e.g.*, chronic renal failure) can revert to the outer leaflet (8). This is associated with many changes, including increased RBC interactions with thrombospondin and adhesion to endothelial cells. It is tempting to speculate that adhesion of RBC to the endothelium circumvents the flow-mediated RBC-free zone, thereby allowing endothelial NO to be scavenged. In turn, this would lead to a proinflammatory/aggregatory state and may provide a mechanism through which RBC contributes to the endothelial dysfunction that is a major component of hemolytic disorders.

Normal RBC and adhesion molecules

Little is known about the role of normal RBC in inflammation or physiological cell–cell communication. The general concept is that normal RBC are innocent bystanders that become physically entrapped in the matrix of a developing inflammatory lesion or thrombus. However, emerging data show that the presence of adhesion molecules on RBC is not restricted to hemolytic disease states. In fact, normal mature RBC express a variety of adhesion molecules that, upon activation, allow adhesion with monocytes, neutrophils, and platelets (25, 32, 72). Again, key questions surrounding function remain. Interactions between RBC intercellular adhesion molecule-4 and platelet $\alpha_{IIb}\beta_3$ may regulate thrombosis responses (32). Alternatively, studies describing complement complex clearance and modulation of T-cell proliferation via RBC-expressed lymphocyte function antigen-3 suggest immunomodulatory roles that impact on transplantation independent of blood group antigen expression (20).

Modulation of inflammation by the RBC: pro- and antiinflammatory effects

The possible effects of the RBC on inflammation are not restricted to expression of adhesion molecules. As discussed above, NO is an integral mediator of vascular homeostasis. In addition to vasodilation, down-regulating inflammatory reactions are key in this function. Regulated release of NO by the RBC therefore could be postulated to tonically down-regulate inflammatory processes. Furthermore, as hypoxia has been shown to stimulate endothelial adhesion molecule expression and the formation of a proinflammatory state (75), NO release under such conditions may represent antiinflammatory function. On the other hand, recent studies have shown that arginase is present in the RBC and the levels of this enzyme are increased in diabetic subjects (39). Arginase removes the substrate for NO production, L-arginine, contributing to endothelial dysfunction and a proinflammatory state.

An alternative antiinflammatory mechanism involves reactions of the RBC with peroxynitrite [ONOO(H)], the product of reactions between NO and superoxide (6, 13, 67). ONOO(H), a potent oxidizing agent, can nitrate aromatic amino acids and has been implicated as an initiating agent in several vascular pathologies, including sepsis and atherosclerosis (5). ONOO(H) rapidly reacts with RBC Hb to form nitrate, an inert product, and ferric or metHb (67). The latter is readily reduced back to the oxyferrous form, providing a catalytic ONOO(H) detoxification mechanism. Given the relatively high concentration of circulating Hb, the RBC represents a significant target for ONOO(H) reactivity in the intravascular compartment, and may limit ONOO(H)-dependent reactions that underlie inflammation-induced tissue injury (13, 67).

In addition to effects on reactive species metabolism, an intriguing antiinflammatory function of the RBC is in regulating levels of inflammatory cytokines. Specifically, the duffy antigen receptor for chemokines (DARC) is a receptor expressed on the endothelium and on RBC (30, 47). This receptor is used by malarial parasite *Plasmodium vivax* to infect RBC, but also is a relatively promiscuous receptor for "CC" and "CXC" subclasses of chemokines. These include a variety of chemokines that regulate diverse vascular functions from inflammation to angiogenesis. The exact function of DARC in the RBC remains to be elucidated, but could act as a sponge to soak up proinflammatory cytokines, thereby limiting tissue injury.

RBC aggregation

In a variety of diseases in which vascular inflammation plays a role, including insulin resistance, obesity, and hypercholesterolemia, RBC aggregates have been detected (41, 68, 69). Furthermore, these aggregates correlate positively with different inflammatory markers, suggesting that the RBC is a target for inflammatory reactions. Consistent with this concept, fibrinogen was found to stimulate RBC aggregation (7). The functional effects of RBC aggregation and how this im-

pacts on inflammation are not clear, but altered rheological properties and subsequent dysregulation of capillary perfusion are likely to play roles. Coupled with concepts proposed in the sickle cell literature and as discussed above, it is interesting to speculate that this would set up a vicious cycle, whereby inflammatory reactions would first promote RBC aggregate production and, subsequently, these would further stimulate inflammation by vasoocclusion and compromising blood flow.

Hemolysis and inflammation

As with effects on blood flow, releasing cell-free Hb into the circulation has the potential to promote significant vascular inflammation through a variety of mechanisms. Firstly, cell-free Hb will rapidly inhibit NO function, including its antiinflammatory effects. In fact, acute inhibition of endothelial NO synthase in the postcapillary vasculature rapidly leads to neutrophil adhesion and activation (43). It is expected that similar effects would be observed with cell-free Hb also. Data shown in Fig. 2 extend these concepts and show that chronic treatment of aortic endothelial cells with cell-free Hb stimulates subsequent adhesion of monocytes. Cell-free Hb also increases endothelial permeability and synthesis of endothelin-1 (3, 29, 78). The latter would compound vasoconstrictive effects associated with NO scavenging. In addition, cell-free Hb is a potent oxidant toward lipids (2, 59, 66). This initiates and propagates the lipid peroxidation cascade and has the effect of increasing membrane leakiness and promoting oxidative damage to membrane proteins. In addition, oxidized lipids have a variety of biological effects, including proinflammatory and vasoconstrictive effects (54), which emphasize the potential for cell-free Hb to mediate vascular dysfunction.

Many studies have documented the adverse effects of Hb in the context of administering Hb-based blood substitutes in resuscitation fluids, and indeed the lack of success of such compounds has been attributed to the prooxidant and inflammatory effects described above (1, 29). In addition, the role of cell-free Hb in mediating vascular dysfunction during hemolytic diseases is now being appreciated in the clinical arena (65). As discussed above, many of these effects arise from scavenging of NO and may underlie vasoocclusive crisis and pulmonary hypertension (64). Interestingly, mechanisms that remove cell-free Hb from the circulation (specifically haptoglobin-dependent chelation of Hb) appear to be dysfunctional or are overwhelmed in sickle cell patients (65). However, cell-free Hb may arise during normal inflammatory processes in the absence of an overt hemolytic disease or therapeutic administration. This is indicated from studies on hemodialysis patients who are more susceptible to vascular injury (84). In these patients, levels of a proatherogenic form of low-density lipoprotein (LDL^-) are also elevated, and it appears to be formed by oxidative reactions mediated by cell-free Hb. The latter can arise from RBC lysis caused by reactive species formed from leukocytes that are activated as a consequence of the dialysis procedure. These studies led to the concept that hemolysis is an ongoing event during inflammation, and if unchecked, the released Hb can propagate inflammation-induced tissue injury.

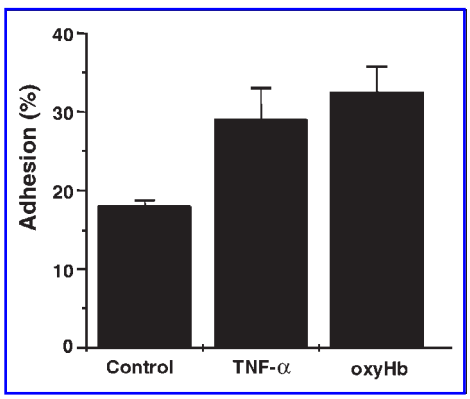

FIG. 2. Cell-free Hb stimulates monocyte adhesion to aortic endothelial cells. Mouse aortic endothelial cells were exposed to cell-free oxyHb, or the proinflammatory cytokine tumor necrosis factor-α (TNF-α), for 6 h and subsequent adhesion of monocytes (WEHI) was determined. Both oxyHb and TNF-α alone increased adhesion by ~10–15%. These data illustrate the proinflammatory potential of cell-free Hb.

SUMMARY

Under physiologic conditions, the intact RBC modulates vascular NO biology. Although the mechanisms are still being elucidated, hypoxia, the architecture of the vessel wall, and RBC itself are important in controlling NO flux in the vasculature. These functions of the RBC may participate in the multiple redundant mechanisms that match blood flow to the metabolic demands of respiring tissues. In addition to regulating NO under physiologic conditions, emerging data from basic to clinical studies highlight a role for disruption in these regulatory mechanisms in the pathogenesis of inflammatory disease. The pathologic mechanisms include adhesion and hemolysis. Whereas little is known on the effects of the former, hemolysis disrupts vascular homeostasis promoting a proinflammatory state. A significant component of this includes scavenging and prevention of NO-dependent function. It is important to note that hemolysis can be a relatively common event *in vivo*, as evidenced by significant hemolysis in the avid runner (77). Certainly multiple, redundant mechanisms exist *in vivo* to limit damage caused by such events. However, these fail-safe mechanisms can be overwhelmed, as seen in sickle cell disease, emphasizing the need for a more detailed understanding of how the RBC modulates the vascular functions of NO in physiology and how disruption of these pathways results in disease.

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ABBREVIATIONS

ATP, adenosine triphosphate; DARC, duffy antigen receptor for chemokines; Hb, hemoglobin; HbS, sickle hemoglobin;

NO, nitric oxide; ONOO(H), peroxynitrite; RBC, red blood cell(s); SNOHb, S-nitrosohemoglobin.

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