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Updated Role of Nitric Oxide in Disorders of Erythrocyte Function

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Abstract

Nitric oxide is a potent vasodilator that plays a critical role in disorders of erythrocyte function. Sickle cell disease, paroxysmal nocturnal hemoglobinuria and banked blood preservation are three conditions where nitric oxide is intimately related to dysfunctional erythrocytes. These conditions are accompanied by hemolysis, thrombosis and vasoocclusion. Our understanding of the interaction between nitric oxide, hemoglobin, and the vasculature is constantly evolving, and by defining this role we can better direct trials aimed at improving the treatments of disorders of erythrocyte function. Here we briefly discuss nitric oxide's interaction with hemoglobin through the hypothesis regarding S-nitrosohemoglobin, deoxyhemoglobin, and myoglobin as nitrite reductases. We then review the current understanding of the role of nitric oxide in sickle cell disease, paroxysmal nocturnal hemoglobinuria, and banked blood, and discuss therapeutics in development to target nitric oxide in the treatment of some of these disorders.

Keywords

Banked blood; erythrocytes; hemoglobin; nitric oxide; nitrite reductase; sickle cell disease

INTRODUCTION

Nitric oxide (NO) is a critical molecule in states of erythrocyte dysfunction including sickle cell disease (SCD), paroxysmal nocturnal hemoglobinuria (PNH) and the use of banked blood for transfusion. Our group previously published a review of the role and targets related to NO in SCD, malaria and banked blood. Since then, this role has been further defined in relation to these disease states. In SCD, therapies targeting the NO pathways through inhaled NO and the use of phosphodiesterase inhibitors are being studied in trials. The hope for these therapies is that they can relieve the various hypoxic complications from sickle cell vasoocclusion mediated by the vasodilatory and the platelet antiaggregatory properties of NO. In the study of PNH, an increased understanding of the role of NO with relation to platelets has emerged. Finally, there have been developments in the understanding of RBC dysfunction in banked blood. It is now believed that NO bioavailability plays an important role, along with known banked blood defects, and

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CONFLICT OF INTEREST

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ongoing trials may improve our knowledge of the true morbidity related to banked blood use.

NO and the Erythrocyte

Our previous review presented two models regarding the interaction of the red blood cell with NO [1]. One model, that of S-nitrosohemoglobin, proposes that NO and hemoglobin form a bonded compound when NO, nitrite, or S-nitrosothiols (SNOs) react with deoxygenated hemoglobin with the molecule in the tense state [2]. In the relaxed state, the NO molecule is transferred from heme to a cysteine residue on the hemoglobin protein (Cysβ93), forming S-nitrosohemoglobin. When oxygen is released in the periphery, hemoglobin returns to the tense state and S-nitrosohemoglobin is believed to release an NO equivalent, likely NO or SNO, through a sequence of reactions ultimately resulting in release of a vasoactive NO equivalent to the vascular endothelium. NO/SNO will then interact with soluble guanylate cyclase in the vascular smooth muscle, ultimately leading to decreased intracellular calcium, smooth muscle relaxation, and vasodilatation. An alternate model, that pertaining to deoxyhemoglobin, states that in peripheral tissue, once oxygen is released from hemoglobin, the deoxygenated hemoglobin acts as a nitrite reductase to produce vasoactive NO from nitrite [3]. Hypoxic tissue, where increased levels of deoxyhemoglobin exist physiologically, would then benefit from preferential local vasodilatation mediated by NO. An additional recent hypothesis postulates that myoglobin acts as a nitrite reductase in hypoxic tissue, mediating vasodilation and matching O_2 delivery to metabolic demand. This is supported by evidence demonstrating decreased reduction of nitrite to NO in myoglobin deficient mice [4].

HEMOLYTIC STATES

Sickle Cell Disease (SCD)

Background—The substitution of the amino acid valine for glutamate, due to a single nucleotide point mutation within the β-globin gene of hemoglobin A, results in sickle hemoglobin S (HbS), sickling and vasoocclusion. When this mutation is present in both copies of the β-globin gene, SCD results. The deoxygenated state of HbS renders these erythrocytes prone to polymerization, resulting in vasoocclusion and complications from local ischemia. Additionally, sickled erythrocytes are less able to internalize phosphotidyl serine in the erythrocyte membrane due to high ATP consumption which impairs aminophospholipid translocase [5]. Sickled erythrocyte phospholipid asymmetry may lead to an increased risk for thrombosis [6]. Common complications related to SCD include vasoocclusive pain crisis, acute chest syndrome, priapism, and vasculopathy induced chronic organ failure [7]. Unlike other hemolytic states such as PNH, cell-free hemoglobin concentrations in sickle cell disease are 10-fold lower [8]. This may explain some of the discrepant findings between studies involving patients with sickle cell disease and those studies involving patients with other hemolytic disorders.

Inhaled Nitric Oxide—A multicenter, randomized controlled phase 2 trial completed in 2011 compared inhaled NO with inhaled nitrogen placebo in 150 individuals presenting with the vasoocclusive crisis (VOC) of sickle cell disease [9]. The overall median age of participants was 24.2 years, with well-matched placebo and treatment groups. The primary endpoints were freedom from opioid use for 5 hrs, pain scale score of 6 or lower, ability to walk, and ability to manage pain at home. Secondarily, other measured endpoints were length of hospitalization, overall pain relief, cumulative opioid usage, and rate of acute chest syndrome or pneumonia requiring transfusion, proportion of patients discharged in the first 24 hours, proportion of patients returning to the ED or hospital within 30 days, and changes in nitrate/nitrite and methemoglobin levels. This latter outcome was a surrogate measure of

nitric oxide metabolism and reactions within blood. The authors observed no significant difference in primary or secondary measures between the two groups, with an overall median time to VOC resolution of 73 hrs. Interestingly, previous studies of inhaled NO in ischemia have shown an association between systemic nitrite generation in the circulation and organs, and an observable effect of NO [10, 11]. In this trial, there was no significant difference in the measured levels of nitrite in the plasma or whole blood, between placebo and treatment groups. Of note in this phase two trial, no NO toxicities were observed. Prior to this trial, two trials, the larger having 20 participants, had demonstrated modest reductions in overall pain and opioid use in children and adults with inhaled NO regimens [12, 13].

While anecdotal benefits have been shown for inhaled NO in sickle cell patients with acute stroke [14], clinical trials have yet to show consistent improvement in clinical outcome. Therefore, the precise role of inhaled NO in the treatment of the complications of SCD remains unclear until the proper clinical trials can provide evidence for more definitive utility.

Phosphodiesterase-5 Inhibitors—The PDE-5 inhibitor sildenafil has been shown to improve exercise capacity and hemodynamics in pulmonary arterial hypertension, and exercise capacity alone in pulmonary venous hypertension related to left heart failure [15, 16]. Tricuspid regurgitation velocity (TRV), as measured by Doppler echocardiography, is an important measure of pulmonary artery pressure. In patients with sickle cell disease, elevated TRV is associated with increased mortality [17–19]. A recent multicenter, randomized controlled trial by Machado *et al.* examined the use of sildenafil in individuals with SCD, TRV ≥ 2.7m/s, and a 6-minute walk distance between 150 and 500m. The primary endpoint was change in exercise capacity at 16 weeks. Secondarily, the authors examined changes in TRV, hemodynamics by right heart catheterization, Borg Dyspnea Score, WHO functional class for pulmonary hypertension, N-terminal pro-brain natriuretic peptide, quality of life assessment, self-reported clinical pulmonary hypertension outcomes, initiation of additional pulmonary hypertension therapies, and assessment of pain. Following randomization of 74 individuals, baseline hemodynamics with administration of the first treatment were measured with right heart catheterization. The authors observed a significant decrease in mean systemic blood pressure, right atrial pressure, mean pulmonary artery pressure, and pulmonary capillary wedge pressure with sildenafil administration. At 16 weeks there was no significant decrease in TRV or improvement in exercise capacity in the sildenafil treated group versus placebo. This study was stopped early due to a significant increase in severe adverse events in the sildenafil treated group. Specifically, an increase in hospitalizations due to pain (35% sildenafil vs. 14% placebo; $P = .03$) was observed. The authors postulated that the role of NO and cyclic guanosine monophosphate (cGMP) in the processing of inflammatory and neuropathic pain may be related to these increased pain episodes. This NO pain pathway has been described in animal models, with multiple studies showing that inhibition of NO, nitric oxide synthase (NOS), or cGMP significantly reduces inflammatory and neuropathic pain [20–23].

Paroxysmal Nocturnal Hemoglobinuria (PNH)

Background—PNH is a clonal hematopoietic disorder characterized by erythrocyte hypersensitivity to complement, hemolysis, esophageal spasm, thrombosis, and marrow aplasia. The incidence of PNH is estimated at less than 1 per 100,000. Affected patients typically present with dark urine and varying degrees of hemolysis, thrombosis and pancytopenia. As a clonal disorder of hematopoiesis, PNH can progress to acute leukemia. Patients with PNH were originally described with “nocturnal” hemoglobinuria due not only to urinary concentration during sleep, but also due to the physiologic acidity that accompanies sleep and accelerates hemolysis.

The molecular basis of PNH is related to mutations in the *PIGA* gene. *PIGA* encodes synthesis of proteins involved in the first step of synthesis of the glycosylphosphatidylinositol (GPI) anchor, which serves as an attachment molecule for many cell surface proteins. Two such proteins, CD55 and CD59, are directly responsible for protecting the erythrocyte against complement-mediated destruction. Documenting deficiency of CD55 and CD59 on leukocytes and erythrocytes by flow cytometry is diagnostic of PNH. Loss of erythrocyte CD55 and CD59 leads directly to complement-mediated lysis of erythrocytes. In addition to hemolysis, marrow failure is a common finding in patients with PNH. Some investigators suggest that marrow failure in PNH is signaled through GPI-linked apoptotic receptors [24]. As such, normal cells in the PNH marrow undergo apoptosis and the remaining PNH clones are able to populate and replace the diseased hypocellular marrow. Thrombosis in PNH is presumably related to effects on endothelium and platelets by NO scavenging by cell-free hemoglobin. An additional etiology of thrombosis involves the urokinase-type plasminogen activator receptor. The urokinase receptor is normally GPI linked on platelets and neutrophils. In patients with PNH, the urokinase receptor is absent on cells resulting in increased soluble serum urokinase receptor levels. Selective binding of urokinase to soluble receptors, rather than platelets, contributes to the thrombotic tendency observed in PNH patients [25]. Recently, the monoclonal antibody eculizumab, directed against complement component 5 (C5), has proven effective in both decreasing hemolysis and decreasing thrombosis associated with PNH [26]. C5 is normally cleaved into C5a and C5b by the enzyme C5 convertase. The anaphylatoxin C5a is important in chemotaxis. C5b forms part of the membrane attack complex. Eculizumab is a recombinant humanized monoclonal $IgG_{2/4}$ antibody that binds to C5 and inhibits its conversion into C5a and C5b by the convertase enzyme. Because C5b generation is inhibited in patients treated with eculizumab, complement-mediated erythrocyte destruction is prevented. As a result, cell-free hemoglobin levels are markedly reduced in PNH patients treated with eculizumab.

Relationship to NO—It has been shown in a CD59 knockout mouse PNH model that NO inhibits the complement mediated activation of platelets [27]. This suggests that thrombosis in PNH may not only be due to NO depletion and its effects on the vasculature, but also due to NO depletion as a cause of platelet activation. As mentioned previously, eculizumab has benefit in the treatment of PNH by preventing hemolysis. Analysis of patient data from the TRIUMPH trial, which established eculizumab as an effective treatment in PNH showed that eculizumab treatment was effective not only in significantly reducing NO consumption but also in improving pulmonary artery pressure [28]. Additionally, eculizumab has been shown to decrease smooth muscle dystonia in patients with PNH due to its effect on NO consumption [29].

Damage to Banked Blood

The “storage lesion” is defined as the change in RBC morphology and physiology that occurs over time in banked blood leading to impaired *in vivo* function and accelerated clearance from the circulation following transfusion [30]. Loss of ATP, depletion of 2,3-BPG, loss of membrane integrity, SNOHb deficiency and generation of free Hb have all been associated with storage lesions in banked blood [1, 31, 32]. The FDA recommends that non-frozen RBCs be stored for a maximum of 42 days, while 15 days is currently the median duration of storage for a transfused unit [33]. Increased incidence of pneumonia, mortality, length of stay (LOS), and multi-organ failure have been associated with transfused units stored for more than 14 days [34–37]. However, in 2006 a Dutch study following 2732 patients who had undergone CABG surgery showed that there was no significant difference in 30 day survival, hospital stay, or ICU stay between patients that had received RBCs stored for 13 days compared to patients transfused with RBCs stored for 24

days [38]. Conversely, in a meta-analysis of 21 different studies examining risk of death in patients transfused with older versus newer stored blood, older blood was associated with increased risk of death and it was calculated that 91 patients would need to be treated with only new blood to save one life (NNT) [39]. In this study, older blood was stored for at least 14 days with the exception of one study in premature infants where older blood was 9 days old.

The Red Cell Storage Duration Study (RECESS) is a multicenter prospective randomized controlled trial that is currently underway to assess the effect of storage age on banked blood [40]. The investigators are comparing transfusions of RBC units stored less than or equal to 10 days versus those stored greater than or equal to 21 days in patients undergoing complex cardiac surgery. The primary endpoint is change in multi organ dysfunction score, with secondary endpoints including major in-hospital complications and all cause mortality. Estimated to be completed in October 2013, this trial is poised to provide important data to help answer the question of whether standard storage practices of banked blood influence patient outcomes.

In a recent study, 14 healthy human volunteers were transfused with a fresh unit (3–7 days following phlebotomy) of autologous red blood cells and with an additional unit after 42 days of storage. Routine laboratory measurements taken at set timepoints following tranfusion showed a significant difference in iron parameters and markers of hemolysis after transfusion with the older units. The increased concentration of non-transferrin bound iron seen after transfusion with the older units was correlated with *in vitro* proliferation of a pathogenic strain of *E. coli*, suggesting that increased free iron following transfusion with older units may predispose patients to greater incidence of transfusion related-infection [41]. In another recent study, eight healthy human subjects donated a double RBC unit *via* apheresis, which was leuko-reduced and processed in an AS-3 anticoagulant/preservative solution. Subjects were transfused with 1 unit of RBCs at 7 and 42 days after collection. Sublingual microvascular blood flow was measured before and after transfusion using a video microscope. This study reported no detrimental effect on tissue oxygenation or the microcirculation in patients transfused with the 42 day old erythrocytes [42]. Although there has been no causal relationship proven between adverse clinical outcome and transfusion of older erythrocytes, evidence exists to suggest maintaining erythrocyte integrity and viability is clinically beneficial and further research is necessary.

Despite some controversy, there is reasonable evidence to suggest that nitric oxide scavenging by free hemoglobin and red cell microparticles is a major mechanism behind the red cell storage lesion [43, 44]. It has been shown that banking erythrocytes results in both cell-free and microparticle encapsulated hemoglobin which reacts with and scavenges NO [43]. Further evidence for the role of NO scavenging as the cause of the erythrocyte storage lesion comes from an animal model where the deleterious effects of transfusion of stored blood were alleviated by treatment with inhaled NO [45]. Roback *et al.* support this notion through their hypothesis known as the insufficient nitric oxide bioavailability hypothesis (INOBA), which aims to explain morbidity and mortality in transfusion recipients. The authors suggest that factors related to both transfused RBC units and donor endothelial dysfunction contribute to insufficient NO levels in the periphery. This regional deficiency of NO leads to vasoconstriction and inadequate oxygen delivery to tissues. The known RBC storage lesions, discussed earlier, further contribute to decreased oxygen delivery by transfused RBC units.

CONCLUSION

NO, as a potent vasodilator, may play a critical role in the future treatment of disorders of erythrocyte function. Our evolving understanding of the role of NO in these disease states and of therapeutic targets within the NO pathways continues to stimulate new trials. Inhaled NO, phosphodiesterase inhibitors, and soluble guanylate cyclase targeted therapies have provided mixed results in terms of therapeutic benefit in disease states involving the erythrocyte. However, NO has a well-established functional role in SCD, PNH, banked blood and other disorders of erythrocyte function. We look forward to trials both currently ongoing and on the horizon, to define and exploit the role of NO and benefit patients having a broad spectrum of disorders.

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