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Research Paper

Mechanism of faster NO scavenging by older stored red blood cells ☆

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ABSTRACT

The blood storage lesion involves morphological and biochemical changes of red blood cells (RBCs) that occur during storage. These include conversion of the biconcave disc morphology to a spherical one, decreased mean corpuscular hemoglobin concentration, varied mean corpuscular volume, reduced integrity of the erythrocyte membrane with formation of microparticles, and increased cell-free hemoglobin. We studied the extent that older stored red blood cells scavenge nitric oxide (NO) faster than fresher stored red blood cells. Using electron paramagnetic resonance spectroscopy and stopped-flow absorption spectroscopy to measure the rate of NO uptake and reaction with hemoglobin in red cells, we found that older stored red blood cells scavenge NO about 1.8 times faster than fresher ones. Based on these experimental data, we simulated NO scavenging by fresher or older stored red blood cells with a biconcave or spherical geometry, respectively, in order to explore the mechanism of NO scavenging related to changes that occur during blood storage. We found that red blood cells with a spherical geometry scavenges NO about 2 times slower than ones with a biconcave geometry, and a smaller RBC hemoglobin concentration or volume increases NO scavenging by red blood cells. Our simulations demonstrate that even the most extreme possible changes in mean corpuscular hemoglobin concentration and mean corpuscular volume that favor increased NO scavenging are insufficient to account for what is observed experimentally. Therefore, RBC membrane permeability must increase during storage and we find that the permeability is likely to increase between 5 and 70 fold. Simulations using a two-dimensional blood vessel show that even a 5-fold increase in membrane permeability to NO can reduce NO bioavailability at the smooth muscle.

Background: Transfusion of older stored blood may be harmful.

Results: Older stored red blood cells scavenge nitric oxide more than fresher cells.

Conclusion: As stored red blood cells age, structural and biochemical changes occur that lead to faster scavenging.

Significance: Increased nitric oxide scavenging by red blood cells as a function of storage age contributes to deleterious effects upon transfusion.

Introduction

Blood transfusion is one of the most common medical therapies, with about 14 million units of red blood cells (RBCs) having been transfused in the United States in 2011 [1]. The average age of red blood cells at transfusion is approximately 18 days. According to the blood banking standard, RBCs preserved in ADOSL can be stored up to 42 days. However, a growing body of literature has demonstrated an association between an increased incidence of adverse clinical outcomes of blood transfusion and the storage of RBCs [2–7]. The adverse effect of blood transfusion is also suggested to be related to the number of units transfused [8,9].

A number of chemical and morphological changes in RBCs occur during blood storage including a reduction in levels of 2, 3-diphosphoglycerate, ATP, and pH values, as well as an increase of potassium and lactate [10]. These changes result in a reduced deformability, increased osmotic fragility, spheroechinocyte formation, reduced integrity of the erythrocyte membrane with

Abbreviations: NO, nitric oxide; RBC, red blood cell; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular hemoglobin volume; PBS, phosphate buffered saline; MetHb, methemoglobin

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formation of microparticles, and increased cell-free hemoglobin (Hb) in plasma [11–17]. Numerous studies have found that the geometry of the red blood cell tends to become more spherical during storage thereby having a smaller surface area to volume ratio [10,18–20]. The mean corpuscular hemoglobin concentration decreases, and the structure of RBC membrane changes significantly during storage [21,22]. In addition, RBC volume varies during blood storage but the type of change depends on which additive preservation solutions is used [23,24]. Changes in RBCs during storage are referred as blood storage lesion. Precise mechanisms that explain how the blood storage lesion is associated with adverse effects of blood transfusion remain unclear.

Nitric oxide functions as the endothelial derived relaxing factor, decreases platelet activation and vascular cell adhesion, influences oxidative and nitrosative stress, functions in host defense, and influences a large number of cellular functions through protein modification [25–29]. We hypothesize that the blood storage lesion is associated with a loss of NO signaling. It has been well established that oxygenated cell-free Hb reacts with NO at a high rate of $6–8\times10^7\ M^{-1}\ s^{-1}$ to form nitrate and methemoglobin [30–32]. Microparticles are small phospholipid vesicles that contain hemoglobin and they react with NO only about 3 times slower than cell-free Hb, but still 1000 times faster than RBCs [33–35]. Several mechanisms are responsible for the reduced rate of NO scavenging by hemoglobin encapsulated in RBCs. NO diffusion to the RBC is limited by an unstirred layer around the RBC which is due to the fast scavenging of NO close to RBCs [36–39]. A RBC cell-free zone is created when RBCs are pushed to the center of vessel during flow and it separates RBCs from endothelial cells where NO is produced [40,41]. Another mechanism that accounts for slower NO scavenging by RBCs is that the RBC membrane has a finite permeability to NO, like a physical barrier which slows down the diffusion [38,42]. These diffusion barriers are regulated by RBC size, shape, and surface area; biophysical properties that are changed during RBC storage [22]. Since hemoglobin and red cell microparticles scavenge NO close to 1000 times faster than red cell encapsulated hemoglobin, red cell breakdown in storage or post transfusion substantially reduces NO bioavailabiliy [34,35]. In addition, recent studies show that the effects of storage will lead to an increased intrinsic NO scavenging by older stored RBCs, as measured by nitric oxide competition experiments and inhibition of aortic vasodilation [20,43,44]. However, the extent of the increase in NO scavenging by older red blood cells compared to fresh ones was not fully explored and the mechanism for this phenomenon was not satisfactorily provided.

In this study, we further examine the rate of NO scavenging by both fresh and old stored RBCs using time-resolved stopped-flow absorption spectroscopy and electron paramagnetic resonance spectroscopy. Computational simulations are also conducted to explore the effects of morphological changes, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and permeability to NO on the rate of NO scavenging using 3D single RBC models. We use results of these simulations to account for the observed increase in NO scavenging by RBCs as a function of storage age.

Experimental procedures

Old and fresh, leukodepleted packed red blood cells were collected from packed red blood cell segments stored in the University of Alabama at Birmingham blood bank in AS-1 storage solution. The average storage length of old and fresh red blood cells in this study were 8.5 ± 1.4 days and 37.5 ± 2.9 days. Red blood cells were washed in phosphate buffered saline (PBS) at least three times or until the supernatants were clear. The hemoglobin concentration of the red blood cell solution (in heme) was determined by absorption spectroscopy using a Cary 100 spectrometer equipped with an integrating sphere detector. Spectra of cell-free hemoglobin were taken on a Cary 50 Bio spectrometer (Varian Inc.). Cell-free hemoglobin was prepared as described previously [45]. All experiments were performed in PBS buffer and all chemicals were purchased from Sigma unless otherwise stated.

Competition experiments

Experiments were performed similarly to those described previously [38,46]. Briefly, oxygenated cell-free Hb and Hb encapsulated in RBCs compete to react with NO to form methemoglobin (MetHb). The relative rates of NO uptake by RBCs compared to cell-free Hb can be determined by examining the concentrations of MetHb produced from each fraction using electron paramagnetic resonance (EPR) spectroscopy. In the experiments, cell-free Hb (100 μM in heme) was mixed with either old or fresh stored red blood cell solutions (50% hematocrit). The mixtures were stirred with a magnetic bar at a slow speed to keep the sample homogeneous throughout the experiment. The NO donor DEANONOate was prepared in a deoxygenated 0.01 M NAOH solution and added to the mixture to a final concentration of 20 μM. The first EPR tube was filled with the mixture at 45 min and the rest of the mixture was centrifuged at 3000*g* for 2 min. The supernatant was put in another EPR tube and the third EPR tube was filled with the RBC pellet. Control experiments were performed as described previously to account for any autoxidation or MetHb reductase activity [46]. All the EPR tubes prepared in the experiment were frozen simultaneously at 1 h. The amounts of MetHb were measured by electron paramagnetic resonance using a Bruker EMX 10/12 spectrometer as described previously [45]. Hemolysis during the experiment was checked by examining the hemoglobin concentrations in the supernatant prior to the addition of NO donor and after the centrifugation.

The ratio of the bimolecular rate constant of NO reacting with cell-free Hb (k_f) to the bimolecular rate constant of NO uptake by RBC-encapsulated Hb (k_r) was determined by the relative amount of MetHb formed in each fraction as shown in the equation:

$$\frac{[\mathrm{MetHb}]_f}{[\mathrm{MetHb}]_r}=\frac{K_f[\mathrm{Hb}]_f}{K_r[\mathrm{Hb}]_r} \quad (1)$$

where the subscripts "*f*" and "*r*" stand for cell-free Hb and RBCs encapsulated Hb respectively. The concentrations indicated by brackets are the moles of the species over the total volume. Natural log terms on both sides of the equation were also calculated as previously described [47] in order to account for the fact that the total amount of oxygenated hemoglobin in the free and RBC fractions may decrease with time from its initial amount. The ratio k_f/k_r calculated using Eq. (1) had less than 2% difference from the result calculated with natural log terms. Three methods were used to calculate k_f/k_r using two MetHb concentrations of those in the mixture, supernatant, and RBC pellet in order to avoid errors as described previously [45].

Stopped-flow analysis

Stopped-flow time-resolved absorption experiments of the reaction between NO and RBCs were conducted using a Molecular Kinetics three-syringe mixer (Indianapolis, IN) coupled to an Olis RSM spectrometer (Bogart, GA) as described previously [47]. The NO donor PROLINONOate (10 mM, Cayman Chemicals) was prepared in 0.01 M NAOH and loaded in the first syringe. Either old or fresh stored red blood cells (100 μM in heme) were filled in the second syringe and the third syringe was loaded with deoxygenated PBS. Mixing was accomplished as follows: the NO donor was diluted to 500 μM by PBS and aged for 20 s in the first mixer so