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Red blood cells stored for increasing periods produce progressive impairments in nitric oxide–mediated vasodilation

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Abstract

BACKGROUND—Clinical outcomes in transfused patients may be affected by the duration of blood storage, possibly due to red blood cell (RBC)-mediated disruption of nitric oxide (NO) signaling, a key regulator of vascular tone and blood flow.

STUDY DESIGN AND METHODS—AS-1 RBC units stored up to 42 days were sampled at selected storage times. Samples were added to aortic rings *ex vivo*, a system where NO-mediated vasodilation could be experimentally controlled.

RESULTS—RBC units showed storage-dependent changes in plasma hemoglobin (Hb), RBC 2,3-diphosphoglycerate acid, and RBC adenosine triphosphate conforming to expected profiles. When freshly collected (Day 0) blood was added to rat aortic rings, methacholine (MCh) stimulated substantial NO-mediated vasodilation. In contrast, MCh produced no vasodilation in the presence of blood stored for 42 days. Surprisingly, the vaso-inhibitory effects of stored RBCs were almost totally mediated by RBCs themselves: removal of the supernatant did not attenuate the inhibitory effects, while addition of supernatant alone to the aortic rings only minimally inhibited MCh-stimulated relaxation. Stored RBCs did not inhibit vasodilation by a direct NO donor, demonstrating that the RBC-mediated vaso-inhibitory mechanism did not work by NO scavenging.

CONCLUSIONS—These studies have revealed a previously unrecognized vaso-inhibitory activity of stored RBCs, which is more potent than the described effects of free Hb and works through a different mechanism that does not involve NO scavenging but may function by reducing endothelial NO production. Through this novel mechanism, transfusion of small volumes of stored blood may be able to disrupt physiologic vasodilatory responses and thereby possibly cause adverse clinical outcomes.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Blood transfusion is the most commonly employed procedure for hospitalized patients in the United States, based on discharge codes.¹ Optimal functioning of this system depends on the ability to store blood for up to 42 days before transfusion. Studies used to support 42-day post-donation storage include biochemical measurements (2,3-diphosphoglycerate acid [2,3-DPG] and adenosine triphosphate [ATP]), measures of red blood cell (RBC) integrity (plasma free hemoglobin [Hb]), and quantification of survival of stored RBCs in autologous transfusion recipients at 24 hours after transfusion.^{2,3} However, there are no specific measurements performed to show that RBCs stored up to 42 days achieve minimal standards of efficacy or produce acceptably low rates of adverse events in transfusion recipients.

Despite the undeniable therapeutic benefits of blood transfusion, numerous studies have demonstrated significant biochemical, structural, and morphologic changes in RBCs during pretransfusion storage.⁴⁻⁶ These changes (the “RBC storage lesion”) may be of negligible impact after short storage periods (“fresh RBCs”), but longer-term storage approaching 42 days (“storage-aged RBCs”; saRBCs) may have deleterious effects on the recipient.

Tinmouth and coworkers⁷ and Wang and coworkers⁸ have performed systematic reviews of dozens of studies that investigated the relationship between blood storage and adverse transfusion events. Meta-analyses showed worse recipient outcomes after transfusion of saRBCs. Since the largest clinical studies included in these reviews were retrospective, further elucidation of the possible adverse effects of saRBCs may be provided by prospective randomized trials. The largest to be published to date, ARIPI,⁹ compared fresh blood (stored up to 7 days; median, 5 days) with standard of care (stored up to 42 days; median, 13 days) in low-birthweight neonates. Although the outcomes showed no difference between study arms, the relatively short storage times in the standard-of-care arm do not allow an assessment of the efficacy of saRBCs stored for long periods (21-42 days). This issue may be better addressed in the ongoing RECESS,¹⁰ ABLE,¹¹ and Red Cell Storage Duration and Outcomes in Cardiac Surgery studies.¹²

As an adjunct to biochemical, molecular, and clinical outcomes studies, investigations of the acute physiologic effects of saRBCs may identify potential mechanisms by which saRBC transfusions could cause adverse outcomes. In addition to oxygen and carbon dioxide transport, another significant physiologic role of RBCs is in the process of hypoxic vasodilation. This activity, which regulates local blood flow to preferentially perfuse and provide oxygen for the most hypoxic tissues, involves combined activities of RBCs and endothelial cells to regulate arteriolar smooth muscle tone.¹³ Disruption of hypoxic vasodilation by saRBCs represents a viable physiologic hypothesis to explain an association between blood storage and adverse transfusion events.¹⁴

Although hypoxic vasodilation is not yet fully understood at a biochemical level, it is likely to involve regulation of nitric oxide (NO) signaling. Theoretically, if saRBCs did not produce or stimulate sufficient NO, then replacement of a patient’s normal RBCs with transfused cells could result in an NO synthesis defect. There is some evidence for this possibility. For example, studies show that *S*-nitrosylation of Hb (SNO-Hb) may produce a storage pool of NO within RBCs that can be released in hypoxic environments,¹⁵⁻¹⁸ and *S*-nitrosylation of Hb has been shown to decline with RBC storage.¹⁹ Other investigators have

shown that nitrite (which is present at high levels in plasma) can be converted to NO by Hb.²⁰⁻²³ In this model, deoxygenated Hb, which is at high concentrations in hypoxic tissues, binds nitrite and a proton to generate NO and methemoglobin. If blood storage altered the nitrite reductase activity of Hb, this NO source would be disrupted. With either of these models, NO must diffuse from the RBC to the smooth muscle to produce vasodilation.^{24,25} In a third model that could potentially explain reduced NO production with saRBCs, ATP release by RBCs (a robust mechanism for stimulating endothelial NO production²⁶⁻²⁹) may be impaired by storage.³⁰

As an alternative mechanism that could also underlie reduced hypoxic vasodilation after transfusion, infused saRBCs could produce an inhibitory factor that interrupts normal NO signaling between endogenous RBCs, endothelium, and the underlying smooth muscle. This mechanism, which may function in conjunction with (or instead of) the synthesis defects described above, is attractive because it could allow a small volume of transfused saRBCs to broadly impair hypoxic vasodilation. Disruption of NO signaling by saRBCs could be mediated by free Hb (or other RBC constituents) released into the plasma as a consequence of RBC hemolysis or by Hb or other factors encapsulated in RBC-derived microparticles.^{4,24,25} The free Hb mechanism is supported by clinical and experimental studies showing that plasma free Hb can scavenge NO, reducing its bioavailability and causing vascular sequelae.³¹⁻³³ Microparticles may be important because they can flow closer to the endothelium than intact RBCs, bringing Hb close to the sites of NO synthesis, which may further accentuate NO scavenging after transfusion.³⁴ Although intact saRBCs could also potentially interfere with NO-mediated vasodilation, there is currently little evidence for this mechanism.

In these studies, we used an ex vivo system where endothelium-dependent, NO-mediated vasodilation can be experimentally controlled and sensitively monitored to assess the impact of fresh blood versus saRBCs on vasodilation. This system has been previously employed to probe interactions between RBCs and the vasculature and to demonstrate how RBC-derived ATP and deoxyhemoglobin-catalyzed NO production can stimulate vasodilation.¹⁴ Using this model, we have instead focused on determining whether saRBCs can interfere with normal NO-mediated vasodilatory activities and to what extent the inhibitory effect is mediated by substances, such as Hb, released from saRBCs, or by an alternate mechanism.

MATERIALS AND METHODS

Blood collection, preparation, and sampling

All protocols were approved by the Emory University Institutional Review Board. Recruited research donors were screened by health history questionnaire and vital signs. 500 mL ($\pm 10\%$) was drawn by peripheral venipuncture into CPD-containing blood collection sets (Fenwal, Inc., Lake Zurich, IL). Bag sets were stored at 2 to 6°C for 1 to 3 hours and then leukoreduced using the integral filter according to manufacturer's instructions. Filtered blood was then centrifuged at $4530 \times g$ for 10 minutes at 4°C. Platelet-rich plasma was expressed into a satellite bag and discarded. The residual RBC pellet was mixed with AS-1 and resuspended with gentle agitation. The RBCs were then stored at 2 to 6°C for up to 42

days in a monitored refrigerator. Additional blood samples obtained at the time of collection were used for infectious disease testing.

At selected time points during the storage period, RBC bags were gently but thoroughly agitated to assure homogeneity, and then 10-mL samples were aseptically removed by syringe through a site coupler. Five milliliters of each aliquot was used for aortic ring bioassays. The remaining sample volume was used for biochemical measurements, as described below.

Aorta preparation

All animal studies were completed in compliance with protocols approved by the Atlanta VA Institutional Animal Care and Use Committee. Rats were euthanized and aortas were carefully excised, cleaned of loose fat and connective tissue, and maintained in physiologic saline solution (Krebs-Henseleit buffer: 118 mmol/L NaCl, 4.73 mmol/L KCl, 1.2 mmol/L MgSO₄, 0.025 mmol/L EDTA, 1.2 mmol/L KH₂PO₄, 2.5 mmol/L CaCl₂, 11 mmol/L glucose, and 25 mmol/L NaH₂CO₃, pH 7.4, in 95% to 5% CO₂ at 37°C). Care was taken to ensure that aortas were free of blood. All chemicals were of highest purity and purchased from Sigma (St Louis, MO).

Blood preparation for ex vivo aortic relaxation

For initial studies, unmanipulated blood samples were added directly to the aortic ring baths at the selected concentration. In subsequent studies, plasma and RBC fractions were tested separately for their effects on aortic ring vasoresponsiveness. For example, to model clinical volume reduction approaches, samples were centrifuged at 5000 × *g* for 5 minutes, the supernatant was removed, and then the resulting RBC pellet was added to the organ baths. To model cell washing approaches, after centrifugation the supernatant anticoagulant and preservative was removed and replaced with saline (0.9% NaCl). This washing step was repeated three times before adding the washed RBCs to the aortic rings.

Endothelium-dependent relaxation

Studies examining endothelium-dependent relaxation were performed as we have described previously.³⁵ Briefly, 5-mm segments of thoracic aorta were mounted between stainless-steel wires in an organ chamber containing Krebs-Henseleit buffer and connected to a Harvard apparatus differential capacitor force transducer. For each aorta, resting tension was adjusted to 50 mN over a 1-hour period. After precontraction with 300 nmol/L phenylephrine, a concentration that yields 80% maximum contraction, relaxation was examined after addition of the endothelium-dependent vasorelaxant methacholine (MCh; 0.1 nmol/L to 10 μmol/L) and the endothelium-independent NO-donor sodium nitroprusside (SNP; 0.1 to 300 nmol/L). To determine the effects of whole blood or blood fractions, stored for varying durations, aortas were incubated with whole blood at a final concentration of 1% (or equivalent amounts of separated RBC or plasma) for 30 minutes before the addition of each vasorelaxant. In other studies, MCh was added first to induce vasodilation, followed by the addition of RBCs to determine their effects on relaxed vessels. Data were recorded using digital acquisition and analyzed using software (PowerLab and Chart, respectively, AD Instruments, Colorado Springs, CO). Vasodilation was expressed as a mean percentage

relaxation relative to the precontracted vessel (defined as 0% relaxation) $\pm$ standard error of the mean. Unpaired t tests and Tukey's post hoc analysis tests were used to compare changes in mean relaxation of vessels in response to graded MCh concentrations in the presence of blood stored between 0 and 42 days.

Hb quantitation

Total Hb was measured in aliquots of the RBC samples using a photometer (HemoCue instrument, HemoCue, Inc., Cypress, CA) according to manufacturer's instructions. In addition, plasma free Hb was determined by spectrophotometry. Standards utilized were from cyanmethemoglobin standard set (Count-A-Part, Diagnostic Technology, Inc., Hauppauge, NY). Drabkin's reagent was used to make 1:2 dilutions of the Hb standards (24.0 and 77.4 mg/dL) to generate a standard curve. Samples were centrifuged at $1940 \times g$ for 10 minutes at 4°C, and the plasma supernatant was removed to another tube. The plasma was centrifuged again under the same conditions, and the supernatant was removed to a clean tube and then diluted in Drabkin's reagent and concentrations were calculated in reference to the standard curve. The assay was performed at an absorbance of 540 nm with standards and samples being run simultaneously within the same 96-well plate. Drabkin's reagent was used as a blank to correct for background and subtracted from the resultant value of each well.

2,3-DPG assay and ATP assays

To stabilize 2,3-DPG and ATP in blood samples for subsequent quantitation, perchloric acid extraction was performed. A 1-mL blood sample was added to a tube containing 2 mL of 5% perchloric acid, which was being continuously mixed. The solution was maintained at 4°C for 20 minutes and then centrifuged at 4°C for 10 minutes at $1940 \times g$. The clear supernatant was transferred to a clean tube and centrifuged again. Two milliliters of the resulting supernatant was transferred to a clean tube and, while vigorously mixing, 300 μ L of 3 mol/L K_2CO_3 was added. The resulting precipitate was allowed to settle for 20 minutes at 4°C. The extract was then centrifuged at $1940 \times g$ (4°C) for 10 minutes. The resulting clear, neutralized supernatant was transferred to a clean tube; a 0.3-mL aliquot was set aside at 4°C for 2,3-DPG quantitation and the remaining volume was capped tightly and stored frozen at -80°C in aliquots. The perchloric acid extract was tested for 2,3-DPG and ATP content utilizing commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. 2,3-DPG content of the blood samples was assayed within 24 hours of extraction and the concentration was calculated using correction factors provided by the manufacturer. ATP content was calculated using an internal standard curve.

Analysis of nitrite and nitrate levels in stored blood

Blood samples were collected in distilled water-rinsed centrifuge tubes containing 100 μ L of 100 mmol/L *N*-ethylmaleimide and 5 μ L of 0.5 mmol/L EDTA. Blood samples were then centrifuged to obtain a plasma sample. Plasma samples were flash frozen and stored at -80°C until further analysis. At the time of measurement, plasma samples were thawed on

ice and nitrite and nitrate concentrations were quantified by ion chromatography using an analyzer (ENO20, Eicom USA, San Diego, CA) as previously described.³⁶

RESULTS

RBC units collected, leukoreduced, and stored according to standard clinical protocols were periodically sampled to confirm that they exhibited the expected biochemical changes with storage. Figure 1 shows alterations in plasma Hb (a marker of hemolysis; Fig. 1A), 2,3-DPG (Fig. 1B), and ATP (Fig. 1C) over the 42-day storage period ($n = 8-15$ samples per data point). Hemolysis, while increasing progressively during storage (Fig. 1A), remained low during the entire storage time, with mean plasma Hb levels less than 0.45 g/dL (approx. 1% hemolysis) at all time points. Changes in 2,3-DPG and ATP concentrations were comparable to those seen in previous studies.³⁷⁻⁴⁰ We also quantified plasma nitrate and nitrite during RBC storage (Fig 2). Since nitrite can be enzymatically converted to NO by deoxyhemoglobin,²² changes in nitrite concentrations during storage could alter the ability of transfusions to influence NO-mediated vasodilation. However, although there was a trend toward reduced nitrite levels with longer storage times (Fig. 2A), it did not reach significance ($p > 0.05$); similarly, changes in nitrate levels during storage were not significant (Fig. 2B).

To determine whether fresh versus saRBCs exerted differential effects on NO-mediated endothelium-dependent vasodilation, rat aortic rings were tested in the presence of samples collected at different times from RBC units. Before addition of blood, all rings were confirmed to contract and dilate appropriately in response to phenylephrine and MCh, respectively. RBC samples by themselves (in the absence of MCh) did not stimulate vasorelaxation or vasoconstriction (data not shown).

As shown in Fig. 3A, aortic rings in the presence of fresh unstored blood (Day 0) exhibited up to 60% mean relaxation in response to 10^{-5} mol/L MCh concentrations ($n = 14-19$ separate experiments per data point). However, in the presence of RBCs stored for 42 days, there was no significant relaxation even at the highest MCh concentrations. The magnitude of MCh-stimulated relaxation was significantly different between fresh versus Day 42 blood ($p < 0.001$) at MCh concentrations of 1 μ mol/L and greater. We next investigated in more detail how the duration of blood storage altered the effects of blood on vasodilation. As seen in Fig. 3B, the mean maximal MCh-stimulated relaxation of vessels in the absence of whole blood was $81.6 \pm 6.4\%$, while the addition of unstored blood (Day 0) modestly attenuated maximal relaxation to $56.9 \pm 3.4\%$ ($n = 13-19$). As blood storage time increased, maximal relaxation markedly decreased. Compared to Day 0 blood, blood storage times of 28 to 42 days produced significantly greater inhibition of vasodilation ($p < 0.01$). The maximal relaxation in the presence of Day 42 stored blood was only $11.3 \pm 4.9\%$.

The studies described above showed that stored blood could inhibit subsequent MCh-stimulated endothelial relaxation. To determine if blood could also reverse existing relaxation (cause contraction), blood was added at increasing concentrations to vessels that were precontracted with phenylephrine and then relaxed with 10 μ mol/L MCh (Fig. 3C).

Samples from Day 42 blood more effectively reversed vasorelaxation than fresh blood samples ($p < 0.01$; $n = 6$).

To begin to dissect the vaso-inhibitory effects of saRBC samples, we investigated whether the activity was associated with the supernatant or RBC fractions. Published data suggested the former as degenerating stored RBCs release Hb and microparticles, which can reduce NO bio-availability.³⁴ RBC samples were washed to replace the plasma-buffer supernatant with saline and then added to precontracted vessels. Washing has been shown to remove both soluble substances such as Hb and also microparticles (N. Blumberg, S. Spinelli, and R. Phipps, unpublished observations, 2012). MCh dose-response curves for blood at Days 0 and 42 of storage are shown in Figs. 4A and 4B, respectively ($n = 5-11$). The results for unmanipulated blood (not washed) were similar to those previously shown in Figs. 3A and 3B. Compared to these results, there was no significant effect of washing of RBCs on MCh-stimulated vasodilation. Maximal relaxation was not different between unmanipulated ($57.1 \pm 5.8\%$) and washed ($42.3 \pm 8.7\%$) Day 0 blood (Fig. 4A; $p = 0.178$). Values of maximal relaxation were also similar with Day 42 unmanipulated and washed blood, 16.2 ± 2.1 and $29.3 \pm 4.6\%$, respectively (Fig. 4B; $p = 0.063$). Thus, washing of fresh or Day 42 RBCs does not alter their effects on NO-mediated vasodilation. As an alternative approach to remove the supernatant, which might be less traumatic to RBCs than repetitive washing, we briefly centrifuged additional samples only once and then removed the supernatant before adding the concentrated RBCs to the ring baths (modeling the clinical practice of volume reduction). This approach was likewise ineffective at abrogating the inhibitory effects of Day 42 blood on vasodilation (not shown). To rule out the possibility that washing injured RBCs and the cells then hemolyzed in the organ baths releasing Hb during the assay, we quantified free Hb in the organ baths after the MCh dose-response analysis ($n = 10$). Only one sample showed detectable free Hb in the bath, and it was an unwashed sample (the amount of free Hb was calculated to represent approx. 1% total RBC hemolysis).

As an alternative approach to identify vaso-inhibitory activity in the supernatant fraction, plasma supernatants were removed from blood units on Day 0 or Day 42 and then added to aortic rings before performing MCh dose-response curves (Fig. 4C; $n = 6$). The results were compared against aortic rings to which no supernatant was added. Vasodilation in the presence of Day 0 supernatant (80.5%) was not different from the responses seen without supernatant present (88.5%; $p > 0.05$). Addition of Day 42 supernatant to aortic rings did produce a small decrement in MCh-stimulated vasodilation (63.8% maximal relaxation), which was not different from Day 0 plasma ($p > 0.05$) but was significantly different from the no-supernatant control ($p < 0.01$). This inhibitory activity was quite small, however, compared to Day 42 blood (Figs. 3A-3C) and washed RBCs (Fig. 4B), which completely or strongly blocked MCh-stimulated vasodilation (10%-30% maximal relaxation).

As an additional approach to further probe the role of free Hb or other NO scavengers in vaso-inhibition, we quantified vasodilation in the presence of blood samples and increasing concentrations of the direct NO donor SNP (Fig. 4D). If saRBC samples scavenged NO, by free Hb or other mechanisms, we would expect them to strongly inhibit SNP-initiated vasodilation. Aortic rings were exposed to increasing concentrations of SNP in the presence of 1% saRBC samples. SNP caused profound vasorelaxation, and there was no effect of

blood storage time on vasorelaxation at any SNP dosage: mean maximal relaxations in the presence of Day 0 and Day 42 blood at 5×10^{-6} mol/L SNP were 99.6 ± 4.9 and $93.5 \pm 1.6\%$ ($p = 0.283$), respectively ($n = 4$). These results indicated that the inhibitory effects of stored blood were mostly accounted for by the RBC fraction, and that the saRBC inhibitory effect did not involve NO scavenging.

DISCUSSION

We and others previously proposed a “double-hit” hypothesis to explain how stored RBC units may cause deleterious outcomes in some transfusion recipients, which we termed the insufficient NO bioavailability (INOBA) hypothesis.⁴¹⁻⁴⁴ This hypothesis postulates that both changes occurring in the RBC unit during storage and pathologic processes in the transfusion recipient (e.g., endothelial dysfunction) can independently regulate local NO levels. Together, the sum of these factors determines NO bioavailability, which controls vasodilation and blood flow. When NO bioavailability declines below a critical threshold, local blood flow and O₂ delivery are insufficient to meet tissue demands, resulting in end-organ complications. Recent studies support this double-hit mechanism to explain adverse effects of old blood.^{43,45}

Regarding the role of RBCs in the INOBA hypothesis, a number of studies have suggested that saRBCs transport and/or synthesize lower amounts of NO than fresh RBCs.^{14,17,19,43} However, less work has been done to investigate the alternative mechanism, also consistent with the INOBA hypothesis, that saRBCs actively disrupt normal ongoing vascular NO signaling.³⁴ Such a dominant inhibitory effect would be physiologically significant and could be mediated by relatively small volumes of transfused RBCs. For this reason, we focused on investigating whether transfused saRBCs could disrupt vasoregulation through interference with normal NO signaling.

After addition of fresh RBC samples (Day 0 of storage) to the aortic ring baths, MCh produced marked vasodilation. In comparison, the addition of stored samples led to significant inhibition of MCh-stimulated vasodilation. These findings were consistent with the idea that banked blood is altered during storage in such a way that it markedly interferes with normal NO-mediated vasodilatory activity. The storage changes were progressive: longer periods of RBC storage significantly increased interference with physiologic vasodilatory signals to the point that no significant relaxation occurred in the presence of Day 42 saRBCs. These effects could be produced by very small volumes of saRBCs.

From a mechanistic perspective, saRBC samples could interfere with MCh-stimulated vasodilation at a number of distinct signaling points including MCh interactions with endothelial receptors, endothelial production and release of NO, and postendothelial NO signaling in the smooth muscle. While there is ongoing debate about the clinical effects of low levels of plasma free Hb on vasoreactivity,^{34,46} we initially anticipated that free Hb or other substances released from stored RBCs mediated the inhibitory effects by scavenging postendothelial NO. For example, Donadee and colleagues³⁴ showed that free Hb-containing supernatant from stored human RBCs significantly increased mean arterial blood pressure when infused into anesthetized rats. However, in contrast to those results, our

studies indicated that plasma supernatant constituents, including free Hb and microparticles, were not the only cause of the vasoconstrictive effect in our model. Removal of the supernatant from stored RBCs, either through volume reduction or through washing, did not improve NO-mediated vasodilation. While addition of Day 42 supernatant alone to the aortic rings did inhibit NO-mediated vasodilation, the effect was only significant when compared to aortic rings with no supernatant added; there was no difference in ring vasodilation when Day 42 supernatant was compared to Day 0 supernatant. We also considered the possibility that intact RBCs became fragile with storage, subsequently hemolyzing and releasing Hb into the aortic organ baths. However, we excluded this possibility because we could not detect a consistent increase in free Hb in the bath supernatant. Thus, while free Hb exerts some inhibitory effects on NO signaling in this system, it does not appear to account for the majority of the vasoconstrictive activity seen with stored RBC samples. Rather, the saRBCs themselves appear capable of inhibiting NO-mediated vasodilation, as others have recently suggested.⁴⁷

NO is synthesized in the endothelium by NO synthase and then acts in a paracrine fashion by diffusing into the underlying smooth muscle cells to activate guanylate cyclase resulting in smooth muscle relaxation and vasodilation.^{48,49} Although other factors released by the endothelium are able to either relax (endothelium-derived hyperpolarizing factor) or contract (thromboxane and endothelin) smooth muscle,^{48,49} studies have demonstrated that endothelium-dependent relaxation of aortic segments by cholinergic agonists such as MCh is due solely to production of NO.^{50,51} In conjunction with this accepted mechanism, our results suggest that stored intact RBCs interfere with normal NO signaling between endothelial and smooth muscle cells. The inhibitory effect could theoretically occur at the level of NO production by the endothelium or could interfere with postendothelial signaling in the smooth muscle. To begin to dissect these possibilities, we stimulated endothelium-independent vasodilation in aortic rings using the direct NO donor SNP. In contrast to their effects on MCh-stimulated vasodilation, saRBC samples stored for any duration (Days 0-42) did not inhibit SNP-mediated vasodilation. These results indicate that rather than interfering with postendothelial NO signaling by scavenging NO, saRBCs may instead suppress endothelial NO production, although further work will be necessary to confirm this effect and determine the underlying mechanism.

Our results appear to contradict the study of Donadee and colleagues³⁴ particularly since the levels of free Hb in our stored units (Fig. 1A) are comparable to those seen in that study. However, our results do not specifically argue against RBC-derived free Hb or microparticles causing vasoconstriction in transfusion recipients.^{34,52} While we have seen small vasoconstrictive effects with the RBC supernatant, the present results argue for the existence of additional distinct vasoconstrictive activity that appears to be due to the RBCs themselves: it depends on the presence of RBCs (not found in plasma), it reduces NO synthesis (does not scavenge NO), and it can completely prevent (or reverse) vasodilation produced by a strong pharmacologic stimulus. This last point is significant in that the RBC-derived inhibitory activity appears much more potent than the vasoconstrictive activity of free Hb. The blood samples were diluted 1:100 when added to the ring bath. Thus, in our studies the vasoconstrictive activities were mediated by RBCs at a final "hematocrit" of approximately 0.65%. By comparison, the Hb-containing plasma was present at a 50-fold

higher concentration in the circulation of rats in the study by Donadee and colleagues. Thus the RBC activity is at least manyfold more potent than free Hb-mediated vasoconstriction, the latter of which exerted only a small effect on MCh-stimulated aortic rings (Fig. 4). Consistent with our conclusions, we note that Donadee and colleagues³⁴ focused only on the vasoconstrictive effects of the supernatant fraction and thus did not examine whether stored RBCs could also increase blood pressure. However, Stapley and coworkers⁴⁷ did specifically examine RBCs and found that they also appear capable of interfering with NO signaling.

While the aortic ring system has a number of advantages, as an experimental model it does have limitations. For example, in circulation RBCs normally only have access to the endothelial surface of blood vessels, while in these studies RBCs could potentially interact with subendothelial cells including smooth muscle. In addition, interactions between RBC and endothelium in circulation are controlled by flow mechanics, while those forces do not occur in this model. Nonetheless, this reductionist model is very useful in that it will allow the mechanisms of saRBC vasoconstriction to be dissected in ways that are not possible with more complex systems. Furthermore, we have now seen comparable inhibition of NO-mediated vasodilation with Day 35 to 42 saRBCs (but not Day 3-7 blood) in hospitalized transfusion recipients (manuscript in preparation), supporting many of the conclusions derived from the aortic ring model.

In summary, studies using in vitro aortic ring models have revealed the unexpected finding that saRBCs interrupt the normal vasodilatory responses mediated by endothelial-derived NO. These effects increase in direct relation to RBC storage time, appear to be mediated by RBCs themselves, and may occur through a novel direct effect on endothelial cells that impairs NO production. Results from the studies may lead to better methods of storing RBCs before transfusion, as well as assays to identify RBCs that could cause adverse effects after transfusion.

ABBREVIATIONS

INOBA	insufficient nitric oxide bioavailability
MCh	methacholine
NO	nitric oxide
saRBC(s)	storage-aged red blood cell(s)
SNP	sodium nitroprusside

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Fig. 1.

Alterations in plasma free Hb, 2,3-DPG, and ATP during RBC storage. Serial aliquots, removed from saRBC units every 7 days over 42 days of storage, were assayed as described under Materials and Methods ($n = 8-15$ samples per data point). (A) Compared to Day 0, hemolysis was significantly greater on Days 35 and 42 of storage ($*p < 0.05$). However, mean plasma Hb on Day 42 (0.42 g/dL) represents less than 1% hemolysis (approx. 0.45 g/dL). (B) 2,3-DPG levels declined progressively with storage, as previously documented ($*p < 0.05$ vs. Day 0 sample). (C) ATP levels seen in stored blood were comparable to those previously described in the literature; compared to Day 28, the decrease seen on Day 42 was significant ($*p < 0.05$).

Fig. 2.

Quantitation of plasma nitrite and nitrate during RBC storage. Plasma samples obtained during the storage period were tested by ion chromatography to quantify nitrite (A) and nitrate (B) concentrations (n = 9-15 samples per data point). No significant storage-related changes were observed at any of the time points ($p > 0.05$).

Fig. 3.

Blood storage time correlates with decreased endothelium-dependent vasorelaxation.

Vasorelaxation of rat aortic segments was quantified in response to increasing concentrations of the endothelium-dependent vasorelaxant MCh. (A) MCh stimulated marked vasorelaxation in the presence of Day 0 saRBCs ($\blacktriangle$, 1% final concentration), but not Day 42 saRBCs ($\blacktriangledown$, * $p < 0.001$; $n = 14-19$ experiments per data point). (B) Time course analysis shows that blood stored 28 days or longer significantly inhibits vasodilation compared to Day 0 saRBCs (* $p < 0.01$; $n = 62$ [no blood], $n = 10-19$ [data points with added blood]). (C) Day 42 saRBCs ($\blacksquare$) were more effective than Day 0 samples ($\bullet$) at stimulating vasoconstriction when added to prerelaxed vessels (* $p < 0.01$ for two curves by two-way analysis of variance; $n = 6$ samples per data point).

Fig. 4.

The vasoconstrictive effects of saRBCs are primarily mediated by RBCs, not the supernatant, and do not act by scavenging NO. Day 0 (A) and Day 42 (B) saRBC samples were washed to replace the supernatant with 0.9% NaCl (◆), or left unmanipulated (■), and then added to aortic rings before MCh. Removal of supernatant by washing did not significantly affect the vasoconstriction ($p > 0.05$; $n = 5-11$ samples per data point). (C) Compared to the no supernatant added control (○), addition of Day 0 saRBC supernatant (■, $n = 6$) did not inhibit MCh-stimulated vasodilation ($p > 0.05$; NS). However, Day 42 supernatant (▲) did cause a small but significant inhibition of vasodilation ($*p < 0.01$) at the highest four MCh concentrations when compared to no supernatant, but not when compared to Day 0 supernatant ($p > 0.05$; NS). These results suggest that saRBC supernatant accounts for only a small fraction of the vasoconstrictive activity of unmanipulated saRBC samples. (D) In contrast to the inhibitory activity of saRBCs on endothelium-dependent MCh-stimulated vasodilation, saRBCs had no effect on vasodilation induced by the endothelium-independent NO-donor SNP ($p > 0.05$; $n = 4$). (▲) Day 0; (▼) Day 42.