S-nitrosohemoglobin deficiency: A mechanism for loss of physiological activity in banked blood

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RBCs distribute oxygen to tissues, but, paradoxically, blood transfusion does not always improve oxygen delivery and is associated with ischemic events. We hypothesized that storage of blood would result in loss of NO bioactivity, impairing RBC vasodilation and thus compromising blood flow, and that repleting NO bioactivity would restore RBC function. We report that *S*-nitrosohemoglobin (SNO-Hb) concentrations declined rapidly after storage of fresh venous blood and that hypoxic vasodilation by banked RBCs correlated strongly with the amounts of SNO-Hb ($r^2 = 0.90$; $P < 0.0005$). Renitrosylation of banked blood during storage increased the SNO-Hb content and restored its vasodilatory activity. In addition, canine coronary blood flow was greater during infusion of renitrosylated RBCs than during infusion of *S*-nitrosothiol-depleted RBCs, and this difference in coronary flow was accentuated by hypoxemia ($P < 0.001$). Our findings indicate that NO bioactivity is depleted in banked blood, impairing the vasodilatory response to hypoxia, and they suggest that SNO-Hb repletion may improve transfusion efficacy.

blood preservation | blood transfusion | erythrocytes | hypoxic vasodilation | S-nitrosylation

The first report of a successful human-to-human blood transfusion was published in 1818 (1), but it was the identification in 1901 by Karl Landsteiner of specific human blood groups (2) combined with the use of citrate to prevent coagulation (3) that established blood transfusion as a medical practice. Since that time, administration of RBCs has become the main therapeutic intervention to treat both acute and chronic anemia with an estimated 14 million units of blood transfused yearly in the United States (4). The conceptual strength of the rationale underlying transfusion (i.e., to increase oxygen delivery in patients with low blood oxygen content) is strong, and it has long been recognized that severe anemia poses a significant risk to patient survival and well being (5). Used correctly, transfusion may be lifesaving (6, 7). However, there is a growing appreciation that storage has a negative effect on RBC oxygen delivery (8–10), and emerging evidence suggests that allogenic RBC infusion may actually harm some recipients (5, 11–14). Notably, the Cochrane systematic review of multiple randomized trials found that liberal blood transfusions vs. restrictive strategies is associated with a 20% increase in mortality and a 56% increase in ischemic events (15, 16).

The interval between RBC donation and administration appears to be an independent risk factor for transfusion-associated morbidity and mortality (17). Procured blood undergoes several time-dependent changes including loss of RBC shape and flexibility (18, 19), decreases in the concentration of molecular modulators of oxygen binding such as 2,3-diphosphoglycerate (20), and increases in RBC adhesiveness (21). It has been proposed that the increased affinity for oxygen, combined with alterations in RBC rheology and adhesion, may exacerbate rather than correct ongoing ischemia (and thus account, at least partly, for the adverse effects of blood transfusion). However, it remains to be shown that any biochemical or molecular measure of RBC function is directly correlated with oxygen delivery *in vivo*. Moreover, even fresh processed blood has been observed to decrease tissue oxygenation, an effect that predates many of these storage-related biochemical changes (22). Thus, additional factors likely contribute to the storage-mediated alterations in RBC physiology that underlie the impairment in oxygen delivery.

The principal determinant of oxygen delivery to tissues is blood flow. Within tissues, blood flow is regulated by the O_2 content of blood, which is coupled inversely to vasodilation (23, 24). Thus, hypoxemia results in increases in blood flow and vice versa. In recent years, much progress has been made in understanding the mechanism through which graded changes in blood O_2 content lead to regulated vasodilation or vasconstriction. In particular, it has been established that RBCs mediate a NO-based hypoxic vasodilatory activity (25–27), in which Hb serves as an O_2 sensor and a hypoxia-responsive transducer of NO signals (23, 24, 26, 28–31). Although mechanistic details continue to be debated, it is generally accepted that the S-nitrosylated derivative of Hb (SNO-Hb) is formed *in vivo* and that circulating concentrations are capable of dilating blood vessels (26, 29, 31–34). Furthermore, we and others have described a number of novel reactions [of NO, nitrite, and *S*-nitrosothiols (SNO)] that produce SNO-Hb *in situ* (25, 29, 35–38) and result in release of NO bioactivity under hypoxic conditions (25, 39–44). Vasodilation by SNO-Hb is thus linked to Hb desaturation (29, 31, 32, 39). Collectively, these data provide a mechanistic basis for matching blood flow with metabolic demand in peripheral tissues (24, 29, 31, 33) and for matching ventilation to perfusion within the lungs (30, 32).

Recently, it has been observed that levels of SNO-Hb are altered in several disease states characterized by disorders in tissue oxygenation (40, 44–49). Where examined, RBCs from these patients exhibit impaired vasodilatory capacity (40, 44, 46). These data suggest that RBC-derived NO bioactivity may play an important role in the respiratory cycle and that impairment of this activity might contribute to the pathophysiology of ischemic conditions. Based on these findings, we reasoned that the inability of banked blood to improve oxygen delivery might relate to a deficiency in SNO-Hb. Specifically, we hypothesized that the impaired vasoregulation associated with administration of banked blood (10, 22, 50–52) results, at least partly, from losses in RBC-derived NO bioactivity. As an initial test of this

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Abbreviations: Hb[FeNO], iron nitrosyl Hb; SNO, *S*-nitrosothiol; SNO-Hb, *S*-nitrosohemoglobin.

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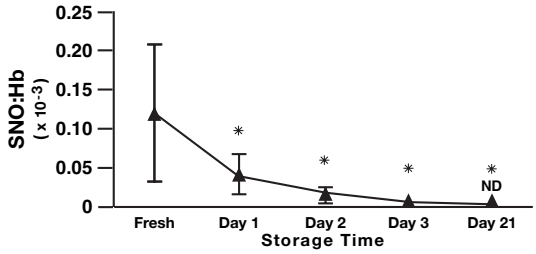

Fig. 1. SNO-Hb in stored human whole blood. Shown is the change in levels over time in human RBCs ($n = 4$) stored in citrate phosphate dextrose/additive solution 1. Packed cells were kept in vacutainers at 4°C and serially sampled by using an aseptic technique at the times indicated. ✳, $P < 0.05$ (significant difference from fresh blood). At day 21, the level of SNO-Hb was below the sensitivity of the assay (ND, not detected).

idea, we screened samples of expired blood (>42 days of storage) and found them to be markedly depleted in SNO-Hb and deficient in hypoxic vasodilatory activity (data not shown). Motivated by these results, we undertook the present study to (*i*) quantitate the effect of storage duration on SNO-Hb content of human packed RBCs prepared and stored under standard American Blood Bank collection conditions, (*ii*) assess the physiological consequences of a potential decline in SNO-Hb concentration, and (*iii*) determine whether SNO-Hb levels and NO bioactivity of banked blood can be revived *ex vivo*.

Results

RBC NO Levels Over Time. In an initial validation of the idea that storage would deplete SNO-Hb, we collected blood from four swine, removed plasma (to yield packed RBCs), and stored them in citrate phosphate dextrose/additive solution 1. After 1 day in storage SNO-Hb levels had declined by 70%, and at 1 week the decline was 83% of the initial levels. Rapid losses in SNO-Hb were also seen in human whole blood stored in vacutainers in citrate phosphate dextrose/additive solution 1 (Fig. 1).

RBC NO and Bioactivity Analyses of Banked Blood. To extrapolate the test tube storage results to the clinical setting, we measured both the RBC NO content and vasodilatory activity (*in vitro* assay) of fresh and leukocyte-depleted banked blood obtained from a commercial supplier [see supporting information (SI) *Text*] (Fig. 2*A*). In addition, we explored the effect of storage on the subcellular distribution (membrane vs. cytosol) and disposition (heme vs. thiol) of NO, which are known to influence RBC vasoactivity (29, 53). Fig. 2*A* shows the levels of SNO-Hb in fresh and banked blood over time as well as the vasodilatory activity of the blood at each time point (cohort A). The mean ratio of SNO to Hb (tetramer) in fresh venous blood was 0.00069 ($n = 10$), consistent with previous reports (40, 44, 54), and fresh RBCs elicited a robust vascular relaxation in organ chamber bioassays (28.5 ± 6.2%). By contrast, SNO-Hb levels in banked blood were reduced by 85–95% at storage days 1, 7, and 43 ($P < 0.009$), and vasodilatory activity of these RBCs was significantly impaired ($P < 0.01$ vs. fresh blood) (Fig. 2*A*). To our surprise, SNO-Hb concentrations were seemingly elevated at days 14 and 21, approaching the levels measured in fresh venous samples (not statistically significant vs. fresh blood), and corresponding RBC bioactivity was also increased at these time points (not statistically significant vs. fresh blood). Overall, total SNO-Hb levels were strongly correlated with RBC vasodilatory activity ($r^2 = 0.90$; $P < 0.0005$), whereas no differences in iron nitrosyl Hb (Hb[FeNO]) concentrations were observed at any time point ($P \geq 0.26$) [consistent with the absence of a direct vasodilatory activity of Hb[FeNO] (53, 55–58)].

SNO-Hb levels are reported to vary substantially among

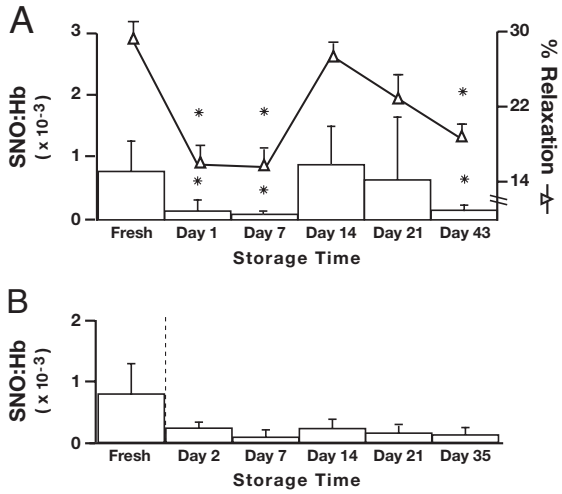

Fig. 2. SNO-Hb and bioactivity of banked RBCs. (*A*) Cohort A: SNO-Hb concentrations ($n = 4$–10), expressed per Hb tetramer, and corresponding vasodilatory activity of RBCs [expressed as changes in tension in rabbit aortic rings (mean ± SD; $n = 11$–22; arc-sine transformed)] for fresh (Day 0) and stored blood. Bars marked with ✳ are time points where SNO-Hb levels were significantly reduced compared with fresh blood ($P < 0.05$). Similarly, vasorelaxations marked with ✳ were significantly less ($P < 0.01$) than those produced by fresh RBCs. Each time point is an independent sampling comprising different individuals. (*B*) Cohort B: Change in SNO-Hb concentration over time from serially sampled blood bags obtained at day 2 ($n = 6$); SNO-Hb is significantly reduced at all time points compared with fresh venous blood. Blood in cohorts A and B was obtained from the same commercial source.

individuals (54, 55), and different individual blood donors constituted the data sets at each time point in Fig. 2*A*. Thus, to determine whether the increases in SNO-Hb at days 14 and 21 represented population variance or a change in cellular physiology [e.g., increased RBC NO synthase activity (59) or perhaps a catalytic action of Hb on nitrite (35, 36), which might accumulate and thereby produce SNO-Hb], a separate cohort of packed cells ($n = 6$ individual donors) was obtained at day 2 of storage from the same commercial source and sampled serially over time (cohort B). SNO-Hb concentrations were, as expected, markedly reduced at day 2 as compared with fresh blood, and levels remained low throughout storage, including days 14 and 21 (Fig. 2*B*). At no time point did the SNO-Hb concentration approach that measured in fresh blood. Furthermore, the amount of SNO in RBC membranes [i.e., a SNO fraction closely identified with vasodilatory activity (29, 53)] was depleted by >90% at days 2–35 compared with historical controls (40) (data not shown). Thus, the variance in SNO-Hb levels seen in cohort A over time is more likely a reflection of a variation in basal SNO levels among different individuals than a real storage phenomenon. We conclude that storage of banked blood rapidly depletes RBCs of bioactive NO, but the data in cohort A emphasize the covariance between SNO-Hb levels and RBC bioactivity, which can vary among individuals. Information on additional blood gases and blood chemistry-related changes during storage can be found in SI Table 1.

Renitrosylation of Blood. Stored RBCs were exposed to a solution of aqueous NO (see *SI Text* for detailed methods) to determine whether SNO-Hb levels could be reconstituted; the effects of renitrosylation are depicted in Fig. 3. At all storage time points, exposure to aqueous NO produced an ≈10-fold increase in both SNO-Hb and Hb[FeNO] (total HbNO) compared with untreated samples. Renitrosylation of stored blood yielded levels of SNO-Hb that were not different from renitrosylated fresh venous blood, with the caveat that after expiration (storage day

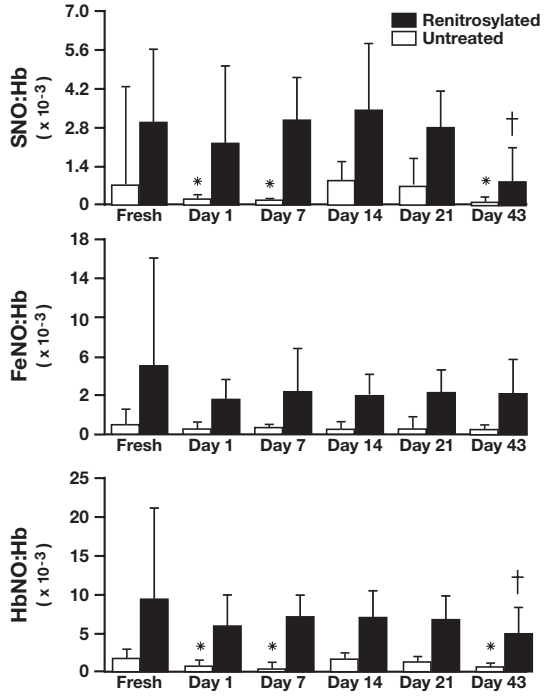

Fig. 3. NO content of human RBCs during storage and after NO repletion. Shown is the effect of renitrosylation on levels of SNO-Hb, Hb[FeNO], and total NO per Hb tetramer (HbNO). At all time points (n = 5–16), renitrosylation, demarcated by the filled bars, significantly increased the levels of both SNO-Hb and Hb[FeNO] vs. untreated cohorts (open bars). ✳, time points where SNO-Hb and HbNO levels in untreated blood were significantly reduced compared with fresh blood ($P < 0.05$); †, the time point (day 43) where SNO-Hb and total NO levels in treated blood remained significantly reduced compared with fresh blood ($P < 0.05$).

43) SNO-Hb levels were not quite as high as other time points, and renitrosylation entailed additional efforts to normalize pH (see *SI Text*). Levels of renitrosylated venous SNO-Hb (1–3 × 10^{-3} per Hb tetramer) were generally equivalent to those reported for fresh arterial blood (54) and were significantly higher than fresh basal venous levels (≈1–5 × 10^{-4} per Hb tetramer) (Figs. 1 and 2).

***In Vitro* Bioactivity of Renitrosylated RBCs.** Fresh and stored RBCs at various time points were assayed for vasodilatory activity in organ chamber bioassays (*in vitro* assay) after exposure to aqueous NO. Fig. 4*A* shows representative relaxation tracings depicting (*i*) the amount of hypoxic vasodilation produced by fresh blood, (*ii*) the relative impairment in vasodilation by stored blood (a decrease in relaxation), and (*iii*) the increase in vasodilatory activity after Hb renitrosylation. Fig. 4*B* shows that renitrosylation of SNO-depleted banked blood (i.e., raising the SNO-Hb level) restored RBC relaxations. Moreover, at all time points, the relaxations produced by renitrosylated stored blood were equivalent in magnitude to those produced by renitrosylated fresh blood.

***In Vivo* Bioactivity of RBCs.** Coronary ischemic events are a principal cause of morbidity and mortality associated with blood transfusions (11–13, 60). Coronary blood flow is an accurate measure of O_2 delivery in the heart and is used routinely to assess ischemic risk in the clinical setting. The *in vivo* assessment of vasodilatory activity by RBCs was therefore measured as real-time changes in canine coronary blood flow in response to infusions of SNO-depleted (stored for 1 day) and renitrosylated human RBCs (Fig. 5) (absolute measures of coronary flow are

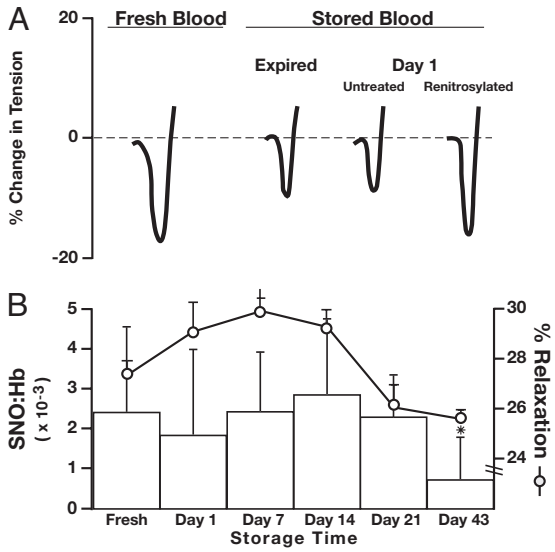

Fig. 4. SNO-Hb content and vasodilatory activity of renitrosylated RBCs. Shown is hypoxic vasodilatory activity of RBCs in bioassays. (*A*) Representative tracings showing the degree of vasorelaxation as percent change in tension in rabbit aortic rings produced by fresh, stored (expired and day 1), or renitrosylated (day 1) RBCs. (*B*) Relaxation data (mean ± SD; n = 11–25) and corresponding SNO-Hb concentrations for renitrosylated fresh and stored RBCs. At all storage time points SNO-reconstituted RBCs exhibited relaxations equivalent to fresh blood.

presented in SI Fig. 6). Under normoxic conditions (21% FiO_2), the infusion of SNO-depleted blood (10–20% residual SNO-Hb) had no effect on coronary blood flow, whereas SNO-replete blood produced a modest but statistically significant increase in flow rate ($P < 0.02$). Under hypoxic conditions (5% FiO_2), infusion of blood increased coronary blood flow; the magnitude of the increase, however, was significantly greater with renitrosylated blood compared with SNO-depleted blood ($P < 0.001$). Moreover, the increase in blood flow elicited by renitrosylated blood was greater under hypoxia than normoxia. Thus, RBCs elicit vasodilation *in vivo* that is potentiated by hypoxia and dependent on SNO bioactivity. In addition, RBCs depleted of SNO-Hb through storage can be renitrosylated to enhance blood flow (i.e., cardiac oxygen delivery), which should, in turn, serve to protect against vasoconstrictor and thrombogenic stimuli.

Discussion

The relationship between the amount of oxygen transported by blood and the amount of oxygen delivered to tissues is not straightforward. Efforts to increase the oxygen content of blood

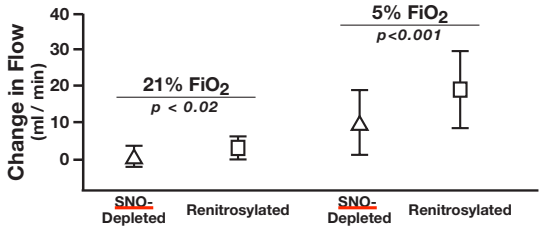

Fig. 5. Hypoxic vasodilation by stored and renitrosylated RBCs *in vivo*. Shown are changes in canine coronary artery blood flow (mean ± SD; n = 7) produced by infusion of SNO-depleted or renitrosylated RBCs. Increases in flow elicited by renitrosylated RBCs were significantly greater than those produced by SNO-depleted RBCs, and the degree of change was greater under hypoxic (5% FiO_2) than normoxic (21% FiO_2) conditions.

often fail to improve oxygen delivery to tissues (61) and, paradoxically, may even make it worse. This problem is exemplified in the administration of packed RBCs, which not only may fail to improve oxygen delivery (5, 22, 62), but has also been associated with an increase in ischemic coronary events (13–15). The disconnect between oxygen content and oxygen delivery underscores the fact that tissue blood flow, rather than blood oxygen content, is the primary determinant of oxygen delivery. In this regard, tissue perfusion is regulated by a physiological response termed hypoxic vasodilation, in which tissue oxygen requirements are directly coupled to blood flow (33, 63). In recent years, important advances toward an understanding of hypoxic vasodilation have been based on the discovery of a central role for RBCs (23, 24, 28, 29, 33, 54, 64, 65), which is supported by physiological studies in humans (23, 24, 28, 44, 54). RBCs react to local changes in tissue oxygenation and adjust NO bioavailability to bring blood flow in line with metabolic demand (26, 29, 33, 65) using Hb as an O_2 sensor and SNO-Hb as a hypoxia-responsive transducer of NO signals (23, 24, 26, 28–30).

It has long been recognized that the affinity of RBCs for oxygen is increased during storage (8, 9) and that this increase can shift the recipient's oxygen dissociation curve to the left, predisposing patients to ischemia (8–10). However, it is difficult to rationalize ischemic events based on small increases in oxygen affinity of a small percentage of the circulating RBC pool contributed by transfused blood, because oxygen availability is rarely limiting *in vivo*. Increases in the affinity of Hb for oxygen are, though, linked directly to increases in the affinity of SNO-Hb for NO (29, 41), and recent studies have reported that increases in Hb O_2 affinity are in fact associated with impaired vasodilation by RBCs (46). Because RBCs traffic through the microcirculation in line, impaired vasodilation by a minor fraction would be expected to adversely influence oxygen delivery. Here we report that storage of blood leads to rapid losses in NO bioactivity, reflected by rapid losses in SNO-Hb, that are precisely paralleled by losses in the ability of RBCs to effect hypoxic vasodilation. We further show that by replenishing SNO-Hb the defect in RBC vasodilation can be corrected, raising the possibility that such intervention might prevent transfusion-associated ischemic morbidity.

Donated blood is stored in an acidic-buffered isotonic solution that contains nutrients and an anticoagulant. In the case of citrate phosphate dextrose/additive solution 1, the pH is ≈6.5, a condition previously shown to accelerate SNO-Hb decay (55). Placement of blood into an acidic mixture may therefore accelerate the decline in RBC function. In a closed oxygen-impermeable environment, a complete loss of SNO-Hb is observed within 21 days (Fig. 1). The environment within a gas-permeable blood bag is not static (66, 67) (SI Table 1), however, and blood from some individuals may retain adequate SNO-Hb to support vasodilatory activity over several weeks [possibly reflecting NO synthase activity in RBCs (59) or a catalytic action of Hb on nitrite (35, 36), which may regenerate SNO-Hb upon oxygenation (25, 29, 35, 44)]. Nonetheless, a significant overall decline in RBC function is noted by day 1 of storage (Fig. 2*A*), and any oxygenation of blood bags during storage (SI Table 1) does not restore SNO-Hb to normal levels (Fig. 2*B*). Moreover, as storage progresses, there is a general deterioration of the erythrocyte's physiologic status, including a loss of the sodium/potassium membrane gradient (SI Table 1), acidification of the extracellular environment (SI Table 1), and an increase in resistance to oxygen transfer across RBC membranes (68). This is accompanied by proteolytic cleavage of band 3 (69), which removes the major site of SNO-Hb interaction with the RBC membrane (53). In sum, conditions within the blood bag during storage are not conducive to maintaining SNO-Hb or RBC hypoxic vasodilatory activity.

Potential benefits of blood transfusions notwithstanding (6, 7), there is currently little doubt as to the inability of stored blood to function like native RBCs: raising hematocrit into the normal range is not advocated, and clinical designs are instead focused on identifying anemic transfusion thresholds that do not produce adverse outcomes (5, 6). The observed losses in RBC SNO that occur with storage are very large relative to the amounts of NO produced *in vivo* (≈1 mmol/day per 70 kg), and thus it is not anticipated that NO-deficient RBCs would rapidly normalize their SNO levels after transfusion. Stored RBCs will therefore act as overall sinks for NO, adversely affecting NO homeostasis *in vivo* and predisposing to vasoconstriction and ischemic insult. In principle, however, raising the hematocrit into the normal range should have beneficial effects if the oxygen delivery function of the transfused blood were preserved. Consistent with this idea, we have demonstrated that replenishing with NO raises SNO-Hb concentrations (Fig. 3) and restores the hypoxic vasodilatory activity of RBCs (Figs. 4 and 5). Moreover, the improvement in coronary blood flow elicited by SNO-Hb repletion was most pronounced during hypoxemia, indicating that vasodilation by RBCs depended on both the oxygen and SNO content of the cells. Thus, our studies not only point to a novel storage defect in RBC that could explain the ischemic consequences of blood transfusions, but they also provide a potential solution to the problem.

In conclusion, we report here newly discovered consequences of storing blood: SNO-Hb levels are depleted during storage, and RBC hypoxic vasodilatory activity is altered (adversely) in parallel with the levels of SNO-Hb. In addition, we show that reconstitution of SNO-Hb can restore RBC vasodilatory activity and improve tissue blood flow. These findings may help to explain why administration of stored blood does not fully correct anemia-associated deficits in tissue oxygen delivery, and they provide preliminary support for a therapeutic intervention to improve transfusion efficacy.

Methods

Details. For details of experimental procedures see *SI Text*.

Banked Blood. Assessments on banked blood before its expiration were done by using packed RBCs purchased from a commercial supplier (Interstate Blood Bank, Memphis, TN). Units of old packed RBCs were obtained from the Duke University Health System Transfusion Services on the day of expiration (storage day 42).

Renitrosylation. RBCs depleted in SNO-Hb were renitrosylated with purified NO solutions (53).

NO-Hb Assays. NO-Hb represents a family of molecules with ranging reactivities (29, 55). Mercury-coupled photolysis–chemiluminescence was used to quantitate NO-Hb levels in fresh and banked human RBCs (55, 70). Mercury-coupled photolysis–chemiluminescence is currently the only method capable of providing quantitative measurements of both SNO-Hbs and FeNO-Hbs (55). [Note that (*i*) the 3C method used in a companion article produces results for SNO-Hb that are qualitatively similar to mercury-coupled photolysis–chemiluminescence; however, 3C does not measure FeNO, and its ability to assay SNO-Hb valency hybrids and membrane-associated NO-Hb is not known; (*ii*) methodology to accurately measure nitrite in RBCs and to differentiate it from SNO or FeNO is not currently available (47, 55).]

RBC Bioactivity. Bioactivity of control and renitrosylated RBCs at the various storage times was measured in organ chamber bioassays at 1% O_2 (54, 71). *In vivo* assessment of infused RBC

vasodilatory activity (coronary blood flow) was made in mongrel dogs. RBCs were depleted of SNO-Hb by overnight storage (up to 24 h) at 4°C.

Statistical Analysis. Detailed explanations of the various statistical methods can be found in *SI Text*. With all analyses, statistical significance was presumed to occur at $P < 0.05$.

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