Supporting Information

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SI Materials and Methods

Blood Procurement, Storage, and Renitrosylation. Rodent whole blood was collected by terminal cardiac puncture of deeply anesthetized mice or rats and then stored in heparin citrate at 4 °C. On the day of study, the RBCs were renitrosylated before transfusion. The stored blood was centrifuged for 3 min at 1,500 × *g* and the buffy-coat removed. Next, the RBCs were suspended in PBS (pH 7.4), gently agitated, and recentrifuged; after removing the supernatant this washing step was repeated. The supernatant was again removed and the RBCs resuspended in PBS to a hematocrit of ∼68% (range 65–72%). All spins were for 3 min at 1,500 × *g*. The mouse RBCs (stored for 1 or 7 d) were reloaded with purified NO (1, 2). These RBCs were transferred to gas-tight vials and deoxygenated with argon. Aqueous NO solution was slowly added to obtain a final NO-to-heme ratio of 1:250 (1–3). The vials were then opened to room air and gently vortexed to achieve rapid oxygenation of the RBCs, thereby inducing S-nitrosylation of Cysβ93 (1). Rat RBCs stored for 7 d were renitrosylated using S-nitrosocysteine (CysNO). The rat cells were incubated with 800 μM CysNO for 10 min at 37 °C before initiating a series of spins and PBS washes to remove excess CysNO and any remaining cellular debris.

Processing of the donated sheep blood followed AABB guidelines (formerly the American Association of Blood Banks); collection and processing was conducted by BioChemed Services. Leukoreduction was accomplished using an in-line filtration system manufactured by Pall Corporation (with the packed RBCs stored in Additive Solution 1). Replicating standard clinical practice, the sheep RBCs were neither washed during storage nor before transfusion. For the required large volume renitrosylation, the packed RBCs were passed over a semipermeable flow-through membrane oxygenator system aerated with the gaseous S-nitrosylating agent ethyl nitrite (4) at 50 ppm in nitrogen (blended by Custom Gas Solutions); for control blood the system was aerated with pure nitrogen. Exposure to ethyl nitrite produced a modest but clinically insignificant rise in metHb levels in the blood exiting the nitrosylating system (from 1.1 ± 0.8% with $n = 15$ bags to 4.9 ± 4.3% with $n = 52$ samples) that did not alter in vivo metHb levels after transfusion.

For all three renitrosylation methods, photolysis-chemiluminescence was used to confirm restoration of RBC S-nitrosothiol (SNO)-hemoglobin (Hb) levels (5, 6). Mean values after renitrosylation ranged between two- and fivefold of the basal storage levels (7, 8): 2.85 ± 0.7 for mice ($n = 4$), 8.7 ± 3.9 for rats ($n = 7$), and 3.5 ± 2.1 for sheep ($n = 13$) expressed as moles SNO-Hb per mole of Hb tetramer × 10^{-3}. Baseline arterial RBC SNO-Hb levels in these species are similar to healthy humans at between 1 and 2 mole of SNO-Hb per mole of Hb × 10^{-3}.

Animal Studies. All of our transfusion paradigms involved the administration of relatively modest amounts of RBCs and three of the studies, conducted under anesthesia, mimicked conditions of intraoperative transfusion where morbidity and mortality (9) may occur after administration of a single unit of blood (10). The fourth study involved administration of blood to conscious animals with extended monitoring, mirroring a nonsurgical setting.

Study 1: Mice. Anesthesia was induced by intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) supplemented with additional injections of ketamine as needed. The tail vein was cannulated and then an Oxylite tissue pO_2 needle probe (Oxford Optronix) was inserted into the hind-limb muscle bed. After probe stabilization and a basal recording interval, 200 μL of packed mouse RBCs [∼10% of the estimated blood volume (11)] were infused into the tail vein. Changes in muscle pO_2 were recorded 60 min after transfusion.

Study 2: Rats. The anesthetic agent for these experiments was ethyl carbamate (urethane; 750 mg/kg, i.p.) to eliminate any potential confounder of ketamine acting upon the NO/cGMP pathway (12). After initiating mechanical ventilation, vascular access of the femoral artery and vein was obtained. Platinum electrodes to monitor muscle pO_2 were inserted into the thigh muscle (13, 14). After a baseline recording session, blood was withdrawn (∼3 mL) until mean arterial pressure (MAP) had declined to 55 mm Hg. This acute anemic state was maintained for 5 min; the animals were then transfused with the human equivalent of one unit of packed rat RBCs (1.4 mL with a hematocrit between 65% and 72%). Rats were monitored for 60 min after the transfusion and then killed; muscle tissue was snap frozen for subsequent determination of energy metabolite levels.

Study 3: Anesthetized Sheep. Moderate anemia was induced by venous blood withdrawal (target Hb of between 8 and 9 g/dL; hematocrit ∼25%); 48 h later these sheep were anesthetized with isoflurane and instrumented for hemodynamic monitoring and blood procurement. In addition to insertion of a Swan-Ganz catheter into the pulmonary artery, both femoral arteries were catheterized. One femoral catheter was advanced into the left ventricle (for subsequent microsphere injections) and the other catheter was positioned in the abdominal aorta for recording hemodynamics and for withdrawal of arterial blood samples. After a baseline recovery period, each sheep received two units of packed RBCs and was then monitored for several hours.

Regional tissue blood flows were quantitated using 15-μm isotope-labeled microspheres (BioPhysics Assay Laboratory). At each experimental time-point the microsphere solution (containing ∼10,000,000 spheres) was sonicated and then quickly injected into the left ventricle followed by a 4.0 mL saline flush. The arterial blood reference sample was withdrawn from the abdominal aorta at a fixed rate of 6.0 mL/min. (Note that the arterial reference samples were withdrawn below the aortic arch so brain blood flow could not be accurately determined.) After the final microsphere injection, the animals were killed and exsanguinated. Blood and tissue samples from the organs of interest were prepared following the manufacturer's guidelines. The analytic method used to quantitate the microspheres (neutron activation) was performed by BioPhysics Assay Laboratory (15). Individual organ blood flows (expressed as $mL \cdot min^{-1} \cdot g^{-1}$) were calculated as $Q_A = (Count_A \times Q_R)/Count_R$ where Q_A is flow per gram of tissue A, $Count_A$ is the number of microspheres in 1 g of tissue A, Q_R is the withdrawal rate of the reference blood sample R in milliliters per minute, and $Count_R$ is the number of microspheres in the reference blood sample R. Utilization of the arterial blood reference samples accounts for any variation in total cardiac output.

In addition to the reference samples, blood was obtained at regular intervals for determination of arterial and venous blood gases and clinical chemistry analyses (Super Chem and CBC conducted by Antech Diagnostic, or at our hospital's clinical chemistry laboratory). GFR was estimated using the following formula: $170 \times [\text{creatinine}]^{-0.999} \times [\text{blood urea nitrogen}]^{-0.170} \times [\text{albumin}]^{+0.318}$, modified from Levey et al. (16).

Study 4: Awake Sheep. Animals were instrumented to secure vascular access under isoflurane anesthesia. After a 48-h recovery period, moderate anemia was induced to the same Hb target as study 3. The transfusion study was conducted the next day where sheep again received two units of packed RBCs and were then monitored overnight. Arterial and venous samples were obtained at regular intervals for determination of blood gases using a GEM 3000 analyzer with an OPL cooximeter (Instrumentation Laboratories). A needle probe (Oxford Optronix) was gently placed into the gluteal muscle of the hip for measuring muscle pO_2.

Data Analyses. Data are presented as means ± SDs except where noted. Standard formulas were used to derive the calculated parameters (systemic vascular resistance, stroke volume, blood O_2 content, and so forth):

Blood oxygen content (CO_2) = (Hb × O_2 saturation × $K_{\text{oxygen capacity}}$) + ($pO_2$ × 0.003). The constant 0.003 is the solubility coefficient for O_2 in blood. The O_2 capacity constant K is 1.36. Arterial-venous O_2 content differences were determined by simple subtraction ($CaO_2 - CvO_2$).

Stroke volume (mL/beat) = cardiac output (CO)/heart rate × 1,000.

Systemic vascular resistance (dynes·s·cm^{-5}) = 80 × (MAP − central venous pressure)/CO.

Pulmonary vascular resistance (dynes·s·cm^{-5}) = 80 × (mean pulmonary arterial pressure − pulmonary capillary wedge pressure)/CO.

Sheep body surface area (BSA; m^2) = weight (kg$^{0.66}$) × 0.121 (17).

- Cardiac index (CI; L·min·m^2) = CO/BSA.
- Systemic O_2 delivery (Do_2; mL/min) = CI × CaO_2.
- O_2 consumption (Vo_2; mL/min) = CI × ($CaO_2 - CvO_2$) × 10.
- O_2 extraction (%) = (Vo_2/Do_2) × 100.

For continuously recorded data, median values were calculated at 30-min intervals and averaged to obtain group data. For normally-distributed data, ANOVA followed by post hoc testing was used to determine within-group differences over time and differences between the experimental groups. Differences in discrete variables were tested for using t tests. Where appropriate, testing was conducted using the equivalent nonparametric test (Kruskal–Wallis, Mann–Whitney). Results were considered significant at $P < 0.05$.

SI Results

SNO Levels in Cysβ93 Genetically Modified Mouse. A report that RBC SNO-Hb was not required to effect hypoxic vasodilation was based on the proposal that mice with humanized Hb with alanine substituted for cysteine at β93 precluded Hb S-nitrosylation and "shifted S-nitrosothiol distribution to lower molecular weight species consistent with the loss of SNO-Hb" (18). However, in our initial assessments of these mice, kindly provided by the authors, we have been unable to find differences in SNO-Hb levels (Fig. S1), consistent with the introduction and expression of additional Hb genes. RBC SNO-Hb levels in the mutant control mice ($n = 5$) were 1.56 ± 0.41 compared with 1.91 ± 0.80 moles SNO-Hb per mole of Hb tetramer × 10^{-3} in the βC93A ($n = 4$; $P = 0.69$) and the percentages of SNO-Hb/Total HbNO (FeNO + SNO) were also similar (51.3 ± 12.4% vs. 62.9 ± 19.6% for the mutant control and βC93A, respectively; $P = 0.62$).

Extended Monitoring. The extended nature of the sheep studies provides some guidance for the design of new clinical studies of blood transfusion. Previous reports of transfusion having marginal impact on measures of organ function or oxygenation have uniformly focused on the period during or immediately following RBC administration (19–21). This timing makes it difficult to parse out the acute benefits of fluid-loading from subsequent RBC effects (adverse or not). Studies 3 and 4 are cases in point: immediate changes in sheep physiologic parameters were indistinguishable between untreated or renitrosylated blood; only during continued monitoring were differences identified. Similarly, some responses to transfusion [e.g., increases in pulmonary arterial pressure (22)] resolved relatively quickly and are thus unlikely to explain persistent decrements in oxygenation or later risk of organ injury and adverse outcome.

Additional Hemodynamic Monitoring in Anesthetized Sheep (Study 3). The time courses of selected hemodynamic parameters are presented in Fig. S2 to complement the data presented in Fig. 3.

Sheep Organ Blood Flows (Study 3). Abdominal organ (liver, spleen, adrenal) blood flow values are presented in Table S1. Each organ demonstrated an initial rise in flow following transfusion of two units of packed ovine RBCs, but there were no differences between the responses to untreated or renitrosylated blood. Heart blood flow (averaged as the flow to the right and left ventricles) was very variable and is therefore not included. This finding is attributed to the measurement technique. The catheter placed within the left ventricle that is used for the microsphere injection is not secured so it can "sway" in response to the blood flowing by it. As a result, at the time of microsphere administration, the catheter tip can be close to the coronary arteries, which will prevent a complete mixing of the spheres with the blood within the ventricle before distribution into the coronary microvasculature.

In sum, unlike the kidneys, there were no liver, adrenal, or spleen blood flow differences in response to untreated or renitrosylated blood. It remains to be determined if flow differences would become apparent under different anemic/transfusion conditions (e.g., trauma-induced acute anemia).

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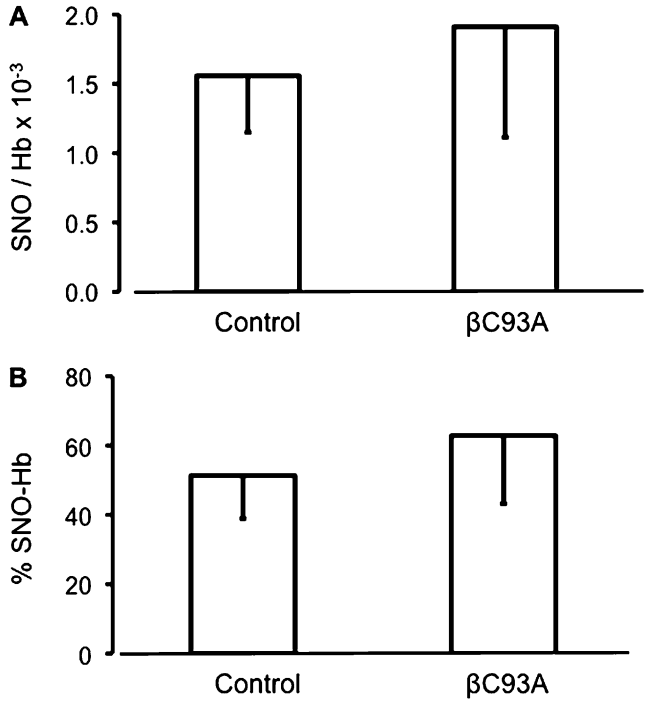

Fig. S1. SNO levels in mutant control and βCys93Ala mutant mice. (*A*) Mean RBC levels of S-nitrosylated Hb (±SEM) in mutant control ($n = 5$) and βC93A mutant mice ($n = 4$) expressed as SNO-Hb per mole of Hb tetramer $\times 10^{-3}$. (*B*) Mean SNO-Hb levels (±SEM) expressed as percent of total HbNO (SNO + FeNO) in RBCs from control and β93 mutant mice. Neither the amount nor the percentage of SNO-Hb were different between the two types of mice ($P = 0.69$ and $P = 0.62$, respectively).

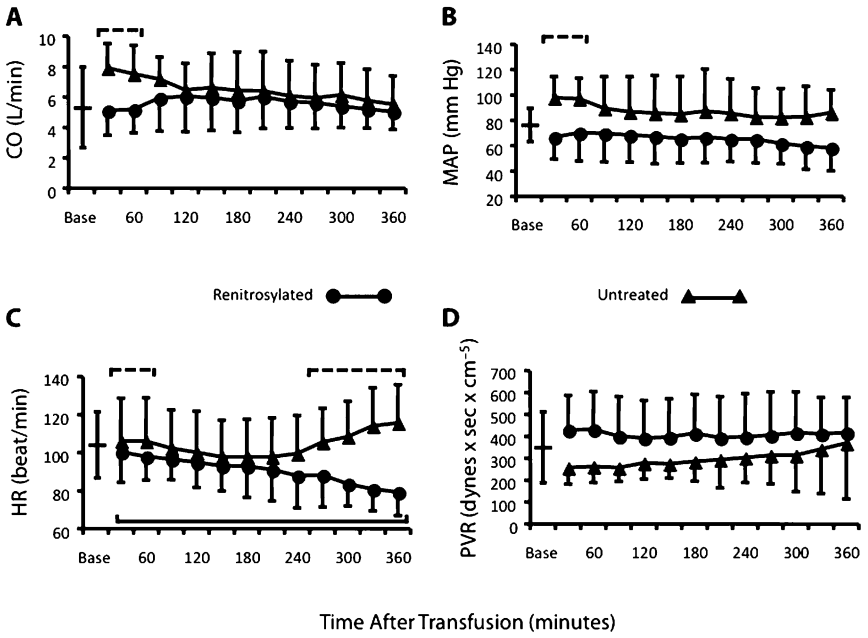

Fig. S2. Additional sheep hemodynamic responses to intraoperative transfusion. Mean time courses (±SD) for various cardiovascular parameters following receipt of two units of untreated (▲) or renitrosylated (●) packed RBCs ($n = 7$). Group mean (±SD) baseline values are demarcated by the freestanding bar in each panel. Within-group datapoints that are significantly different ($P < 0.05$) from their respective baseline values are enclosed in the dashed (untreated) or solid (renitrosylated) brackets. (*A*) Cardiac output (CO) initially rose in the untreated group but returned to baseline levels within 90 min after the end of the transfusion; administration of treated RBCs did not alter CO. (*B*) MAP in the untreated cohort initially rose then returned to baseline, but did not change in the treated group. (*C*) Heart rate (HR) exhibited opposing responses, rising in the untreated cohort and gradually declining in the sheep that received renitrosylated RBCs. (*D*) Pulmonary vascular resistance (PVR) exhibited considerable variability but no values were significantly different from baseline during the posttransfusion monitoring period.

Table S1. Organ blood flows in anesthetized sheep after transfusion

Organ	Base ($mL \cdot min^{-1} \cdot g^{-1}$)	Untreated (% base)		Renitrosylated (% of base)	
		T0	T4	T0	T4
Liver	0.40 ± 0.27	146 ± 58	70 ± 53	165 ± 62	82 ± 34
Spleen	0.41 ± 0.24	142 ± 67	143 ± 62	131 ± 53	147 ± 85
Adrenals	1.65 ± 0.89	206 ± 171	114 ± 70	121 ± 67	113 ± 12

Organ blood flows at baseline (Base), at the end of RBC transfusion (T0), and 4 h after the transfusion ended (T4).