ORIGINAL RESEARCH COMMUNICATION

Peroxiredoxin-2 Recycling Is Inhibited During Erythrocyte Storage

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Abstract

Aims: Transfusion with stored red blood cells (RBCs) is associated with increased morbidity and mortality. Peroxiredoxin-2 (Prx-2) is a primary RBC antioxidant that limits hydrogen peroxide (H₂O₂)-mediated toxicity. Whether Prx-2 activity is altered during RBC storage is not known. **Results:** Basal and H₂O₂-induced Prx-2 activity was measured in RBCs (stored for 7–35 days). Basal Prx-2 thiol oxidation increased with RBC age, whereas H₂O₂-dependent formation of dimeric Prx-2 was similar. However, reduction of Prx-2 dimers to monomers became progressively slower with RBC storage, which was associated with increased H₂O₂-induced hemolysis. Surprisingly, no change in the NADPH-dependent thioredoxin (Trx)/Trx-reductase system, which recycles dimeric Prx-2, was observed in stored RBCs. Using mouse RBCs expressing human wild type (β 93Cys) or hemoglobin (Hb) in which the conserved β 93Cys residue is replaced by Ala (β 93Ala), a role for this thiol in modulating Prx-2 reduction was demonstrated. Specifically, Prx-2 recycling was blunted in β 93Ala RBC, which was reversed by carbon monoxide-treatment, suggesting that heme autooxidation-derived H₂O₂ maintains Prx-2 in the oxidized form in these cells. Moreover, assessment of the oxidative state of the β 93Cys in RBCs during storage showed that while it remained reduced on intraerythrocytic Hb in stored RBC, it was oxidized to dehydroalanine on hemolyzed or extracellular Hb. **Innovation:** A novel mechanism for regulated Prx-2 activity in RBC *via* the β 93Cys residue is suggested. **Conclusion:** These data highlight the potential for slower Prx-2 recycling and β 93Cys oxidation in modulating storage-dependent damage of RBCs and in mediating post-transfusion toxicity. *Antioxid. Redox Signal.* 22, 294–307.

Introduction

TRANSFUSION WITH PACKED RED BLOOD CELL (pRBC) stored for up to 42 days in the blood bank is a currently approved therapeutic strategy to improve tissue oxygenation in multiple clinical settings. However, recent retrospective analyses have highlighted the potential for increased morbidity and mortality associated with transfusion of RBC stored for longer *versus* shorter periods of time (3, 25, 36, 56, 58, 61, 63). This perspective has been supported by preclinical studies demonstrating adverse effects of stored blood in providing a “second” hit that promotes toxicity compounded by the patient’s underlying disease. Proposed mechanisms that constitute the “second” hit include oxidative stress, inhibition

of nitric oxide (NO) signaling, iron overload and related toxicity, immune cell activation, and exacerbation of inflammation (2, 4, 8, 15, 27, 42, 48, 52–54, 64).

Storage causes multiple changes in the RBC, including oxidative damage, loss of ATP, and loss of membrane and volume with an associated transition from biconcave discs to echinocytes and less deformable cells (9, 20, 22, 48, 50, 56, 60). Recent studies are beginning to link these changes with mechanisms that lead to post-transfusion toxicity. For example, alterations in RBC shape, formation of microparticles, and hemolysis accelerate NO scavenging and promote oxidative stress; loss of chemokine binding capacity can promote inflammation; and released free iron can predispose transfusion recipients to infection (2, 4, 15, 27, 42, 54). This

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Innovation

Red blood cell (RBC) hemolysis during storage is associated with adverse effects of transfusion with stored RBC. Mechanisms leading to RBC hemolysis during storage are not known. We show that peroxiredoxin-2 (Prx-2) recycling, a key element in its antioxidant activity, is compromised in stored RBC. We also show that the conserved β 93Cys residue of hemoglobin is a novel regulator of Prx-2 recycling by controlling heme auto-oxidation, and that oxidation of this thiol to dehydroalanine during storage may lead to increased RBC hemolysis. These data provide novel mechanistic insights into how hemolysis occurs in stored RBCs and suggest that Prx-2 recycling and the β 93Cys residue are novel targets to modulate this process.

understanding has fuelled interest in preventing biochemical and morphological alterations in the RBC during storage to avoid post-transfusion toxicity. A potentially common mechanism in both these contexts is increased oxidative stress. Storage is associated with oxidative damage to the RBC indexed by accumulation of oxidized products and/or loss of endogenous antioxidants (20, 49, 50). Specific oxidative stress-derived biomarkers may help identify RBCs that are more susceptible to hemolysis during storage and/or cause injury in the transfusion recipient (20, 49). Moreover, prevention of morphologic and biochemical changes in RBCs stored under anaerobic conditions indicates a causative role of oxidative stress in the storage lesion (18, 62). Finally, RBCs are now appreciated as active participants in modulating redox homeostasis *in vivo* by both promoting (*via* hemoglobin [Hb] redox cycling-derived reactive species) and inhibiting (by detoxifying reactive species *via* erythrocytic antioxidant systems) oxidative injury in the systemic and pulmonary compartments (1, 6, 13, 19, 24, 31, 34, 46, 47, 51, 55, 57). Storage appears to perturb this balance, resulting in more pro-oxidative RBCs.

RBCs are endowed with multiple antioxidant systems that limit peroxide (hydrogen peroxide [H_2O_2], lipid hydroperoxide) and peroxynitrite-dependent effects. Peroxiredoxin-2 (Prx-2) has emerged as the critical antioxidant protecting RBCs from H_2O_2 produced endogenously (by Hb autooxidation and subsequent superoxide dismutation) and exogenously (*e.g.*, from activated neutrophils) at low (physiologic) concentrations (11, 32, 38, 40, 43, 45) and, therefore, may limit oxidative injury to other cells/tissues in the vasculature (6, 57). Prx-2 is a 2-Cys-Prx homodimer, by which the peroxidatic cysteine on one subunit is oxidized to a sulfenic acid by H_2O_2 (a two-electron oxidation). The second or resolving Cys on the other subunit reacts with the sulfenic acid to form a disulfide bridge to complete the reaction cycle (40). At higher fluxes of H_2O_2 , the peroxidatic Cys may undergo further oxidation to sulfinic or sulfonic acid, which relies on sulfiredoxin for reduction (11). Under normal conditions, formation of disulfide-linked Prx-2 dimer is relatively rapid and is followed by reduction back to the monomer by the thioredoxin (Trx)–Trx reductase system, which is fuelled by NADPH (40). The reduction of oxidized Prx-2 is relatively slow compared with the initial oxidation, taking several minutes. While the slow recycling could limit the antioxidant efficacy of Prx-2, since this protein is present at relatively

high concentrations (~ 200 – $300 \mu M$ in pRBC) being the third most abundant protein in the RBC, its capacity to scavenge H_2O_2 remains high (11, 40). Thus, this noncatalytic, but high-capacity pathway has been suggested to limit oxidative damage to RBCs under physiological conditions.

Relatively, little is known on the role of Prx-2 in storage-dependent damage to RBCs nor whether its activity may be regulated by other factors beyond Trx-dependent reduction. Previous reports have documented the formation of oligomeric Prx-2 complexes, and shown increased association of Prx-2 with the RBC membrane, during storage (49, 50). However, whether Prx-2 activity is altered by storage is not known. This is important, as the slow reduction of Prx-2 becomes limiting under conditions of high H_2O_2 flux, as expected during inflammatory stress in transfusion recipients. Here, we show that reduction of disulfide-linked Prx-2 dimer is significantly inhibited in RBCs stored for > 14 days, which occurs without changes in Trx reductase activity. We show an unexpected role for the β 93Cys residue of Hb in affecting storage-dependent changes in Prx-2 recycling and, provide evidence for a novel function for this conserved RBC thiol as well as new insights into how RBC Prx-2 activity is regulated. These data are discussed in the context of a functional assay to assess RBC storage and potential mechanisms for post-transfusion toxicity.

Results

Prx-2 oxidation and recycling during RBC storage

Figure 1A shows that basal Prx-2 oxidation by disulfide formation increased in RBCs stored for 7 or 35 days compared with freshly isolated cells. Notably, the variation in basal Prx-2 oxidation increased proportionally with storage age. An important aspect of Prx-2 activity is to metabolize peroxides and then be recycled back to the reduced form. H_2O_2 -dependent formation of dimeric Prx-2 (oxidized Prx-2) in RBCs can be measured by Western blotting under nonreducing conditions. RBCs of different storage ages were exposed to H_2O_2 , and time-dependent changes in Prx-2 oxidation were measured. Figure 1B presents these data and shows that (i) basal (time 0) Prx-2 oxidation was higher at day 35 relative to day 14 and 28 ($p < 0.05$ by one-way analysis of variance (ANOVA) with Tukey *post hoc* test); (ii) H_2O_2 significantly increased Prx-2 oxidation, which was maximal at the first time point (5 min) measured; (iii) the magnitude of the maximal level of Prx-2 oxidation increased with RBC storage age ($p < 0.05$ by one-way ANOVA with Tukey *post hoc* test for day 7 *vs.* 35 for 5 min data); and (iv) dimeric Prx-2 was slowly reduced back to the monomer over 60 min; however, this was not observed with day 35 RBC, where Prx-2 remained $> 75\%$ oxidized. To determine how RBC storage affected the rate of Prx-2 reduction, Figure 1C replots these data after normalizing to the maximal Prx-2 oxidation. Interestingly, reduction was linear during the first 30 min, with no further reduction occurring thereafter. Linear regression analysis over 30 min showed that the rate of Prx-2 reduction progressively slowed by 30% and 70% for day 28 and 35 RBC, respectively, with no differences with RBC day 14 or younger. Finally, slower Prx-2 reduction correlated with increased H_2O_2 ($10 \mu M$)-induced hemolysis of day 35 RBC compared with day 7 RBC (Fig. 1D). Figure 1E shows that H_2O_2 is rapidly consumed (< 5 min) after addition to RBC,

FIG. 1. Effect of storage time on Prx-2 oxidation and recycling kinetics. (A) Basal Prx-2 oxidation in RBC freshly isolated or after storage for 7 or 35 days. Each symbol represents an RBC preparation from a distinct donor. p -Value shown determined by one-way ANOVA (nonparametric assuming non-Gaussian distribution). (B) H_2O_2 ($10 \mu M$) was added to human RBCs stored for different times (7–35 days) in isotonic saline buffer (pH 7.4) and Prx-2 oxidation measured at 5, 15, 30, or 60 min as described in Materials and Methods section. Representative Western blots and quantitation of Prx-2 oxidation expressed as the fraction of the Prx-2 dimer relative to total Prx-2 are shown. Data are mean $\pm$ SEM ($n = 6$). * $p < 0.001$ compared with respective basal (time 0) by one-way RM-ANOVA with Tukey's *post hoc* test. (C) Time-dependent changes in Prx-2 relative to the maximal amount formed (at 5 min). Solid lines show linear regression fits ($p < 0.001$ for all slopes being not zero) for data between 5 and 30 min and were $y = -0.021x + 1.09$ (day 7, ■); $y = -0.02x + 1.09$ (day 14, ○); $y = -0.014x + 1.07$ (day 28, △); $y = -0.0064x + 1.04$ (day 35, ▼). Slopes were significantly different between day 14 and 28 ($p < 0.01$), between day 7 and 35 ($p < 0.001$), and between day 14 and 35 ($p < 0.0001$). (D) Hemolysis induced by addition of H_2O_2 ($10 \mu M$) to day 7 or 35 RBCs (after 60 min in isotonic saline buffer (pH 7.4)). Each data point represents a distinct RBC preparation. * $p < 0.02$ by t -test. (E) H_2O_2 ($10 \mu M$) was added to phosphate-buffered saline (■), day 7 RBC (▼), or day 35 RBC (○) and H_2O_2 concentrations were measured at indicated times. Data shown are mean $\pm$ SEM ($n = 3-4$). No differences between day 7 and 35 RBC-dependent consumption of H_2O_2 were observed. H_2O_2 , hydrogen peroxide; Prx-2, peroxiredoxin-2; RBC, red blood cell; RM-ANOVA, repeated measures-analysis of variance.

with no differences between young and old RBC, suggesting that slower reduction of oxidized Prx-2 observed with day 35 RBC (Fig. 1C) is not due to slower metabolism of added H_2O_2 .

Effects of RBC storage on Trx reductase activity

The Trx and NADPH-dependent Trx reductase system is rate limiting for reduction of oxidized Prx-2 in RBC. However, no differences in basal Trx-reductase activities, Trx protein levels, or NADPH levels were observed in day 7 versus 35 RBC (Fig. 2), suggesting that this was not the basis of differential Prx-2 reduction kinetics.

Effects of glucose on Prx-2 cycling in stored RBC

Since glucose levels decline during RBC storage (28), which could limit NADPH recycling, we next tested whether

addition of glucose could rescue the defect in reduction of oxidized Prx-2 after H_2O_2 addition. Figure 3 shows time-dependent changes in Prx-2 oxidation normalized to maximal (at 5 min) after addition of H_2O_2 . Inclusion of glucose increased the rate of Prx-2 reduction for both day 7 and 35 RBC compared with the reaction without glucose (compare to Fig. 1). For example, ~85% reduction back to monomer was observed by 15 min for day 7 RBC compared with only ~25% for day 7 RBC without glucose (Fig. 1). However, while glucose accelerated Prx-2 reduction for day 7 and 35 RBC, the latter was still slower compared with the former (Fig. 3), indicating that the inhibited rates of Prx-2 recycling in older RBC were not due to differences in glucose concentrations.

Effect of $\beta 93Cys$ on Prx-2 recycling in stored RBC

Our recent studies showed that H_2O_2 -dependent oxidation of Prx-2 was greater in RBC expressing human Hb in which

FIG. 2. Effect of storage time on Trx reductase system in RBC. Thioredoxin reductase activity (A), thioredoxin levels (B), and NADPH levels (C) were measured in RBCs stored for 7 or 35 days as described in Materials and Methods section. Data are mean $\pm$ SEM [$n=8-9$ for (A), and $n=3$ for (B, C)]. In all cases, differences were not significant by unpaired *t*-test. Trx, thioredoxin.

the $\beta 93$ Cys residue was replaced with Ala (57), suggesting a role for this conserved residue in erythrocyte redox homeostasis mechanisms. We, therefore, compared storage-dependent changes in Prx-2 oxidation and reduction in murine RBC expressing wild-type human Hb ($\beta 93$ Cys) or human Hb in which an Ala residue has been substituted in the $\beta 93$ position ($\beta 93$ Ala). Our previous studies have confirmed the loss of Hb $\beta 93$ thiol in $\beta 93$ Ala RBC without any compensatory changes in other Hb subunits or RBC thiols (30, 39, 57). RBCs were leukoreduced and stored for 1 or 10 days (a time frame that mimics storage of human RBCs for approximately 42 days) (7, 23, 42), and H_2O_2 -dependent Prx-2 oxidation was measured. Figure 4A shows that there was no difference in basal Prx-2 oxidation between $\beta 93$ Cys and $\beta 93$ Ala RBCs consistent with our earlier study (57). H_2O_2 oxidized Prx-2 with kinetics similar to that observed in human RBCs. Maximal levels of oxidized Prx-2 were observed at 5 min followed by a time-dependent reduction to the monomer, which was linear over 20 min. The change in maximal increase in Prx-2 oxidation (*i.e.*, 5 min Prx-2 dimer fraction minus basal) was greater for 10 days compared with 1 day for both RBC.

FIG. 3. Effect of storage time on Prx-2 recovery after H_2O_2 exposure with added glucose. Human RBCs stored for 7 ($\square$) or 35 days ($\blacktriangledown$) were exposed to $10 \mu M H_2O_2$ for 5, 15, 30, or 60 min in the presence of glucose (5 mM) and Prx-2 oxidation was measured as described in Materials and Methods section. Data show values normalized to maximal levels of oxidized Prx-2 (at 5 min) and are mean $\pm$ SEM ($n=3$). * $p<0.05$ and # $p<0.01$ by two-way ANOVA with Bonferroni *post hoc* test.

Figure 4B plots the time-dependent reduction of dimeric Prx-2 normalized to maximum (5 min). Linear regression analysis showed that reduction was linear over 20 min, and Prx-2 reduction was significantly slower in 10 days RBC compared with the respective 1 day RBC (by ~ 10 -fold) for $\beta 93$ Cys RBC. However, no storage-dependent effect on Prx-2 reduction rates were observed with $\beta 93$ Ala RBC. Notably, Prx-2 reduction was significantly slower (approximately twofold) in $\beta 93$ Ala RBC compared with $\beta 93$ Cys stored for only 1 day. Figure 4C shows $\sim 90\%$ of added H_2O_2 was rapidly consumed (< 2 min) by all RBC groups. However, a slight but significant increase in steady-state H_2O_2 levels after the initial consumption was observed with $\beta 93$ Ala RBC stored for 10 days compared with $\beta 93$ Ala RBC stored for 1 day; no differences between $\beta 93$ Cys RBC stored for 1 or 10 days were observed.

Carbon monoxide accelerates Prx-2 recycling

In the next series of experiments, the effects of carbon monoxide (CO) added before H_2O_2 or 5 min after H_2O_2 (*i.e.*, after attaining maximum Prx-2 oxidation) on Prx-2 oxidation and reduction kinetics were tested. No differences in H_2O_2 -dependent oxidation of Prx-2 with or without CO treatment in either cell were observed (not shown). Figure 5A and B show the effects of CO on the rate of Prx-2 reduction (after initial oxidation) in $\beta 93$ Cys and $\beta 93$ Ala RBC, respectively. Pre-saturation of RBC with CO affected neither initial H_2O_2 -dependent Prx-2 oxidation nor Prx-2 reduction in either RBC, although a trend toward decreased Prx-2 oxidation over 5 min was noted in CO-treated cells ($p=0.08$ between with and without CO groups for $\beta 93$ Cys RBC). Notably, however, when administered 5 min post H_2O_2 , CO significantly increased Prx-2 reduction rates in $\beta 93$ Ala RBC (Fig. 5B), with no effect of CO observed in $\beta 93$ Cys RBC (Fig. 5A). Figure 5C shows that pretreatment of human RBC stored for 35 days with CO, or CO-added 5 min after H_2O_2 , significantly accelerated Prx-2 reduction from the dimer to monomer (by $\sim 35\%$ relative to control at 20 min).

Oxidation of $\beta 93$ Cys during storage

RBC storage is associated with oxidative stress characterized in part by glutathione and protein thiol oxidation (16, 20, 33). Whether the $\beta 93$ Cys residue is a target for oxidation during storage is not known. To evaluate this possibility, BODIPY N-ethylmaleimide (BD-NEM) labeling experiments

FIG. 4. Effect of storage time and β 93Cys on Prx-2 recovery after H_2O_2 exposure. RBCs from transgenic mice expressing human Hb with β 93Cys or β 93Ala were leukoreduced and stored as described in Materials and Methods section. After 1 or 10 days of storage, H_2O_2 ($10 \mu M$) was added and time-dependent (0–30 min) changes in Prx-2 oxidation were determined. **(A)** Prx-2 oxidation expressed as the fraction of the Prx-2 dimer relative to total Prx-2. Data are mean $\pm$ SEM ($n=4$). * $p<0.01$ # $p<0.05$ compared with respective basal (time 0) by one-way RM-ANOVA with Tukey's *post hoc* test. ** $p<0.05$ by *t*-test for comparison of maximal levels of Prx-2 oxidation at day 1 versus 10. **(B)** Time-dependent changes in Prx-2 relative to the maximal amount formed (at 5 min) with lines showing fits by linear regression. * $p<0.01$ between day 1 β 93Cys and day 10 β 93Cys RBC, # $p<0.05$ between day 1 β 93Cys and day 1 β 93Ala. **(C)** H_2O_2 concentrations (normalized to initial) after addition to β 93Cys or β 93Ala RBC stored for either 1 or 10 days. * $p<0.05$ relative to β 93Ala RBC stored for 1 day by two-way RM-ANOVA with Bonferroni *post hoc* test ($n=3-5$). Hb, hemoglobin.

were performed. Freshly isolated RBCs or cells stored for 7 or 35 days were collected and separated from the supernatant (SN) by centrifugation. Pelleted RBCs were then hemolysed by hypotonic lysis. BD-NEM was then added to the initial SN and hypotonically lysed RBC to label available cysteine residues. Each fraction was resolved by Western blotting, and degree of BD-NEM binding was determined and normalized to the Hb β -chain. Figure 6 shows that storage did not affect the relative binding of BD-NEM to erythrocytic Hb. However, BD-NEM binding to cell-free Hb arising from hemolysis during storage (*i.e.*, SN fraction), was significantly decreased by $\sim 50-60\%$ compared with Hb present inside the RBC. It should be noted that hemolysis was higher at 35 days indicated by higher levels of β -chain in day 35 SN. However, the relative loss of β 93Cys reactivity to BD-NEM in SN Hb was similar between samples collected from RBCs stored for 7 or 35 days (Fig.

6A). To assess whether the β 93Cys modulated storage-dependent RBC damage, hemolysis and formation of microparticles was compared in β 93Cys and β 93Ala RBC. Figure 6B shows that hemolysis increased after storage of either RBC, but the degree of hemolysis was significantly higher with β 93Ala RBC, suggesting that this cysteine protects the RBC during storage. Figure 6C shows that storage also resulted in increased microparticle levels; however, no difference between the magnitude of this increase between β 93Cys and β 93Ala RBC was observed. Finally, to assess the fate of β 93C on SN Hb LC-MS analyses of trypsin digested peptides were performed. Surprisingly, no evidence for formation of disulfides, sulfinic or sulfonic acids was observed. Figure 7 shows that, in fact, the β 93Cys was oxidized to dehydroalanine (DHA) in 35 days SN Hb compared with 7 days SN Hb. No evidence of other thiol modifications was observed.

FIG. 5. Effects of CO on Prx-2 recycling in $\beta 93\text{Cys}$ and $\beta 93\text{Ala}$ RBC. RBCs (stored for 1 day) were untreated, saturated with CO before, or 5 min after addition of H_2O_2 ($10\ \mu\text{M}$), and time-dependent (0–30 min) changes in Prx-2 oxidation were determined as described in Materials and Methods section. (A, B) Time-dependent changes in Prx-2 relative to the maximal amount formed (at 5 min) for $\beta 93\text{Cys}$ and $\beta 93\text{Ala}$ RBC, respectively. Data are mean $\pm$ SEM ($n = 3$). * $p < 0.05$ by two-way RM-ANOVA with Bonferroni post-test. (C) Effects of CO on H_2O_2 -dependent Prx-2 oxidation relative to without CO in human RBC stored for 35 days. * $p < 0.05$ by one-way RM-ANOVA relative to control. Data are mean $\pm$ SEM, $n = 4$. CO, carbon monoxide.

Discussion

Numerous biochemical and morphologic changes occur in RBCs during cold storage, resulting in cells that contribute to transfusion-related toxicity. Increased oxidative stress is ap-

preciated to play a key role in storage-dependent changes to the RBC. With regard to Prx-2, previous studies have shown that during storage, oligomeric complexes between Prx-2 and other RBC proteins are formed, and that there is an increased association between Prx-2 dimers and the RBC membrane. This is consistent with increased Prx-2 oxidation and a role for this enzyme in protecting RBC membrane constituents from storage-dependent oxidative stress (49, 50). Whether Prx-2 activity and specifically reduction after an initial oxidation is affected during storage is not clear. This is an important consideration, as the reduction step is rate limiting in the Prx-2 reaction cycle in RBCs. We show here that RBC storage results in increased basal Prx-2 oxidation, which did not affect the initial oxidation of Prx-2 mediated by exogenously added H_2O_2 . However, oxidized (dimeric) Prx-2 is readily reduced back to the monomeric form in younger RBC, whereas this reduction process is significantly slowed in older RBC.

The potential significance of this finding is underscored by the fact that Prx-2 is considered the primary antioxidant system to negate H_2O_2 -mediated oxidative damage in the RBC (41). In the model proposed, Prx-2 oxidation is rapid, but its reduction is slow (relative to Prx-2 reduction in other cell types). However, since Prx-2 is the third most abundant protein in the RBC, an efficient antioxidant function is evident. With RBC storage, reduction of oxidized Prx-2 is slowed even further, which could account for the progressive accumulation of oxidized Prx-2 in RBC during storage observed here, and potentially other oxidation epitopes reported by others (33). Moreover, RBCs with compromised Prx-2 recycling are less likely able to deal with oxidative stress encountered post-transfusion, indicated by increased H_2O_2 -mediated hemolysis (Fig. 1D). In turn, this may be compounded by the fact that patients receiving transfusions are typically under heightened oxidative stresses (due to inflammatory cell activation, for example). We propose that attenuated Prx-2 activity is one mechanism that results in a more pro-oxidative environment in the vascular compartment and provides a second hit that underlies the pathology associated with transfusion of older RBCs.

Current FDA guidelines stipulate that storage procedures should result in RBCs that have $\geq 75\%$ survival 24 h post-transfusion. Previous studies have noted variability in post-transfusion survival between distinct RBC units, leading to the concept that some donors are “good” storers *versus* some are “poor” storers (17). This has led to recent interest integrating current understanding of mechanisms of storage-related changes, with identifying donor-specific factors affecting the cells. Oxidative stress-dependent markers have been discussed in this context, and accumulation of Prx-2 in the RBC membrane has been proposed as one possible biomarker (20, 50). Our data (Fig. 1A) also show variance in basal Prx-2 oxidation status, which is amplified as units are stored for longer times. We speculate that assessment of basal Prx-2 oxidation and/or reduction kinetics after H_2O_2 challenge may provide an additional marker/activity to identify which RBCs are better candidates for storage and transfusion. Future studies will need to determine whether RBCs with higher basal Prx-2 oxidation correlate with poorer post-transfusion recovery and/or increased toxicity.

The mechanism through which Prx-2 reduction was slower in older RBC remains unclear. H_2O_2 was consumed equally

FIG. 6. Role of β 93Cys in RBC degradation during storage. (A) RBCs were collected from healthy volunteers, (designated fresh) or after 7 or 35 days of storage, and β 93Cys levels were measured by BD-NEM labeling. For stored RBCs, BD-NEM labeling to Hb in cell-free fraction and inside RBCs is shown; no cell-free Hb was present in freshly prepared RBCs. Representative Western blot images show signals for β -chain Hb and matched BD-NEM labeling. Shown images are from the same gel (albeit not from adjacent lanes as indicated). Quantitation shows BD-NEM binding normalized to β -chain Hb and mean $\pm$ SEM ($n = 3-6$). $*p < 0.0001$ by unpaired t -test. NS, not significant by one-way ANOVA. (B) Hemolysis was measured after storage of β 93Cys or β 93Ala RBC for 1 or 10 days. $*p < 0.001$ by Wilcoxon-matched paired t -test, $\#p < 0.03$ by the Mann-Whitney t -test. (C) Microparticles were measured after storage of β 93Cys or β 93Ala RBC for 1 or 10 days. $*p < 0.05$ by paired t -test. No difference in the magnitude of storage-dependent increases in microparticles between either RBC was observed. BD-NEM, BODIPY N-ethylmaleimide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

rapidly (< 5 min) after addition to day 7 or 35 RBCs, consistent with previous reports using freshly isolated cells (40), excluding variable H_2O_2 levels as mediating the observed differences in Prx-2 recycling kinetics. Surprisingly, no differences in Trx activity or Trx levels were observed. In addition, NADPH concentrations, which provide reducing equivalents to the Trx repair system, were not different between young and old RBCs, consistent with earlier reports that showed increases in NADPH during storage in saline, adenine, glucose, mannitol (SAGM) solutions (14). Interestingly, glucose supplementation increased the rate of Prx-2 recycling, presumably by increasing flux through NADPH formation pathways. However, significant differences between older and younger RBCs remained, suggesting that other mechanisms for Prx-2 recycling, beyond Trx reductase, are present in the RBC.

Our recent study indicated that the conserved Hb cysteine residue (β 93Cys) is also a modulator of reactions between erythrocytes and H_2O_2 (57). Specifically, the reaction between oxyferrous heme (of oxyHb) and H_2O_2 was approximately twofold faster in RBCs lacking the β 93Cys, which resulted in a greater degree of H_2O_2 -mediated Prx-2 oxidation in β 93Ala RBCs (57). Data presented here suggest that the β 93Cys is also involved in Prx-2 reduction. In RBCs stored for 1 day, rates of Prx-2 reduction were approximately

twofold slower in RBC lacking the β 93Cys residue. Interestingly, Prx-2 reduction rates did not change on storage of β 93Ala RBCs, but decreased in β 93Cys RBCs to levels similar to those observed in β 93Ala RBCs. In other words, RBC storage led to a loss of β 93Cys-dependent control over Prx-2 reduction. With regard to the mechanism underlying this result, one consideration is the different reaction kinetics between β 93Cys and β 93Ala and H_2O_2 . β 93Ala Hb reacts faster, and thus the H_2O_2 concentration available to oxidize Prx-2 is predicted to be lower with β 93Ala RBC, which should, in turn, be observed as faster reduction of Prx-2 dimer to monomer. However, the opposite was observed. Another possibility is that secondary generation of peroxides (H_2O_2 or lipid peroxides) derived from Hb redox cycling reactions will be greater with β 93Ala; in this case, continued Prx-2 oxidation would be observed as slower rates of Prx-2 reduction, consistent with the observed data. To test this, we treated RBC with CO to ligate and prevented ferrous heme redox cycling and autoxidation. A recent study suggested that blocking ferrous heme prevented Prx-2 hyperoxidation induced by H_2O_2 generated by Hb autoxidation (12). While pre-CO-treatment had no effect on added H_2O_2 -dependent Prx-2 oxidation or reduction, CO added 5 min after H_2O_2 , a time point, when all added H_2O_2 has been consumed and Prx-

FIG. 7. Formation of DHA at the β 93 position of extracellular Hb during storage. Extracellular Hb was collected by centrifugation from human RBC stored for 7 or 35 days, and fate of the β 93Cys was determined by tandem mass spectrometry. Shown is a representative mass spectrum (A), b and y ions from detected trypsin fragments; highlighted values indicate ions detected and non-highlighted values are predicted ions (B) and chromatogram depicting relative intensities of DHA in day 7 versus 35 sample (C). DHA, dehydroalanine. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

2 oxidation is maximal, significantly accelerated Prx-2 reduction, but only in β 93A1a RBC. This suggests that higher autoxidation rates of Hb lacking the β 93Cys, and/or inability of this Hb to react with superoxide/ H_2O_2 generated from autoxidation, results in increased intracellular H_2O_2 that reacts with Prx-2, maintaining a higher steady state concentration of oxidized Prx-2. Blocking ferrous heme with CO

prevents the secondary and endogenous generation of H_2O_2 , resulting in the observed time-dependent reduction of Prx-2. These data also suggest that autoxidation of Hb lacking the β 93Cys is stimulated by an initial challenge with H_2O_2 , a possibility currently under investigation. CO also accelerated Prx-2 reduction in stored human RBC, suggesting increased autoxidation-derived H_2O_2 also sustains higher Prx-2

peroxidation levels in this setting. We also note that there remains a final possibility for explaining slower Prx-2 reduction in $\beta 93\text{Ala}$ RBC, such as direct protein–protein interactions between Hb and Prx-2. This premise has been suggested by reports of oligomeric Prx-2 binding to and stabilizing Hb, thereby preventing hemichrome formation (26).

Irrespective of the mechanism, the proposed role of the $\beta 93\text{Cys}$ residue in Prx-2 recycling prompted us to evaluate whether this residue is oxidized during RBC storage. No change in $\beta 93\text{Cys}$ oxidation on intraerythrocytic Hb was observed in stored RBC (Fig. 6). However, a significant decrease in $\beta 93\text{Cys}$ available for BD-NEM labeling for extracellular (hemolysed) Hb was noted. This result suggests two scenarios. First is that the $\beta 93\text{Cys}$ is oxidized only after hemolysis. However, the fact that the extent of $\beta 93\text{Cys}$ loss in cell-free Hb did not increase with storage time ($\sim 50\text{--}60\%$ oxidation was observed with day 7 and 35 samples), despite the fact that cell-free Hb increases with storage (due to ongoing hemolysis), suggests that little further oxidation of the $\beta 93\text{Cys}$ on extracellular Hb occurs during storage. The second possibility is that RBCs, in which significant $\beta 93\text{Cys}$ oxidation occurs, are more prone to hemolysis under storage conditions. This is further supported by increased storage-dependent hemolysis of $\beta 93\text{Ala}$ RBC compared with $\beta 93\text{Cys}$ RBC (Fig. 6B). While the precise mechanisms leading to hemolysis during RBC storage are not known, our data suggest that the $\beta 93\text{Cys}$ plays a role. We note that no differences in microparticle formation were observed, suggesting a selective effect of the $\beta 93\text{Cys}$ for controlling hemolysis. LC-MS analysis indicated that the $\beta 93\text{Cys}$ on extracellular Hb was oxidized to DHA. Formation of DHA on selenocysteine of glutathione peroxidase in RBC treated with H_2O_2 has been documented, suggesting an oxidative stress mechanism for DHA formation (11). Consistent with this finding, we also observed significant increases in carboxyethyllysine-modification, which is a product of increased oxidative stress in the presence of glucose (not shown). Several mechanisms for DHA formation from cysteine in model peptides have been discussed, albeit under relatively extreme conditions of heat, pH and involve β -elimination of $\text{HS}^\bullet$ from Cys-disulfide radicals (10, 35, 44). That said, DHA has been detected on albumin *in vivo* (5). How formation of DHA occurs on the $\beta 93\text{Cys}$ during RBC storage remains to be elucidated. Of additional interest is the fact that DHA is electrophilic; in other words, a transition from a nucleophilic to electrophilic center occurs at the $\beta 93$ site on the released Hb during storage. Whether this significantly alters the reactivity of Hb, and specifically alters its propensity to form adducts with nucleophiles after transfusion is an intriguing possibility that may have effects on transfusion toxicity. This possibility is under investigation.

Finally, we note that most of our studies were performed with human RBCs collected from segments of donor RBC units. Emerging data indicate that biomarkers for storage-dependent RBC damage (including hemolysis and complement fragments) can differ in magnitude between segments and matched bags. For example, hemolysis is higher in segments (37), but complement fragments are higher inside the bags (29), suggesting that currently no generalizable trend can be concluded. That said, we acknowledge this limitation in our study design, and note the need for future studies evaluating kinetics of Prx-2 oxidation in RBCs collected

from the actual donor units rather than attached segments. An additional limitation includes using mouse RBCs stored in the presence of a gas head-space, which is not the case for human RBC storage, and which may result in an elevated oxidative stress. That said, we note that Prx-2 recycling differences were still observed between $\beta 93\text{Cys}$ and $\beta 93\text{Ala}$ RBC stored for only 1 day. A final consideration is that we only investigated Prx-2 in the cytosol. It is evident that membranes and their constituents play important roles in the RBC redox state. Recent studies, for example, demonstrate an active NADPH oxidase in RBC membranes that modulate RBC aging in sickle cell disease (21). In addition, oligomeric Prx-2 accumulates in the membrane during storage (50, 51). Whether membrane constituents affect slower Prx-2 recycling in stored RBC and the role of the $\beta 93\text{Cys}$ residue in this process remain to be determined.

Conclusions

In summary, we demonstrate that Prx-2 activity is compromised during storage. Given the critical role for this RBC protein in protecting against endogenous and exogenous H_2O_2 -induced oxidative damage, we propose that loss of Prx-2 activity plays a role in storage-dependent damage to the RBC, and to post-transfusion redox imbalance and vascular oxidative stress in patients receiving older RBC units. Evidence is also provided to suggest that the Hb $\beta 93\text{Cys}$ residue maintains Prx-2 recycling, and we propose that its oxidation leads to storage-dependent hemolysis.

Materials and Methods

Materials

NEM was purchased from Pierce. Duo-Lux chemiluminescent/fluorescent substrate was purchased from Vector Laboratories, Inc. Sephadex G25 (fine) was purchased from GE Healthcare Life Science. Antibodies against peroxiredoxin, Trx, and Hb β -subunit were purchased from Abcam. Goat anti-rabbit IgG antibody conjugated to alkaline phosphatase was purchased from Millipore. BD-NEM (B-10250) was from Invitrogen. Nitrocellulose (162-0112, $0.2\ \mu\text{m}$ pore size) and micro Bio-Spin 6 columns were purchased from Bio-Rad. All other materials were purchased from Sigma-Aldrich.

Animals

Hb $\beta 93\text{Cys}$ -positive ($\beta 93\text{Cys}$) and -negative ($\beta 93\text{Ala}$) mice were generated and maintained as previously described (30). Age-matched males between 12 and 18 weeks of age that do not contain detectable Hb gamma chain and display less Hb-reduced thiol content were used (30, 39, 57). Mice were anesthetized with isoflurane (5%) and maintained at 2–3% with lack of responsiveness assessed by loss of toe-pinch reflex. Blood was collected by cardiac puncture with $50\ \mu\text{L}$ (trisodium citrate [22.0 g/L], citric acid [8.0 g/L], and dextrose [24.5 g/L]). As in earlier studies (30, 39, 57), no differences in reticulocyte counts were observed between $\beta 93\text{Ala}$ and $\beta 93\text{Cys}$ mice ($10.9\% \pm 2.6\%$ and $8.6\% \pm 2.5\%$ [mean $\pm$ SEM, $n = 4$]). Leukoreduction was performed under sterile conditions by filtering whole blood through a Sephadex G25:Microcellulose column (1:3 w/w). Columns were then washed with 10 times the volume of phosphate-buffered saline (PBS). Preliminary studies demonstrated $>99\%$ loss

of leukocytes by FACS analyses (not shown). Collected RBCs were centrifuged at 1500 g for 5 min at 4°C, washed thrice with cold Adsol, and concentrated to a hematocrit of 60% with Adsol in sterile Eppendorf tubes. RBCs (0.4 ml total volume at 60% Hct) were stored at 4°C for 1 or 10 days in 0.7 ml Eppendorf tubes. All experiments involving animals were conducted according to protocols approved by the University of Alabama at Birmingham (UAB) IACUC.

Human RBC collection and storage

Leukoreduced pRBCs of different storage ages (7–10, 14, 28, 35, and 42 days) were collected from segments (~0.7 ml) attached to units from the UAB blood bank. Only pRBCs stored in Adsol were used for these studies. On the day of experiments, RBC were collected from segments, washed thrice with ice-cold isotonic phosphate buffer (80 mM NaP_i, 40 mM NaCl, 10 mM KCl, and 100 μ M DTPA), and centrifuged at 4°C (1500 g for 10 min per wash and the SN discarded). An RBC aliquot was taken and hemolysed in water (1:10). Hb concentration was determined by visible spectroscopy (450–700 nm) and measurement of oxyHb using the extinction coefficient 14.6 mM⁻¹ cm⁻¹ for absorbance at 577 nm. In all cases, oxyHb represented >99% of the total Hb as assessed by spectral deconvolution as described (54). All protocols were approved by the UAB Institutional Review Board.

CO-treatment or RBC

Where indicated, the effects of CO on Prx-2 oxidation kinetics were tested by gently bubbling CO gas (20 ml) through RBC (50 μ M final heme, 5 ml) in isotonic phosphate buffer (as described next for Prx-2 oxidation) either immediately before addition of H₂O₂ or 5 min after H₂O₂ addition. Two microliters of pRBC were collected immediately after bubbling CO gas and at the end of the experiment, hemolysed in water (1:10) and the concentration of COHb was determined by visible spectroscopy. In every experiment, >95% of the Hb remained in the CO-bound ferrous (COHb) state (not shown). Prx-2 oxidation and hemolysis were determined as described next.

Prx-2 oxidation

RBCs were diluted to 50 μ M heme using isotonic phosphate buffer (80 mM NaP_i, 40 mM NaCl, 10 mM KCl, and 100 μ M DTPA) and treated with H₂O₂ (10 μ M) for approximately 60 min at 37°C. RBCs were mixed by inversion every 5–10 min to limit settling. Prx-2 oxidation was measured as previously described (57). Briefly, at each indicated time, RBCs were treated with 100 mM NEM for 20 min at 37°C to prevent further Prx-2 oxidation. RBC were then pelleted (2000 g, 30 s) and lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without β -mercaptoethanol (BME). Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Membranes were blocked with 1–5% nonfat dried skim milk powder (w/v) in TBS-T (137 mM NaCl, 2.6 mM KCl, and 0.05% Tween-20 [v/v] in 25 mM Tris-HCl pH 7.4) for 1 h at room temperature and then washed with TBS-T for 30 min. Membranes were incubated with rabbit poly or monoclonal antibody against Prx-2 followed by secondary alkaline

phosphatase-linked goat anti-rabbit IgG antibody (AP132A; Millipore). Blots were developed with Duo-Lux chemiluminescent/fluorescent substrate (Vector Laboratories, Inc.) and exposed to film. Prx-2 oxidation was assessed by the amount of dimeric Prx-2 (MWt ~38 kDa) divided by the total Prx-2 (dimeric + monomeric [19 kDa] forms).

H₂O₂ measurement

RBCs (50 μ M heme) were incubated with H₂O₂ (10 μ M) in PBS containing 10 μ M DTPA, at 37°C. Five hundred microliters aliquots were collected at indicated times, and RBC was rapidly pelleted (2000 g for 15 s). Four hundred microliters of the SN were collected, and H₂O₂ levels were determined using the FOX assay as described (40) with modifications to increase sensitivity. Specifically, 400 μ l SN was added to 100 μ l FOX reagent (excluding BHT) (9 ml methanol with 1.5 mM ferrous sulfate and 0.6 mM xylene orange in 1 ml 125 mM sulfuric acid) for 30 min at room temperature in the dark, and absorbance at 560 nm was measured. H₂O₂ concentrations were calculated using $\epsilon_{560\text{nm}} = 43,970 \text{ M}^{-1} \text{ cm}^{-1}$. All experiments included buffer-alone blanks and H₂O₂-alone group (with no RBC added).

Trx reductase activity

Trx reductase activity was measured as previously described (40). Briefly, RBCs were washed with isotonic phosphate buffer and counted using a hemocytometer. 5×10^7 RBCs were treated with 60 μ M 2,4-dinitrochlorobenzene (DNCB) or dimethyl sulfoxide vehicle alone for 30 min. Cells were pelleted, lysed in 5 mM phosphate buffer, pH 7.4 + 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and incubated at 37°C for 1 min. NADPH (200 μ M final concentration) was then added and incubated for 5 min at 37°C. Lysates were then applied to Micro Bio-Spin 6 size-exclusion column (Bio Rad, Inc.), and eluted with 5 mM phosphate buffer, pH 7.4. The absorbance at 412 nm of the eluate was measured using a DU 800 Spectrophotometer (Beckman Coulter). Fold changes in activity were determined by a comparison of DNCB (Trx reductase inhibitor)-treated samples with vehicle control.

Trx measurement

RBCs were lysed in RIPA buffer (0.5% Na-deoxycholate [w/v], 0.1% SDS [w/v], 150 mM NaCl, EDTA 0.5–1 mM, and 1% NP-40 [v/v] in 50 mM Tris-HCl, pH 7.4) and 15 μ g protein mixed 1:1 (by volume) with 2 $\times$ reducing sample buffer (2% BME [v/v], 4% SDS [w/v], 10% glycerol [v/v], and 0.2% [v/v] bromophenol blue in 100 mM Tris HCl, pH 6.8) and resolved by Western blotting (SDS-PAGE) on an 18% gel. After transfer, membranes were blocked with 5% nonfat dried skim milk powder (w/v) in TBS-T (137 mM NaCl, 2.6 mM KCl, and 0.05% Tween-20 [v/v] in 25 mM Tris-HCl pH 7.4) for 1 h at room temperature and washed with TBS-T for 30 min. Membranes were incubated with rabbit polyclonal antibody against Trx (ab26320; Abcam) diluted 1:1000 in 5% nonfat dried skim milk (w/v) in TBS-T overnight at 4°C followed by secondary alkaline phosphatase-linked goat anti-rabbit IgG (AP132A; Millipore) diluted 1:1000 in 5% nonfat dried skimmed milk (w/v) in TBS-T at room temperature for 2 h. Membranes were then developed

by chemiluminescence using Duo-Lux chemiluminescent/fluorescent substrate (Vector Laboratories, Inc.), and sequential images by AlphaEaseFC™ (Protein Simple, Inc.) were taken with quantification that was only performed on bands which had not reached saturation.

NADPH(H) measurement

NADPH was measured as previously described (59). Twenty microliters pRBC were diluted in 1.98 ml extraction buffer (20 mM nicotinamide, 20 mM NaHCO₃, and 100 mM Na₂CO₃), then subjected to freeze-thaw lysis, and centrifuged briefly to pellet cellular debris. Extracts were maintained on ice and shielded from light. An aliquot of each extract was heated for 30 min at 60°C. Fifty microliters of extract was mixed with 480 μ l of cycling buffer (100 mM Tris-HCl pH 8.0, 0.5 mM MTT, 2 mM PES, 5 mM EDTA, and 1.3 U/ml G6P dehydrogenase) in quartz cuvettes and incubated at 37°C for 2 min. Fifty microliters of 10 mM glucose-6-phosphate was added, and the sample was mixed. The absorbance at 570 nm was measured at 20 s intervals over 5 min, and concentrations were calculated by a comparison to calculated rates from NADPH standards.

Measurement of the β 93Cys using BD-NEM

Available thiol of the β 93Cys residue was measured on Hb present in the RBC and Hb present after storage-dependent hemolysis. RBCs were centrifuged at 1500 *g* for 10 min at 4°C to separate the hemolyzed SN fraction from the intact RBCs. Intact RBCs were then lysed 1:5 in ice-cold distilled water containing DTPA (100 μ M), that is, the intraerythrocytic fraction. To measure the reduced thiols, BD-NEM (500 μ M) was added to both fractions (100 μ M heme final) in PBS + DTPA (100 μ M) and in the dark for 15 min at room temperature (20 mM BD-NEM stocks were dissolved in dimethylformide, and concentration was determined using $\epsilon_{504\text{nm}} = 76,000\text{M}^{-1}\text{cm}^{-1}$). The reaction was terminated by addition of 5 mM L-Cysteine. Proteins were then resolved by SDS-PAGE on 10–18% gradient gels (under limited light exposure conditions and without bromophenol blue). After electrophoresis, BD-NEM-labeled proteins in the glass plates were developed on a Typhoon Imager (520BP 40, emission filter, blue laser [488 nm]). Proteins on the gel were transferred onto nitrocellulose membranes for 3 h at 80V in a wet transfer system. Membranes were blocked with 5% nonfat dried skimmed milk powder (w/v) in TBS-T (137 mM NaCl, 2.6 mM KCl, and 0.05% Tween-20 [v/v] in 25 mM Tris-HCl pH 7.4) for 1 h at room temperature and then washed with TBS-T for 30 min, followed by incubation with mouse monoclonal antibody against Hb β -subunit (ab55081; Abcam) diluted 1:1000 in 5% nonfat dried skim milk (w/v) in TBS-T (overnight, 4°C) followed by rabbit anti-mouse IgG alkaline phosphatase-conjugate (ab6729; Abcam) diluted 1:1000 in 5% nonfat dried skim milk (w/v) in TBS-T for 2 h. After washing with TBS-T for 30 min, membranes were developed by chemiluminescence using Duo-Lux chemiluminescent/fluorescent substrate (Vector Laboratories, Inc.), and sequential images by AlphaEaseFC (Protein Simple, Inc.) were taken with quantification that was only performed on bands which had not reached saturation. BD-NEM staining was normalized to anti- β -hemoglobin antibody binding.

Assessment of β 93C oxidation by MS analysis

In-gel digestion. SN fractions of RBC stored for 7 or 35 days were collected in urea (10 mM) and NEM (100 mM) dissolved in water. After 30 min incubation at room temperature, proteins were resolved by nonreducing SDS gel electrophoresis. Gels were stained with Coomassie blue to visualize protein bands. Bands corresponding to Hb were excised, and excess stains were removed by an overnight wash of 50% 100 mM ammonium bicarbonate/50% acetonitrile. After destaining, disulfide bonds were reduced by 25 mM dithiothreitol at 50°C for 30 min. Alkylation of any remaining free thiols groups was carried out with 55 mM iodoacetamide for 30 min in the dark. The excess alkylating agent was removed, and the gel pieces were washed twice with a 100 mM ammonium bicarbonate for 30 min. The gel pieces were evaporated to dryness in a SpeedVac (Savant) before the addition of trypsin, which was added at 12.5 ng/ μ l (Promega Gold Mass Spectrometry Grade), and incubated overnight at 37°C. Peptides were extracted from the gel pieces using a 1:1 mixture of 1% formic acid and acetonitrile twice for 15 min. Extracts were pooled and evaporated to dryness. The samples were then resuspended in 30 μ l of 0.1% formic acid before mass spectrometry analysis.

Nano cHiPLC-tandem mass spectrometry. An aliquot (5 μ l) of each digest was loaded onto a Nano cHiPLC 200 μ m $\times$ 0.5 mm ChromXP C18-CL 3 μ m 120Å reverse-phase trap cartridge (Eksigent) at 2 μ l/min using an Eksigent autosampler. After washing the cartridge for 4 min with 0.1% formic acid in ddH₂O, the bound peptides were flushed onto a Nano cHiPLC column 200 μ m $\times$ 15 cm ChromXP C18-CL 3 μ m 120Å (Eksigent) with a 45 min linear (5–50%) acetonitrile gradient in 0.1% formic acid at 1000 nl/min using an Eksigent Nano1D+LC. The column was washed with 90% acetonitrile-0.1% formic acid for 10 min and then re-equilibrated with 5% acetonitrile-0.1% formic acid for 10 min. The Applied Biosystems 5600 Triple-ToF mass spectrometer (AB-Sciex) was used to analyze the protein digest. The IonSpray voltage was 2300 V, and the declustering potential was 80 V. Ionspray and curtain gases were set at 10 and 25 psi, respectively. The interface heater temperature was 120°C.

Eluted peptides were subjected to a time-of-flight survey scan from 400 to 1250 *m/z* to determine the top 20 most intense ions for MSMS analysis. Product ion time-of-flight scans at 50 ms (over the range from *m/z* 100–1500) were carried out to obtain the tandem mass spectra of the selected parent ions. Spectra are centroided and de-isotoped by Analyst software, version TF (Applied Biosystems). A β -galactosidase trypsin digest was used to establish and confirm the mass accuracy of the mass spectrometer.

Protein Pilot 4.5 search queries. The tandem mass spectrometry data were processed to provide protein identifications using an in-house Protein Pilot 4.5 search engine (AB-Sciex) using the *Homo sapiens* Sprot protein database and using a trypsin digestion parameter. Possible modified peptides on the Hb- β subunit were evaluated by setting the software to analyze biological modifications with an emphasis on β 93Cys modifications. *De novo* inspection of the data was then used to validate the potential modification sites.

Hemolysis measurement

At indicated times, RBCs were collected and pelleted by centrifugation (1500 g, 10 min, 5°C). Cell-free Hb in the SN was measured by visible spectroscopy (400–700 nm) and concentration and oxidation state were determined by deconvolution as previously described (54).

Microparticle measurement

Stored mouse RBCs were left unwashed (60% Hct) and incubated with anti-Ly76 antibody (TER-119)-FITC conjugated antibody (ab93587; Abcam at 0.1 µg/ml) for 60 min in PBS and in the dark at room temperature. Samples were then analyzed by flow cytometry using a Beckman Coulter 3500, and events were acquired using Beckman Coulter native software. Approximately 100,000 events were collected per measurement. All analyses were done with Kaluza software (Beckman Coulter, Inc.).

Statistical analysis

Storage time and H₂O₂ time-dependent changes in Prx-2 oxidation were analyzed by one-way or by two-way repeated measures (RM)-ANOVA. Rates of Prx-2 reduction were determined by linear regression as indicated. Changes in hemolysis were assessed by unpaired and paired *t*-test as appropriate. Data were also assessed for normal distribution by the Kolmogorov-Smirnov normality test or D'Agostino and Pearson normality test; if data were not normally distributed, Mann-Whitney (unpaired) or Wilcoxon (paired) *t*-tests were used. *p*-Values less than 0.05 were considered significant. All analyses used GraphPad Prism Software.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL092624 and HL095468 to R.P.P.) and Cardiovascular T32 Pre-doctoral Training Grant to R.S. The AB Sciex 5600 Triple-ToF mass spectrometer used in this study was purchased with funds from a Shared Instrumentation grant from the National Center for Research Resources (S10 RR027822-01, S.B., Principal Investigator). The operation of the UAB Targeted Metabolomics and Proteomics Laboratory is additionally supported by federal grants P30 AR050948-10 Skin Disease Research Center; DK079337-06 O'Brien Acute Kidney Injury Center.

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, April 14, 2014; date of final revised submission, September 10, 2014; date of acceptance, September 29, 2014.

Abbreviations Used

ANOVA = analysis of variance
BD-NEM = BODIPY N-ethylmaleimide
BME = β -mercaptoethanol
CO = carbon monoxide
DHA = dehydroalanine
DNCB = 2,4-dinitrochlorobenzene
H ₂ O ₂ = hydrogen peroxide
Hb = hemoglobin
NO = nitric oxide
PBS = phosphate-buffered saline
pRBC = packed red blood cell
Prx-2 = peroxiredoxin-2
RBCs = red blood cells
RM-ANOVA = repeated measures-analysis of variance
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SN = supernatant
Trx = thioredoxin
UAB = University of Alabama at Birmingham