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Glutathione Loading Prevents Free Radical Injury in Red Blood Cells After Storage

U.J. DUMASWALA[a,*], M.J. WILSON[b], Y.L. WU[c], J. WYKLE[a], L. ZHUO[a], L.M. DOUGLASS[a] and D.L. DALEKE[b]

[a]*Hoxworth Blood Center, University of Cincinnati Medical Center, Cincinnati, OH 45267–0055,* [b]*Department of Biochemistry and Molecular Biology, Medical Sciences, Indiana University, Bloomington, IN 47405 and* [c]*Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, MD 20892*

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We have previously demonstrated that the loss of glutathione (GSH) and GSH-peroxidase (GSH-PX) in banked red blood cells (RBCs) is accompanied by oxidative modifications of lipids, proteins and loss of membrane integrity[1]. The objective of this study was to determine whether artificial increases in antioxidant (GSH) or antioxidant enzyme (catalase) content could protect membrane damage in the banked RBCs following an oxidant challenge. RBCs stored at 1–6°C for 0, 42 and 84 days in a conventional additive solution (Adsol®) were subjected to oxidative stress using ferric/ascorbic acid (Fe/ASC) before and after enriching them with GSH or catalase using a hypotonic lysis-isoosmotic resealing procedure. This lysis-resealing procedure in the presence of GSH/catalase raised intracellular GSH and catalase concentrations 4–6 fold, yet produced only a small reduction in mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentrations (MCHC). Indicators of oxidative stress and membrane integrity were measured, including acetylcholinesterase (AChE) activity, GSH concentration, phosphatidylserine (PS) externalization (prothrombin-converting activity) and transmembrane lipid movements (^{14}C-lyso phosphatidylcholine flip-flop and PS transport). GSH-enrichment protected AChE activity in fresh (0 day) and stored (42 and 84 days) RBCs from Fe/ASC oxidation by 10, 23 and 26%, respectively, compared with not-enriched controls. Following oxidative stress, the rate of transbilayer lipid flip-flop did not increase in fresh cells, but increased 9.3 % in 42-day stored cells. Phosphatidylserine exposure, as measured by prothrombinase activity, increased 2.4-fold in fresh and 5.2-fold in 42-day stored cells exposed to Fe/ASC. Previous studies have shown that 42-day storage causes a moderate decrease in PS transport (~ 50 %), whereas transport rates declined by up to 75 % in stored RBCs when challenged with Fe/ASC. GSH-enrichment prevented the increase in passive lipid flip-flop and the increase in prothrombinase activity, but offered no protection against oxidative damage of PS transport. In contrast to these effects, catalase-enrichment failed to protect GSH levels and AChE activity upon oxidative stress. Membrane protein thiol oxidation was assessed by labeling reactive protein thiols with 5-acetalamidofluorescein followed by immunoblotting with antifluorescein antibodies. Significant oxidation of membrane proteins was confirmed by a greater loss of thiols in stored RBCs than in fresh RBCs. These results demonstrate that it may be possible to prevent storage-mediated loss of AChE, increased lipid flip-flop, and increased PS exposure, by maintaining or increasing GSH levels of banked RBCs.

* Corresponding Author: Umakant J. Dumaswala, Ph. D, Hoxworth Blood Center, University of Cincinnati, PO Box 670055, 3130 Highland Avenue, Cincinnati, Ohio 45267–0055. Telephone 513–558–1521; Fax 513–558–1522; email umakant.dumaswala@uc.edu

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Abbreviations: Adenosine-5′-triphosphate (ATP); Adsol® commercial additive solution; citrate-phosphate-dextrose (CPD); dilauroylphosphatidylserine (DLPS); ferric ammonium sulfate/ascorbic acid (Fe/ASC); glutathione (GSH); oxidized glutathione (GSSG); GSH-peroxidase (GSH-PX); horseradish peroxidase (HRP); hydrogen peroxide (H_2O_2); 5-iodoacetalamidofluorescein (5-IAF); lysophosphatidylcholine (LPC) malondialdehyde (MDA); mean cell hemoglobin (MCH); mean cell hemoglobin concentration (MCHC); mean corpuscular volume (MCV); morphological index (MI); phosphate-buffered-saline (PBS); phosphatidylethanolamine (PE); phosphatidylserine (PS); red blood cells (RBCs); saline-adenine-glucose-mannitol (SAG-M); superoxide dismutase (SOD)

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INTRODUCTION

Oxidation is a significant cause of cellular damage and results in the accumulation of reactive end products of lipid oxidation, modification of membrane protein and nucleic acid structure and reduction of enzymatic activity. In healthy erythrocytes, significant oxidative damage is prevented by a very efficient antioxidant system, consisting of small antioxidant compounds such as glutathione (GSH), vitamin E, vitamin C, and enzymes such as GSH-peroxidase (GSH-PX), catalase, and superoxide dismutase (SOD). Reduced GSH, together with GSH-PX, is one of the major scavengers of activated oxygen species in red blood cells (RBCs). GSH accounts for 90% of the intracellular non-protein thiols and is therefore the most important intracellular reducing agent[2]. Reports from our laboratory have suggested an inverse relationship between RBC-GSH content and the extent of membrane damage during blood bank aging of RBCs[1,3,4]. Specifically, we observed that banking of RBCs induces a time-dependent loss of GSH, altered phosphatidylethanolamine (PE) asymmetry, and a decrease in phosphatidylserine (PS) transport activity. More recently, we demonstrated that a decline in GSH concentration in banked RBCs is accompanied by a decrease in GSH-PX and an increase in the oxidative modification of membrane lipids and proteins, including malondialdehyde (MDA) formation, protein aggregation, and carbonylation of protein band 4.1[1].

These phenomenological correlations support the hypothesis that oxidative stress may have an important etiological role in oxidative damage of the membrane of banked RBCs and that GSH has a protective role. Thus, maintenance of GSH may improve quality and shelf-life of banked RBCs.

RBCs lack a fully operative γ-glutamyl cycle and related enzymes, especially γ-glutamyltranspeptidase. Therefore, the synthesis of RBC-GSH is dependent upon the availability of substrate amino acids and the release of oxidized glutathione (GSSG) is correlated with GSH turnover[5]. Since RBCs are equipped with the GSH synthetic pathway enzymes, GSH levels can be easily manipulated by adding the GSH precursor amino acids to the medium[3].

In view of several recent reports suggesting that catalase is important for disposing of hydrogen peroxide (H_2O_2) and protecting GSH in human erythrocytes[6–8], the objective of this study was to evaluate critically the relative contribution of GSH and catalase in providing antioxidative defense against oxidative challenge, and to confirm whether oxidative modifications, specifically of lipids, in blood bank aged RBCs were a result of cellular GSH loss. This information is vital for the development of new blood bank additives that will prevent the premature aging of banked RBCs by free radical mediated events. The results demonstrate that it may be possible to prevent storage-mediated loss of AChE, increased lipid flip-flop, and increased PS exposure, by maintaining GSH levels of banked RBCs.

MATERIALS AND METHODS

Blood was collected from volunteer donors selected using criteria established by the American Association of Blood Banks and the Food and Drug Administration. The protocol was approved by the University's Institutional Review Board. Standard units of blood (450 ±

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45 mL) were collected into polyvinyl chloride bags (PL 146; Baxter Healthcare Corporation, Deerfield, IL) containing 63 mL citrate-phosphate-dextrose (CPD) anticoagulant and were centrifuged (RC-3B; Sorvall; Dupont, Wilmington, DE) at 835 × *g* for 10 minutes. The platelet rich plasma was expressed into a satellite bag. The packed RBCs were stored with 100 mL Adsol per unit of blood at 1–6°C. The experiments were carried out at 0 (fresh), 42 and 84 days of storage.

Preparation of erythrocytes and membranes

RBCs were purified from contaminating leukocytes and platelets using alpha cellulose-microcrystalline cellulose by the method of Beutler[9]. The purified RBCs were lysed in 30 volumes of 5 mmol/L Na_2HPO_4, pH 8.0[1,3,4], and the hemoglobin free white ghosts were isolated by centrifugation at 38,000 × *g* for 20 minutes followed by five washes in the phosphate buffer. Total proteins were determined using the bicinchoninic acid method (Pierce, Rockford; IL).

GSH/catalase loading of RBC by lysis-resealing

The antioxidant status of the fresh and stored RBCs (42 and 84 days respectively) was assessed by measuring GSH concentration and the activities of the enzymes GSH-PX and catalase, using a modification of the procedures described by Beutler[9]. Acetylcholinesterase (AChE) activity was evaluated as an important measure of RBC membrane vesiculation and integrity[1]. To investigate the relative importance of GSH and catalase in RBC antioxidant defense, fresh and banked RBCs (42 and 84 days) were enriched with GSH or catalase as described by Scott et al.[8] with slight modifications. Briefly, washed packed RBCs (80% to 85% hematocrit) were mixed with either GSH (final concentration 16 mmol/L) or catalase (final concentration 50 mg/mL) and sealed in dialysis tubing (11.5 mm diameter; relative molecular weight cut-off of 1000). The tubes were dialyzed against a lysis buffer (5 mmol/L potassium phosphate buffer (pH 7.4) and 2 mmol/L EDTA) for 1 h at 4°C and then transferred to a resealing buffer (160 mmol/L NaCl, 5 mmol/L potassium phosphate buffer (pH 7.4) and 5 mmol/L glucose) for 30 min at room temperature. After resealing, cells were washed with PBS until the supernatant was clear (five to seven times). Control samples were subjected to identical lysis-resealing procedure in the absence of GSH or catalase.

Mean cell volume (MCV), hemoglobin, and hematocrit of control and resealed RBCs were analyzed with an automated cell counter-MaxM (Coulter Electronics, Hialeah, Fl.). RBC morphology was examined by light microscopy. The control, control-resealed, GSH-enriched and catalase-enriched RBCs were then challenged with 100 µmol/L ferric chloride or ferric ammonium sulfate and 25 mmol/L ascorbic acid[1] (Fe/ASC) in PBS, pH 7.4 for l h at 37°C. GSH or catalase-loaded /resealed RBCs were compared with control-resealed and not-manipulated (before lysis-resealing) RBCs.

The pre- and post-challenged RBCs were analyzed for AChE, adenosine 5′-triphosphate (ATP) content, and GSH-levels using procedures described previously[1,3,4]. Prothrombin-converting activity was assayed as described previously[3,4,10]. Released thrombin was measured using the chromogenic substrate sarcosine-Pro-Arg-*p*-nitroanilide (500 µmol/L final concentration).

Phosphatidylserine transport

Phosphatidylserine transport was measured using dilauroylphosphatidylserine (DLPS) and a cell morphological method[3,4,11]. When incubated with erythrocytes, short chain (< C14) phospholipids transfer rapidly ($t_{1/2}$ ~ minutes) between membranes and lipids induce changes in cell shape characteristic of the monolayer loca-

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tion of the lipid; lipids that remain in the outer monolayer induce spiculation due to the increase in monolayer surface area, whereas lipids that accumulate in the inner monolayer induce membrane invagination. A conversion from discoid or echinocytic shapes to invaginated shapes is indicative of transbilayer transport. RBCs were treated with sonicated vesicles composed of DLPS. At appropriate time intervals, aliquots of the cell suspension were fixed in 10 volumes of 0.5% buffered glutaraldehyde and viewed by light microscopy. Cell shape was scored according to a morphological scale: discocytes were scored 0, echinocytes were given positive values depending on degree of spiculation and stomatocytes were given negative values depending on the degree of invagination. The average score of a field of 100 cells was defined as the Morphological Index (MI). The rate of PS transport was expressed as the rate of reversion of DLPS-induced echinocytes to stomatocytes. In the range of the MI scale used in these experiments (±1 – ±3), the response of cell shape to monolayer concentration of lipid was linear.

Flip-flop measurements

Lysophosphatidylcholine (LPC) flip-flop was measured using a modification of the method of Mohandas et al.[12]. Washed RBCs were suspended at 5% hematocrit (600 µL final volume) in PBS containing glucose (5 mmol/L), penicillin (50 IU/mL), and streptomycin (50 µ g/mL). A solution of 1-[1-^{14}C]-palmitoyl-L-lyso-3-phosphatidylcholine (^{14}C-LPC) was diluted with unlabeled LPC in toluene, dried under a stream of dry argon, dried further under vacuum to remove traces of solvent, and reconstituted in ethanol to give the desired final concentration (0.4 mmol/L) and specific activity (28 mCi/mmol). The dissolved lipid (3 µL) was added to the sample while vortexing and incubated at 37°C. At the designated time points, two aliquots of 25 µL were taken. One was added to 225µL of PBS plus glucose (5 mmol/L). The second was added to an identical solution containing 1% (w/v) bovine serum albumin. The samples were centrifuged (16,000 × *g*; 5 min) to pellet the cells. Aliquots of the supernatant and pellet were transferred to scintillation vials and the samples were bleached with H_2O_2 (250 µL, 30%) in a small amount (100 µL) of scintillation fluid at 80°C for 12 h. After cooling to room temperature, the amount of radioactivity was determined by scintillation counting. Counts associated with the pellet after PBS washes represent the total lipid in both leaflets in the bilayer. Counts remaining in the pellet after albumin washes represent lipid which was inaccessible due to prior translocation to the inner leaflet. The difference between these two values represents lipid label distributed in the outer leaflet. Rates were calculated from the change in the distribution of the labeled lipids between monolayers as a function of time.

Analysis of protein thiol oxidation in fresh and stored RBCs

All the manipulations in this procedure were carried out in O_2-free atmosphere. This was achieved by equilibrating reagents with an O_2-free atmosphere overnight or longer in the presence of 5% hydrogen and 95% nitrogen[13]. Fresh and 42-day stored RBCs were washed in PBS and centrifuged. After removal of supernatant, RBCs were rapidly frozen and stored in liquid N_2 until further use. Thiol oxidation was detected as described previously[13] with minor modifications. To label reactive thiols, 0.1mL of RBC suspensions were mixed with 0.5mL of 5-iodoacetalmidofluorescein (5-IAF; final concentration 20 µmol/L from a 40 mmol/L stock in dimethyformamide) in anoxic-lysis buffer and incubated in the dark at 37°C for 10 min. The reaction was stopped by freezing the suspension in liquid N_2. Thirty microliters of 5-IAF-labeled whole cell lysate was mixed with electrophoresis sample buffer containing β-mercaptoethanol, electrophoresed on a 12% SDS-PAGE gel, and

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blotted using standard procedures[1]. Immunoblotting was performed using rabbit-anti-fluorescein as primary antibody and horseradish peroxidase (HRP) conjugated goat-anti-rabbit as secondary antibody. Immunodetection was performed using the Enhanced Chemiluminescence (ECL) Super Signal substrate kit (Pierce, Rockford, IL). Thiol oxidation was indicated by decreased anti-fluorescein signal. The concentration of RBC suspension in each assay was equivalent for each sample. This was achieved by adjusting the hematocrit of the samples prior to analysis and was further confirmed by Coomassie blue staining of the SDS-PAGE gel.

RESULTS

Efficiency of osmotic lysis-resealing on GSH/catalase loading and effect on RBC characteristics

RBC physical characteristics after osmotic lysis-resealing in the presence or absence of GSH/catalase were normal, although there was a slight decrease in MCV, mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). However, similar changes occured in fresh as well as stored RBCs (Table I). This manipulation produced no significant decline in endogenous GSH and catalase levels in control-resealed (buffer-louded), compared to not-manipulated (pre-lysis), samples (Table II). The control pre-lysis reseal and control-reseal ATP levels remained unaltered (data not shown). Resealing lysed RBCs in the presence of 16 mmol/L GSH or 50 mg/mL catalase resulted in a greater than five-fold increase in GSH/catalase content of the RBCs (Table II). The control catalase values rose > 8-fold in fresh and stored RBCs after lysis-resealing manipulations (4.1 ± 0.5 vs. 48.0 ± 13.6 × 10^4 IU/mL RBC). Thus, this method of manipulation of intracellular RBC components to study their physiological role produces minimal alteration of cellular characteristics, while allowing for the loading of compounds to which the RBC membrane is impermeable.

TABLE I Effect of Osmotic Lysis-Resealing on RBC Physical Characteristics

	MCV (fL)	*MCH (pg/RBC)*	*MCHC (g/dL RBC)*
0 days storage			
Pre-lysis	92.5 ± 1.9	30.7 ± 0.7	33.2 ± 0.8
Buffer-loaded	88.7 ± 2.7[a]	25.8 ± 1.4[a]	29.1 ± 1.7[a]
GSH-loaded	89.9 ± 3.1[a]	25.0 ± 1.6[a]	28.1 ± 2.2[a]
84 days storage			
Pre-lysis	91.9 ± 4.2	29.8 ± 1.7	31.2 ± 0.6
Buffer-loaded	86.7 ± 3.3[a]	23.4 ± 1.6[a]	26.9 ± 1.6[a]
GSH-loaded	88.2 ± 2.9[a]	23.0 ± 2.0[a]	26.0 ± 1.9[a]

n = 6; mean ± SD.

a. $p < 0.01$ compared to pre-lysis samples.

TABLE II Efficiency of GSH Loading by Osmotic Lysis-Resealing (μmol/mL RBC)

	0 days	*42 days*	*84 days*
Pre-lysis	2.38 ± 0.7	1.68 ± 0.3	1.25 ± 0.5
Buffer-loaded	1.97 ± 0.3	1.48 ± 0.3	1.02 ± 0.3
GSH-loaded	9.18 ± 2.0[a]	7.16 ± 2.0[a]	7.44 ± 1.2[a]

n = 6 – 15; mean ± SD.

a. $p < 0.001$ compared to pre-lysis cells or buffer-loaded ghosts.

Effect of oxidant stress on GSH/catalase enriched RBCs

To compare their susceptibility to oxidant stress, fresh, 42, and 84-day banked RBCs were enriched with either GSH or catalase using the hypotonic lysis-isoosmotic resealing procedure. The pre-lysis, buffer-louded, GSH-loaded, and catalase-loaded RBCs were then challenged with the Fe/ASC oxidative system. A ferric ion-dependent oxidation system was selected as

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free radical mediated phenomenon in RBC can be attributed to the presence of this oxidant; both hemoglobin bound and free iron are available[14]. The presence of residual oxygen during storage of red blood cells contributes to the formation of methemoglobin and its toxic progenies hemichrome, hemin, Fe^{3+}, and hydroxyl radical. Hemoglobin bound iron can catalyze the Haber-Weiss reaction and the free iron or free heme can act as biological Fenton reagents[14]. Metal-catalyzed oxidation of amino acid residues of protein has been shown[15]. Although ascorbic acid is an antioxidant, under appropriate conditions, it is a promoter of oxidation[16,17]. Ascorbic acid has been shown to enhance iron mediated peroxidative injury in hepatic lysosomes and microsomal suspensions[18,19] and in extracts of RBC[20].

The lysis-resealing procedure produced no significant change in AChE activity compared to not-lysed RBCs (Figure 1A). Not-lysed cells and cells resealed in the absence of GSH showed a significant decline (~ 20 – 40%; $p < 0.001$) in AChE activity upon oxidant challenge, regardless of whether the cells were stored or not (compare Figures 1A and 1B). GSH-loading of RBCs resulted in better maintenance of AChE activity in fresh, 42- and 84-day stored cells after Fe/ASC oxidation, compared to cells lysed and resealed in the absence of GSH (10, 23, and 26 % greater activity, respectively; Figure 1B).

In similar experiments, AChE activity was not protected from oxidation in catalase-enriched RBCs. The percentage AChE activities after oxidant treatment were (± SD; n = 4; enriched/not enriched): 60.9 ± 34.3/54.6±15.3 (fresh), 41.4 ± 1.9/45.4 ± 8.9 (42-day), and 44.3 ± 3.9 / 43.7 ± 2.0 (84-day). Catalase enrichment also failed to protect GSH against oxidative stress. The percentage of GSH remaining after oxidant treatment was not significantly different in catalase-enriched compared with not-enriched RBCs. The percentage GSH values were (± SD; n ≥4; enriched/not enriched): 55.4 ± 19.3/62.3 ± 21.5 (fresh), 74.3 ± 20.1/76.0 ± 24.5 (42-day), and 47.8 ± 23.4/41.3 ± 24.0 (84-day). Scott et al.[8] reported that catalase enrichment protects RBC GSH from H_2O_2 mediated oxidation. However, we did not observe a beneficial effect of catalase enrichment on cellular GSH in either fresh or banked RBCs when challenged with Fe/ASC. The discrepancy in our results and that of Scott

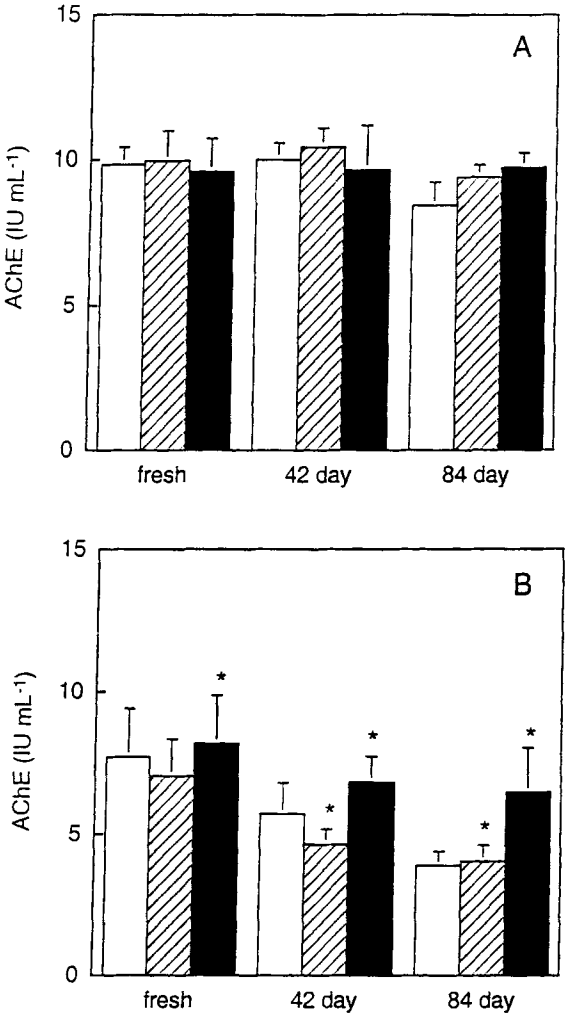

FIGURE 1 GSH protects RBC AChE activity from oxidative stress. Freshly (0 day), 42-and 84-day stored RBCs (open bars) were lysed and resealed in the absence (shaded bars) or presence (filled bars) of GSH, AChE activity was determined prior to (A) and after (B) a 1 h challenge with Fe/ASC. Results are expressed as IU AChE mL^{-1}. The values are mean ± SD for $n > 9$; $p < 0.01$ (GSH enriched vs. not GSH-enriched)

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et al. could be attributed to the choice of oxidant[8]. However, when we used concentrations of H_2O_2 equivalent to that used by Scott et al. (20 mmol/L), significant morphologic changes and clumping of RBC were produced.

Effect of oxidant stress and GSH loading on PS transport, passive flip-flop and prothrombinase activity

In order to comprehend the relationship between GSH concentration, oxidative stress and the maintenance of lipid asymmetry, the markers of lipid asymmetry were measured including, prothrombinase activity (an indicator of PS asymmetry), passive lipid flip-flop and aminophospholipid transport activity (specific indicators of phospholipid scrambling), in fresh and stored, GSH-enriched/GSH-not enriched, control and oxidant treated RBCs. We have previously reported[3,4] a loss of RBC phosphatidylethanolamine (PE) asymmetry and a significant decline in aminophospholipid transport activity in conventionally banked (Adsol) RBCs. In contrast, lipid asymmetry was not disturbed when RBCs were stored in a glutamine plus phosphate (Pi) containing additive that better maintained GSH levels[3]. In the present work, storage of RBCs for 42 days in Adsol decreased the rate of flippase activity by 42 % compared to fresh cells (Figure 2A). A similar trend was observed for RBCs lysed and resealed in the presence or absence of GSH. Subsequent Fe/ASC treatment had no effect on flippase activity in fresh or stored cells (open bars; Figures 2A and 2B). However, a greater decline (~ 50 –75%) in transport rate was observed in stored cells lysed and resealed in the presence or absence of GSH when subjected to Fe/ASC treatment (Figure 2B). Because the level of flippase activity remaining in stored cells is sufficient to maintain lipid asymmetry, the loss of PE asymmetry observed earlier in stored RBCs[3,4] may not have been due to a reduction in aminophospholipid transport as a result of declining GSH concentrations.

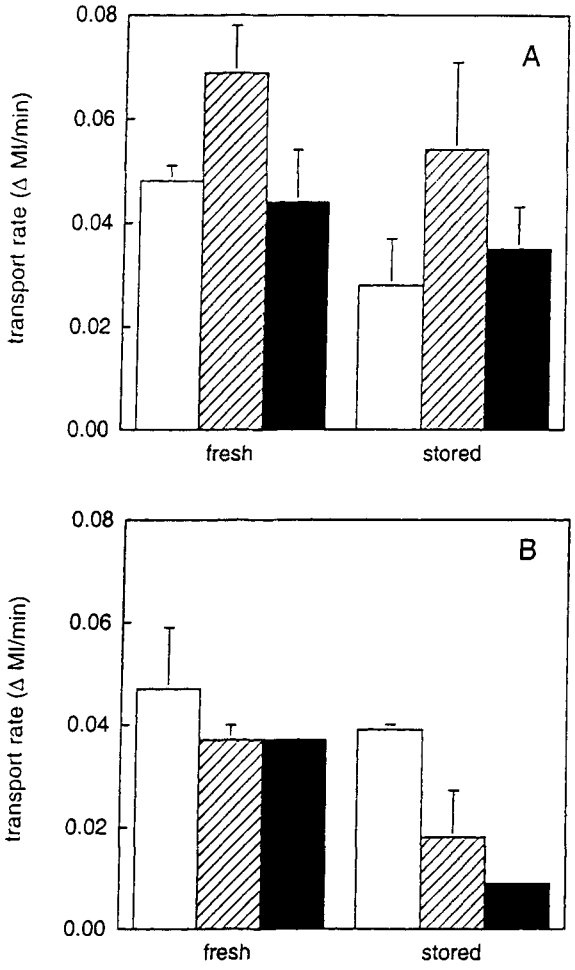

FIGURE 2 Effect of GSH-loading and oxidative stress on RBC flippase activity. Fresh and 42-day stored RBCs (open bars) were lysed and resealed in the absence (shaded bars) or presence (filled bars) of GSH and flippase activity was measured prior to (A) and after (B) a 1 h challenge with Fe/ASC. Flippase activity was determined using the cell morphology assay described in **Materials and Methods**. The results are expressed as Δ MI min^{-1}. The values are mean ± SD for n=3–4; ($p<0.01$ for unchallenged vs. challenged)

It has been proposed that significant PS externalization by an increase in flip-flop rates requires suppression of flippase activity[21–23]. In addition to Ca^{2+}, these processes may be regulated by oxidative insults[21–22]. Consistent with this hypothesis, passive lipid flip-flop activity was increased substantially ($p< 0.01$) by oxidant

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stress in not-manipulated as well as GSH enriched stored cells compared with fresh cells (Figure 3). Part of this effect is due to storage; flip-flop rates in stored cells were 2-fold greater in stored compared to fresh cells not exposed to oxidant. Prior GSH-loading significantly ($p < 0.05$) reduced lipid flip-flop rates in cells challenged with oxidant (Figure 3). Finally, oxidation had no effect on lyso PC flip-flop rates in fresh cells regardless of oxidant exposure(data not shown). A lack of effect of oxidant on passive flip-flop in fresh cells is consistent with the greater amount of GSH and higher flippase activity in these cells as compared to stored cells[1,3,4].

If GSH-loading protects stored RBCs against an increase in lipid flip-flop rates, then PS exposure should also be reduced. To test this hypothesis, fresh cells and cells stored for 42 days were challenged with Fe/ASC, either before or after lysing and resealing in the presence or absence of GSH. The lysing and resealing procedure resulted in a substantial increase in prothrombin-converting activity, even in the absence of oxidant stress (Figure 4A). Oxidant treatment had little further effect on the prothrombin-converting activity of fresh or stored cells prior to lysis or after lysis and resealing in the absence of GSH (Figure 4B). However, GSH-loading resulted in a reduction in prothrombin-converting activity, indicating that this treatment reduced the exposure of PS on the surface of the cell (Figure 4B).

These results suggest that the storage-induced increase in PS exposure is likely to be due to an increase in passive flip-flop combined with a significant drop in flippase activity. Post-storage loading with GSH exerts a protective effect by decreasing lipid flip-flop.

Oxidation of thiols in RBC membranes

Probing Western blots of whole RBC proteins with antifluorescein antibody following reaction with 5-IAF showed significantly greater oxidation in the membrane proteins of stored RBCs compared to fresh RBCs (Figure 5). Labeling with 5-IAF was decreased by 25–48% in several membrane proteins including but not restricted to those of molecular weight 97, 81 and 45 kDa in 42 day stored RBCs compared to fresh RBCs. These molecular mass correspond to bands 3, 4.1 and 5. We have previously confirmed greater oxidative changes, such as aggregation and carbonylation of band 3 and band 4.1 proteins, in 42-day stored RBCs than in fresh RBCs, using a double immunoblot technique[1].

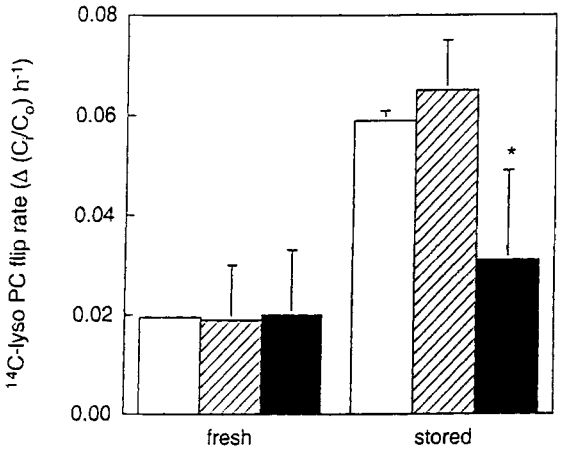

FIGURE 3 GSH loading protects RBC against oxidant-induced increases in passive lipid flip-flop. Fresh (0 day) and 42 day stored RBCs (open bars) were lysed and resealed in the absence (shaded bars) or presence (filled bars) of GSH and passive flip-flop measured after a 1 h challenge with Fe/ASC. Lipid flip-flop was measured with ^{14}C-labeled lyso-phosphatidylcholine as described in the **Materials and Methods**. Data are expressed as the rate of change of the ratio of outer monolayer to inner monolayer radiolabel. The results are reported as mean ± SD for n = 3- 4; $p < 0.03$ (fresh GSH-enriched vs fresh not GSH-enriched) or mean±range; n=3 (fresh not-lysed and all stored samples)

DISCUSSION

Previous work from our laboratory has demonstrated that banked RBCs show a time dependent decline in GSH levels concomitant with

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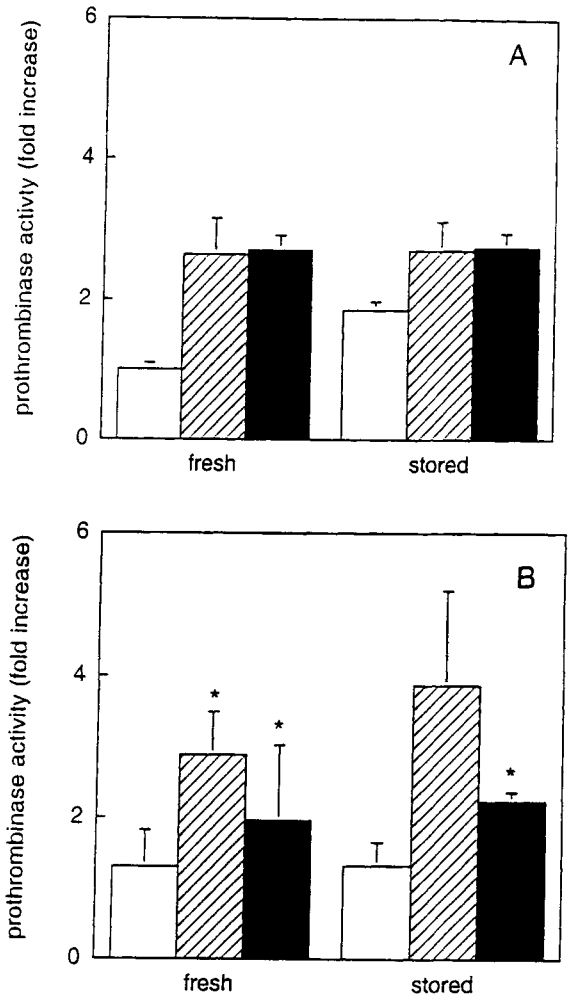

FIGURE 4 RBC GSH-loading reduces oxidant-induced increases in prothrombin converting activity. Fresh and 42 day stored RBCs (open bars) were lysed and resealed in the absence (shaded bar) or presence (filled bars) of GSH and prothrombin converting activity prior to (A) and after (B) a 1 h oxidant challenge with Fe/ASC. Prothrombin-converting activity was measured as described in **Materials and Methods** and is expressed as the fold-increase over freshly isolated cells. The values are reported as mean ± SD for n = 3 – 4; $p < 0.02$ (fresh GSH enriched vs. fresh not GSH-enriched) or mean ±range; n-=2 (all stored and all not-oxidized samples)

increase in oxidative modifications of lipid and protein[1,4]. GSH is an important intracellular antioxidant that regulates major housekeeping biochemical processes within the cell by maintaining reduced conditions of cytoplasmic proteins. GSH may be oxidized to GSSG, as a result of direct radical attack, or during the elimination of H_2O_2 and lipid peroxides by the activity of GSH-PX, which uses GSH as cofactor, or indirectly through GSH-requiring repair processes such as reduction of oxidized protein thiol groups.

Post-storage GSH-loading protects cells against oxidant damage

Conflicting data exist about the importance of GSH/GSH-PX and catalase in combating free radical injury, presumably due to differences in experimental conditions. These include the choice of oxidants used[23,24], use of intact cells versus cell free systems[6–7], augmentation with antioxidant versus depletion using specific inhibitors[25,26] and differences in biological-end points[22–26]. The results are further complicated as interactions between various cellular antioxidants are complex and can play similar roles (i.e. catalase and GSH-PX) or can operate in tandem to decompose free radicals to harmless products (i.e. SOD and catalase/GSH-PX). To circumvent these problems we have used direct augmentation with GSH or catalase to discriminate between the decrease in the scavenging capacity and the GSH-dependent repair processes that may initiate premature aging of the banked RBCs. Loss of integrity of lipid bilayers due to peroxidation, one of the most pertinent effects of oxidative damage[27], was used as a biological end-point in our studies. We selected AChE for evaluation for several biochemical and physiological reasons. Acetylcholinesterase activity can be conveniently measured in intact cells[28]. In addition, AChE activity is cell-age-dependent and we and others have previously observed a decline in AChE activity in banked RBCs[29,30]. We have also previously shown that AChE is enriched in membrane vesicles released during storage relative to parent RBC membrane and vesiculation has been correlated with spectrin oxidation in banked RBCs[3,4,29,31]. This excessive release could have important structural and

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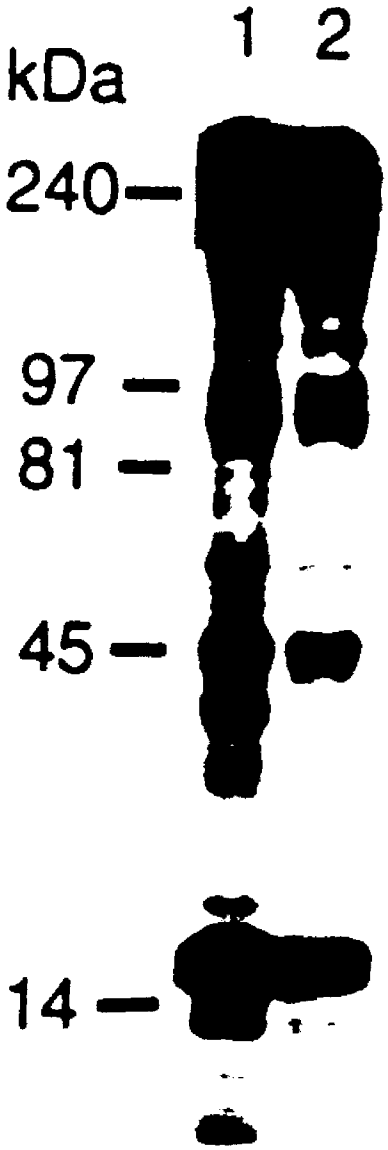

FIGURE 5 RBC membrane thiols were oxidized during storage. Reactive thiols in fresh and 42 day stored RBC membranes were labeled with of 5-IAF, electrophoresed and transferred to nitrocellulose membrane. Labelled thiols were detected by immunoblotting with anti-fluroscein antibody followed by reaction with HRP conjugated secondary antibody. A decrease in signal correspond to oxidation of reactive thiols. Lanes 1 and 2 represents membrane proteins fresh and stored RBC protein patterns respectively. n=4; $p<0.01$ (fresh vs. stored)

functional implications on RBC physical properties. Finally, oxidation might inactivate AChE directly or by generating reactive byproducts of lipids in close proximity to AChE[32].

GSH, rather than catalase, is required to protect cells from post-storage oxidation

Though several studies have documented the importance of catalase in RBC oxidant defense[6–8], we observed no change in catalase activity even in RBCs banked for 84 days or after oxidant challenge. In addition, inhibiting catalase with sodium azide did not increase the severity of the damage following oxidant challenge (UJD, unpublished observations). Furthermore, GSH, but not catalase, could partially protect AChE activity in both fresh and banked cells after oxidant challenge indicating that, by augmenting cellular GSH levels, free radical damage to cellular components can be curtailed. The degree of protection varied with the storage interval indicating that the protection afforded by GSH may be dependent on the balance between ability of RBCs to synthesize and/or regenerate GSSG and loss of GSSG from the cell[2]. In view of these observations, our results are in concert with our previous report and the hypothesis of Eaton et al that GSH and GSH-dependent enzyme GSH-peroxidase are needed to protect against the low levels of free radical that are continuously generated endogenously during storage[1,33]. Therefore, further studies were conducted to ascertain whether augmented levels of reduced GSH could protect banked RBC against oxidative stress, specially markers of lipid asymmetry.

GSH plays a significant role in the maintenance of banked RBC lipid asymmetry

To directly measure the effect of oxidant challenge on banked RBC membrane lipid organization, specific assays for aminophospholipid transport, lipid flip-flop and PS externalization were used to assess oxidant-induced damage. It

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is conceivable that altered aminophospholipid transport and passive flip-flop results in a consequent loss of aminophospholipid asymmetry in RBCs during storage. This membrane perturbation may also be liable for an increase in membrane associated globin[34], loss of membrane lipids[35], oxidation of skeletal proteins, loss of cellular antioxidant capability[36] and the appearance of "senescent antigen" like structures[37], leading to premature aging of the RBCs *in vitro*. Loss of lipid asymmetry and the appearance of PS in the outer leaflet enhances RBC-binding to macrophages or monocytes[38–41]. Thus, the appearance of PS on the surface of stored cells, or the reduced capacity of stored cells to defend themselves against oxidant stress, would result in a decreased lifespan of these cells *in vivo*.

Geldwerth et al.[42] demonstrated that RBC storage alters the kinetics of transbilayer mobility of PE, PS and phosphatidylcholine, and the activity of aminophospholipid translocase. More recently, the same group reported RBC-PS exposure using annexin V-ferrofluid during storage in saline-adenine-glucose-mannitol (SAG-M) preservative[43]. We have previously shown a selective loss of PE asymmetry in RBC stored in Adsol[3,4]. Given the apparent correlation between oxidative events and loss of phospholipid asymmetry in banked RBCs, we investigated the direct effect of GSH on the oxidative stress induced flip-flop of phospholipids across the membrane. Phosphatidylserine exposure, as determined by prothrombin-converting activity, was significantly lower in banked RBCs loaded with GSH than in RBCs not-enriched. Active restoration of PS asymmetry by flippase and or suppression of scramblase activity would counteract the exposure of aminophospholipids and yield lower prothrombinase activation. Our data also imply that flippase activity is not protected by subsequent GSH-loading in banked RBCs and that the major contributing factor for maintenance/loss of asymmetry in banked RBCs may not be attributed to oxidative modification or loss of flippase activity. However, GSH-loading provided an antioxidative defense to stored RBCs by suppressing flip-flop and subsequent PS exposure.

GSH-depletion during storage results in protein sulfhydryl group oxidation

The decline in membrane protein thiol levels in stored RBCs is consistent with the lower GSH levels and the lower GSH-dependent GSH-PX activity reported previously[1]. This decline was found in several RBC proteins, especially those in the 45, 81 and 96 kDa regions, suggesting that bands 3, 4.1 and actin are oxidized during storage. These results are consistent with our previous report in which we observed greater aggregation of RBC protein band 3 and carbonylation of band 4.1. Further work is needed to identify the location of oxidized sulfhydryl-groups and to assess their importance in cellular metabolism. Regardless, better maintenance of RBC GSH-content could prevent protein and lipid oxidation and eventually prevent hemichrome-band 3 aggregation[44].

Role of glucose in storage-induced oxidation

The mechanism by which storage induces oxidative stress, and a reduction in antioxidant capability, is not clear. However, the damage may be due to the effect of hyperglycemia during storage. The storage media used in these studies (Adsol) contains a high concentration of glucose (122 mM). When mixed with RBCs, the final concentration of glucose in the blood-preservative mixture remains high (~35 mM, 600–630 mg/dL) compared to normal plasma glucose concentrations. Previous studies have shown that incubation of RBCs with high concentrations of glucose (20–50 mM) at 37°C induces a time- and concentration-dependent loss of transbilayer lipid asymmetry ($t_{1/2}$= 6 h and EC_{50} = 12 mM, respec-

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tively) and subsequent conversion to a procoagulant state[10]. This lipid randomization is likely due to an increase in passive lipid flip-flop resulting from increased oxidant stress (MJW and DLD unpublished observations). Experimental conditions described in the present work are similar to those used in these studies. The most significant differences are temperature (37°C vs. 1–6°C), hematocrit (5 % vs. ~ 55 %), and buffer composition. It is feasible that the effect of Adsol on RBC oxidative stress may be identical to the effects of acute hyperglycemia at higher temperatures, but that the effect requires a longer time due to the lower temperature of incubation.

CONCLUSIONS

The present results show that Fe/ASC-induced oxidative stress, 1] increases prothrombinase activity and passive flip-flop, indicators of membrane asymmetry, and 2] decreases AChE activity. These effects were countered by GSH-enrichment of fresh and stored RBCs. Surprisingly, catalase failed to prevent loss of GSH and loss of AChE activity in banked RBCs. The significant decline in the reactive thiols of RBC membrane proteins induced by storage supports the hypothesis that thiol oxidation may make RBCs susceptible to further oxidative insults. These results suggest that GSH protects RBCs against the oxidant stress-mediated loss of AChE activity, increased lipid flip-flop and increased PS exposure and disturbed phospholipid asymmetry. Augmenting banked RBCs with GSH may protect RBC membrane from this damage. Studies designed to accentuate RBC GSH levels by non-invasive methods and to evaluate the effect of this treatment on membrane integrity and RBC function are underway.

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