Hemoglobin Oxidation Products Extract Phospholipids from the Membrane of Human Erythrocytes†

Michael S. Moxness,‡ Linda S. Brunauer,§ and Wray H. Huestis*,||

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota 55905, Department of Chemistry, Santa Clara University, Santa Clara, California 95053, and Department of Chemistry, Stanford University, Stanford, California 94305

Received September 11, 1995; Revised Manuscript Received February 19, 1996⊗

ABSTRACT: Hydrogen peroxide oxidation of human erythrocytes induces a transfer of phospholipid from the membrane into the cytosol [Brunauer, L. S., Moxness, M. S., & Huestis, W. H. (1994) *Biochemistry 33*, 4527−4532]. The current study examines the mechanism of lipid reorganization in oxidized cells. Exogenous phosphatidylserine was introduced into the inner monolayer of erythrocytes, and its distribution was monitored by microscopy and radioisotopic labeling. Pretreatment of cells with carbon monoxide prevented both hemoglobin oxidation and the transfer of phosphatidyserine into the cytosolic compartment. The roles of the various hemoglobin oxidation products in lipid extraction were investigated using selective oxidants. Nitrite treatment of intact cells produced almost complete conversion to methemoglobin, but no detectable lipid extraction. Treatments designed to produce the green hemoglobin derivatives, sulfhemoglobin and choleglobin, resulted in cytosolic extraction of phosphatidylserine. Ion exchange and size exclusion chromatography of oxidized cytosolic components revealed a lipid−hemoglobin complex. The interaction between lipid and hemoglobin oxidation products was verified in a model system. Purified hemoglobin, enriched in sulfhemoglobin and choleglobin by treatment with H_2O_2, H_2S, or ascorbate, extracted phospholipid from small unilamellar phospholipid vesicles. Electron paramagnetic resonance studies demonstrated that hemoglobin oxidation products also adsorb fatty acids from solution. This newly described activity of hemoglobin may play a role in the clearance of oxidatively damaged and senescent cells from circulation.

As a result of their oxygen carrying function, erythrocytes accumulate oxidative damage during their life span in circulation. Protein aggregation, enzyme degradation, and lipid peroxidation have been shown to accompany red cell aging (Kannan et al. 1991; Jain, 1988; Jimeno et al., 1991) and are accelerated in conditions such as diabetes and sickle cell anemia (Hebbel, 1990; Wolff et al., 1991). In addition, denser senescent erythrocytes are more susceptible to phagocytosis by macrophages (Bennet & Kay, 1981), but the signal that initiates destruction has not been identified. Studies using hydrogen peroxide to mimic the effects of aging suggest that increased phospholipid spacing in the outer leaflet of the plasma membrane stimulates phagocytosis (Pradhan et al., 1990). Previously, we demonstrated that hydrogen peroxide treatment induces a transfer of phosphatidylserine (PS)[1] from the inner leaflet of the membrane into the cytosol of RBC (Brunauer et al., 1994). This extraction occurs without covalent modification of the lipid or endovesiculation of the membrane and may play a role in the biophysical alterations that accompany senescence.

The current study further characterizes cytosolic extraction of membrane lipid from oxidized RBC. The role of soluble cytosolic components in binding membrane lipids was assessed. Hemoglobin (Hb), the most abundant protein component and a conspicuous target of oxidation, is a likely candidate for the extracting species.

The oxidative sensitivity of Hb has been the subject of extensive study. Hydrogen peroxide is produced intracellularly by autoxidation (Winterbourn, 1990) and extracellularly by neutrophils (Weiss, 1982). Giulivi and Davies (1990) reported that two oxidation equivalents of peroxide convert Hb Fe(II) to Fe(IV) or ferrylHb. The Fe(IV) protein is present only transiently, reacting in a comproportionation reaction with another Fe(II) molecule to produce 2 Fe(III) molecules. The Fe(III) protein can react further with H_2O_2, producing an Fe(IV) species with an oxidized tyrosine in the globin moiety. This species can degrade to the so-called green hemoglobin derivatives, choleglobin or sulfhemoglobin (sulfHb) (Katsumata et al., 1985; Winterbourn, 1990). The role of these Hb oxidation products in PS extraction was investigated.

MATERIALS AND METHODS

Materials. L-[3-^{3}H]serine (sp act. 20 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin (BSA), *Streptomyces* species phospholipase D (Type VII), DEAE Sephadex A-50, and serine were obtained from Sigma Chemical Co. (St. Louis, MO).

† This work was supported by NIH Grant HL 23787, a William and Flora Hewlett grant of Research Corporation, and an Arthur Vining Davis Junior Faculty Fellowship awarded by Santa Clara University.

* To whom correspondence should be addressed.

‡ Mayo Clinic.

§ Santa Clara University.

|| Stanford University.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: bovine serum albumin (BSA), dilauroylphosphatidylserine (DLPS), dilauroylphosphatidyl-L-[3-^{3}H]serine (^{3}H-DLPS), dimyristoylphosphatidylcholine (DMPC), electron paramagnetic resonance (EPR), ferrylhemoglobin (ferrylHb), hemoglobin (Hb), hematocrit (Hct), liquid scintillation counting (LSC), methemoglobin (metHb), morphological index (MI), oxyhemoglobin (HbO_2), phosphate buffered saline (PBS), red blood cells (RBC), sulfhemoglobin (sulfHb), small unilamellar vesicles (SUV).

S0006-2960(95)02167-2 CCC: $12.00

Sephacryl S300 and CM-Sephadex C-50 were purchased from Pharmacia, Inc. (Piscataway, NJ). 12-Doxylstearic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade.

Hb was purified as described previously (Winterbourn, 1990). The Hb fractions were combined and concentrated in Centriprep-30 concentrators (Amicon Division, W. R. Grace, Inc. (Beverly, MA)), assayed by Drabkin's method (Zijlstra & Van Kampen, 1960), and stored in liquid nitrogen.

Dilauroylphosphatidylserine (DLPS) was synthesized as described previously (Hall & Huestis, 1994; Hermetter et al., 1982). Radiolabeled DLPS (^{3}H-DLPS) was synthesized by phospholipase D-catalyzed headgroup exchange (Comfurius et al., 1990) of dilauroylphosphatidycholine with L-[3-^{3}H]serine (1 Ci/mol). DLPS and ^{3}H-DLPS were purified chromatographically using CM52 cellulose (Whatman, Inc., Clifton, NJ).

Cells. Human RBC were obtained from healthy adult volunteers by venipuncture and collection into citrate anticoagulant. RBC were washed by repeated centrifugations (3000*g*, 5 min) in 150 mM NaCl. RBC were stored at 5 °C in 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4 (PBS), and used within 24 h. Carbon monoxide-treated cells were prepared by gently bubbling CO gas through a 20% Hct RBC suspension until conversion to carboxyHb was complete, as indicated by spectral analysis. All buffers were preequilibrated with CO, and cell−vesicle incubations were conducted under low ambient light to minimize CO dissociation.

Vesicle Preparation. Lipid stocks were prepared in chloroform and stored at −20 °C under argon. Small unilamellar vesicles (SUV) were prepared by removing the chloroform under a gentle stream of argon, suspending the lipid in magnesium-free PBS, and sonicating the suspension to clarity, under argon, in a bath sonicator.

Cell−Vesicle Incubations. Incubations were initiated by mixing RBC with suspensions of lipid vesicles to yield a final hematocrit (Hct) of 50%; the concentration of lipid in these mixtures is indicated in the figure legends. Incubations were carried out at 37 °C, and unincorporated lipid vesicles were removed by washing in PBS.

Oxidation. Cells were incubated at room temperature with various oxidants (concentrations as noted in figure legends) at a final Hct of 5%. Cells oxidized with H_2O_2 were pretreated with 1 mM sodium azide to inhibit catalase. Purified Hb (3 mM) was treated with oxidant (concentrations given in figure legends) either at room temperature (H_2O_2 or $K_3Fe(CN)_6$) or at 37 °C (H_2S or ascorbate).

Cell Morphology. Samples were prepared for morphological analysis by fixation in 10−20 volumes of buffered glutaraldehyde (1% w/v in PBS). Cells were examined by light microscopy and scored using a previously described grading scale (Bessis, 1973; Ferrell et al., 1985). Echinocytes were assigned scores of +1 to +5, reflecting the degree of crenation; discocytes were scored 0; and stomatocytes were assigned scores of −1 to −4, depending on the degree of invagination. The average score of a field of 100 cells is defined as the morphological index (MI). The variation observed in counting replicate fields of cells was typically 0.1−0.15 MI unit.

Separation of Membrane and Cytosol. Erythrocytes containing radiolabeled DLPS were incubated at 0 °C for 30 min in 20 volumes of lysis buffer (5 mM Na_2HPO_4, pH 8) and then centrifuged (10 000*g*, 30 min). The bulk of the supernatant (greater than 80%) was removed from the pellet, and aliquots of the two fractions were assayed for radioactivity. Quantitative removal of supernatant from the pellet was not attempted to avoid contamination of the cytosolic fraction (supernatant) with membrane components (pellet).

Lipid Extraction from Model Membranes. Radiolabeled sonicated vesicles were prepared as above, containing 1−5 nCi of ^{3}H-DLPS in 0.5 mM dimyristoylphosphatidylcholine (DMPC) carrier lipid.[2] Vesicles were incubated in PBS containing oxidized or unoxidized Hb (2 mM heme), prepared as above. After 30 min incubation at room temperature, protein−lipid complexes were isolated by size exclusion and cation exchange chromatography (see below).

Assay for Radioactivity. Samples containing Hb and radiolabeled lipids were bleached for 2 h in 0.1 volume of 30% H_2O_2 at 60 °C; 10 mM sodium azide was added 10 min prior to bleaching in order to prevent foaming. Samples were counted in Ecolite (ICN Biomedicals, Inc., Irvine, CA) with a Model LS 3801 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA) using standard quench curves to calculate disintegrations per minute.

Hemoglobin Analysis. Hb samples were diluted into Drabkin's reagent (Zijlstra & Van Kampen, 1960). After 15 min at room temperature, the absorbance was recorded and the Hb concentrations were determined. In separate experiments, Hb solutions of 50 μM were obtained by dilution of purified Hb or by lysis of cell samples in 0.2% Triton X-100 buffers. An absorbance spectrum (400−800 nm) was taken of each sample on a HP 8452 spectrophotometer (Hewlett-Packard Co. (Palo Alto, CA)). Hb species were quantitated by solving simultaneous equations with previously published extinction coefficients for oxyHb, metHb, and either ferrylHb or sulfHb (Zwart et al., 1986; Whitburn, 1982). Choleglobin was estimated from the absorbance at 700 nm using a millimolar absorption coefficient of 4 (French et al., 1978). Since the absorbance spectrum of choleglobin is relatively flat, its contribution was eliminated before multicomponent analysis by subtracting the absorbance at 700 nm across all wavelengths of each spectrum (Winterbourn, 1990).

Size Exclusion Chromatography. Formation of protein−lipid complexes was demonstrated by size exclusion chromatography. Cytosol (800 μL, 1 mM heme) containing radiolabel was applied to a Sephacryl S300 gel filtration column (1 × 45 cm) and eluted with column buffer (40 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). Each fraction was assayed for radiolabel by liquid scintillation counting (LSC) and for Hb by Drabkin's method. In separate experiments, Hb−vesicle mixtures from model studies were subjected to size exclusion chromatography as above, and fractions were analyzed for radioactivity and Hb.

Cation Exchange Chromatography. Cytosol (800 μL, 1 mM heme) containing radiolabel was applied to a CM-Sephadex C50 cation exchange column (1 × 25 cm) equilibrated with column buffer (50 mM Na_2HPO_4, pH 6.5). Hb was eluted with a linear 0−0.5 M NaCl gradient. Each fraction was assayed for radiolabel by LSC and for Hb by Drabkin's method. Hb and vesicles eluted reproducibly in

[2] DMPC has no sites of unsaturation and is insensitive to oxidative damage; in addition, the lipid does not aggregate under the experimental conditions employed, making it an appropriate choice as a carrier lipid.

the expected fractions. In separate experiments, Hb−vesicle mixtures were applied to CM-Sephadex C50 cation exchange columns (1 × 4 cm) equilibrated with column buffer (40 mM NaCl, 10 mM Na_2HPO_4, pH 6). Vesicles were eluted with 6 mL of column buffer at pH 6, and Hb was eluted with 6 mL of column buffer at pH 8. Four fractions were collected and analyzed by LSC.

Electron Spin Resonance Spectroscopy. Separate Hb aliquots were treated with buffer or one of the following reagents: 1.5 equiv (in relation to heme) $K_3Fe(CN)_6$, 1 equiv H_2O_2, 5 equiv ascorbate, 5 equiv H_2S, and 1.5 equiv $K_3Fe(CN)_6$ followed by 4 equiv H_2O_2. Potential spin label reductants (H_2S, ascorbate or $Fe(CN)_6^{4-}$) were then removed by passage through G-25 Sephadex columns. Each Hb species (2 mM heme) was incubated with 12-doxylstearic acid (0.5 mM) for 30 min at room temperature. An aliquot (25 μl) of the mixture was taken up in a capillary tube and examined using a Varian Model E-109 EPR spectrometer (Varian Associates, Inc. (Palo Alto, CA)) at room temperature. The contributions of free and immobilized spin label to each spectrum were analyzed with EPR software provided by the Laboratory of Molecular Biophysics, NIH.

RESULTS

Shape Change and Cytosolic Extraction Assays in RBC Containing Carboxyhemoglobin. Heme oxidation was inhibited by treating cells with carbon monoxide (CO) prior to exposure to oxidants. Carboxygenated and oxygenated cells were incubated with DLPS vesicles and allowed to convert to stable stomatocytic shapes, as the incorporated exogenous lipid was translocated to the membrane inner monolayer. Subsequent treatment with H_2O_2 produced little or no shape change in the CO-treated cells (Figure 1A). Under the same conditions, the oxygenated cells reverted to discoid and echinocytic morphologies (Figure 1B).

Oxygenated and carboxygenated erythrocytes were incubated with ^{3}H-DLPS to permit assay for lipid in the cytosol. H_2O_2 induced the extraction of radiolabeled lipid by cytosolic components in oxygenated RBC (Table 1). Radiolabel extraction into cytosol was 4- to 20-fold lower in CO-pretreated cells. In oxygenated cells, both reversion from stomatocyte to discocyte morphology and appearance of cytosolic radiolabel correlated consistently with the red-to-brown color change of oxidized heme, and with the appearence of choleglobin. At the highest oxidant concentrations tested, CO-pretreated cells exhibited some increases in DLPS solubilization, which correlated well with a small increase in heme oxidation evident in the visible spectrum.

Chromatography of Cytosol from RBC Treated with H_2O_2. The association of ^{3}H-DLPS with Hb was examined by size exclusion and ion exchange chromatography. After incubation with ^{3}H-DLPS, erythrocytes were oxidized with H_2O_2. The cytosolic fraction was isolated by hypotonic lysis and centrifugation to remove membrane fragments. The soluble fraction was analyzed by size exclusion chromatography (Figure 2A) on Sephacryl S300. Residual membrane fragments are expected to elute from this material in the void volume (fractions 19−21), and small molecules such as lipid monomers elute in fractions 44−46. Hb and radiolabel coeluted at an intermediate volume, fractions 30−38.

To verify that radiolabeled lipid is bound to Hb, the cytosolic fraction was analyzed by cation exchange chromatography on CM-Sephadex. The column was developed using a 0−0.5M NaCl gradient (Figure 2B). At pH 6.5, Hb and ^{3}H-DLPS coeluted in fractions 65−71.

Shape Change Assay with Different Oxidants. To identify which oxidation product or products were responsible for

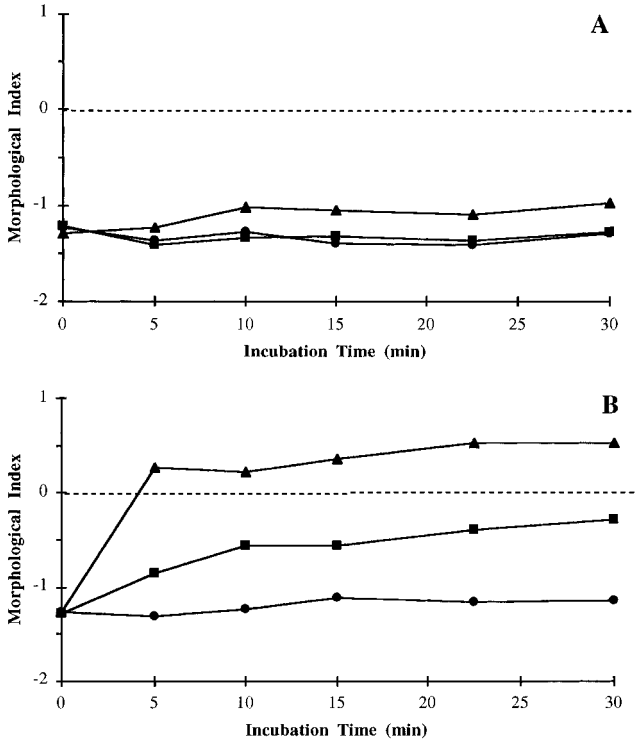

FIGURE 1: Effect of carbon monoxide on H_2O_2-induced shape reversion. RBC were equilibrated with carbon monoxide by bubbling CO through a 20% Hct cell suspension for 30 min at room temperature. RBC (50% Hct) were made stomatocytic by incubation with 100 μM DLPS vesicles at 37 °C for 60 min. Both CO-treated (A) and oxygenated (B) RBC were then incubated with 0 mM H_2O_2 (circles), 0.2 mM H_2O_2 (squares), and 0.8 mM H_2O_2 (triangles). At the times indicated, aliquots of cells were fixed and MI's were calculated. The dotted line indicates a morphological index of 0.

Table 1: Effect of Carbon Monoxide on Cytosolic Extraction of ^{3}H-DLPS and Choleglobin Formation[a]

H_2O_2 pretreatment (no. of heme equiv)	CO pretreatment	% ^{3}H-DLPS in cytosol	% choleglobin (% of total Hb)
0.0	−	13.8 ± 0.1	0.6 ± 0.3
0.1	−	20.5 ± 1.4	5.9 ± 0.9
0.2	−	39.5 ± 0.3	8.9 ± 1.1
0.4	−	53.3 ± 0.2	15.3 ± 0.1
0.8	−	57.6 ± 0.9	20.7 ± 0.7
1.0	−	60.3 ± 0.9	23.3 ± 0.4
2.0	−	62.9 ± 0.7	26.0 ± 0.1
4.0	−	63.2 ± 1.6	27.8 ± 0.1
0.0	+	13.1 ± 0.6	0.7 ± 1.3
0.1	+	13.7 ± 0.2	0.9 ± 1.1
0.2	+	16.6 ± 0.1	1.6 ± 0.9
0.4	+	23.2 ± 0.7	2.7 ± 1.6
0.8	+	28.2 ± 0.6	2.7 ± 0.5
1.0	+	31.5 ± 0.2	2.8 ± 1.3
2.0	+	34.0 ± 1.1	5.5 ± 0.9
4.0	+	33.8 ± 1.1	7.3 ± 0.9

[a] RBC were equilibrated with carbon monoxide by bubbling CO through a 20% Hct cell suspension for 30 min at room temperature. CO-treated and oxygenated RBC were then incubated with ^{3}H-DLPS vesicles at 37 °C for 60 min. Catalase was inhibited with 1 mM sodium azide, followed by incubation with various concentrations of H_2O_2. The cells were lysed, and the cytosolic aliquot was assayed for radiolabel and for choleglobin after separation by centrifugation.

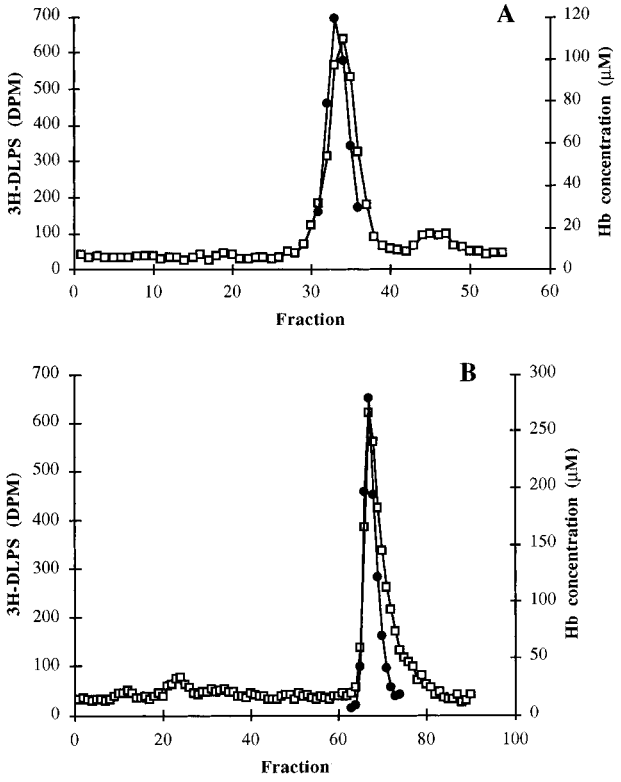

FIGURE 2: Chromatography of cytosol from oxidized RBC. ^{3}H-DLPS was introduced into the inner monolayer of erythrocyte membranes by incubation with lipid vesicles. Cells were then treated with 2 mM H_2O_2 at 5% Hct and subsequently lysed in 5 mM sodium phosphate, pH 8. The cytosol was separated from the membrane by centrifugation, and an aliquot was applied to a Sephacryl S300 gel filtration column (A) or a CM Sephadex column (B). Fractions (0.8 mL) were assayed for radiolabel by LSC (squares) and Hb by Drabkin's assay (circles).

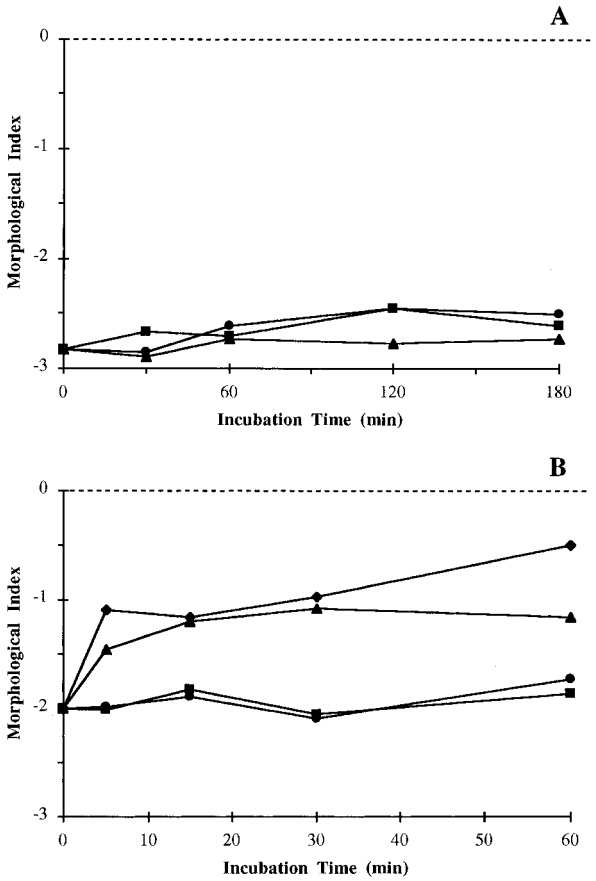

FIGURE 3: Effect of nitrite and H_2S on the morphology of RBC preloaded with DLPS. Erythrocytes (50% Hct) were pretreated at 37 °C with 125 μM DLPS for 1.5 h. Cells at 5% Hct were then treated with (A) 0 mM (circles), 1 mM (squares), or 5 mM (triangles) NO_2^- , or (B) 0 mM (circles), 2 mM (squares), or 5 mM (triangles) H_2S at room temperature. At the times indicated, aliquots were fixed and MI's were calculated. The dotted line indicates a morphological index of 0.

movement of DLPS from the membrane into the cytosol, RBC were treated with oxidants that generate different oxidized heme products. RBC made stomatocytic by incubation with DLPS were treated with sodium nitrite, which converts HbO_2 almost exclusively to metHb (Bunn & Forget, 1986; van Assendelft & Zijlstra, 1965). This oxidation did not induce reversion from stomatocytic morphology, indicating little DLPS extraction by cytosolic components (Figure 3A). No method is known to produce choleglobin exclusive of other oxidation products in the intact cell. However, a protein that has undergone a similar heme modification, sulfHb, can be produced by incubating cells with H_2S (Sigaard-Andersen et al., 1972). H_2S treatment of cells produced partial conversion of HbO_2 to sulfHb (Table 2) and caused shape reversion of DLPS stomatocytes to discocytes (Figure 3B). The reversion was similar to but not as extensive as the shape alterations seen in H_2O_2-treated RBC (Figure 1B).

Cytosolic Extraction by Different Hemoglobin Oxidation Products. In separate experiments, extraction of lipid into the cytosolic compartment was quantified using radiolabeled lipid. Cells were treated with ^{3}H-DLPS under conditions allowing transport to the membrane inner monolayer. Cells were then treated with H_2O_2, nitrite, or H_2S, followed by lysis and separation into cytosolic and membrane fractions by centrifugation. Nitrite treatment (yielding 87% metHb) produced little change in cytosolic PS (Table 2). In contrast, cells treated with H_2S (yielding sulfHb) showed a 3- to 5-fold

Table 2: Effect of Different Oxidants on Cytosolic Extraction of Lipid[a]

treatment	% cytosolic DLPS	% oxyHb	% metHb	% ferrylHb	% sulfHb	% choleglobin
control	6.8 ± 0.33	97	1	1	0	1
2 equiv of H_2O_2	63 ± 1.2	22	34	15		29
2 equiv of NO_2^-	7.2 ± 0.39	78	20	2		0
5 equiv of NO_2^-	7.8 ± 0.35	6	87	4		2
2 equiv of H_2S	22 ± 0.80	83	0		13	4
5 equiv of H_2S	36 ± 1.6	67	0		25	9

[a] RBC were incubated with ^{3}H-DLPS vesicles for 60 min at 37 °C. Cells were then oxidized with the agents shown and subsequently lysed. Oxidant quantities correspond to heme equivalents. The cytosolic and membrane fractions were separated by centrifugation and assayed for radiolabel.

increase in cytosolic lipid. Consistent with the shape reversion result, the most extensive cytosolic extraction was generated by H_2O_2 treatment, which produces choleglobin as well as metHb (Table 2).

Correlation between Choleglobin Generation and DLPS Extraction. Cells preequilibrated with either CO or O_2 were labeled with ^{3}H-DLPS, and then incubated with increasing concentrations of H_2O_2 (0−2 mM; the latter concentration corresponds to 2 heme equiv). After 30 min at room temperature, cells were lysed and isolated supernatant was analyzed for choleglobin and ^{3}H-DLPS. A positivecorrela-

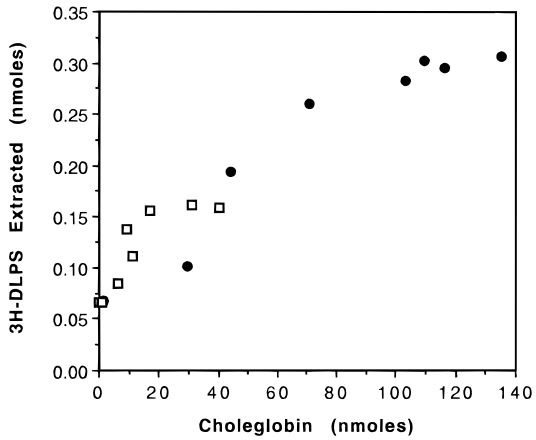

FIGURE 4: Choleglobin concentration and extraction of ^{3}H-DLPS. ^{3}H-DLPS was introduced into the inner monolayer of control and CO-pretreated RBC by incubation with lipid vesicles for 1 h at 37 °C. Control (closed circles) and HbCO (open squares) RBC were then incubated with increasing concentrations (0−2 mM) of H_2O_2 for 30 min at room temperature. Cytosolic fractions were isolated and analyzed for choleglobin and ^{3}H-DLPS as described in Materials and Methods.

tion was found between choleglobin generation and cytosolic DLPS (Figure 4).

Cation Exchange Chromatography of Hb−Vesicle Mixtures. The interaction between protein and phospholipid was examined in a model system consisting of purified human Hb and synthetic sonicated lipid vesicles. Hb was incubated with SUVs containing DMPC and ^{3}H-DLPS. Hb−vesicle mixtures were applied to short cation exchange columns and eluted with a pH step gradient. In samples containing HbO_2 or metHb, less than 10% of the lipid radiolabel coeluted with hemoglobin (Table 3). When Hb was pretreated with H_2O_2, there was a 7-fold increase in radiolabel coeluting with the oxidized Hb. The binding of PS to choleglobin, sulfHb, and ferrylHb was investigated in a similar manner. Choleglobin, produced by incubation of isolated Hb with ascorbate (Lemberg & Lakewood, 1941), extracted 53% of the ^{3}H-DLPS from DMPC vesicles (Table 3). H_2S treatment (Sigaard-Andersen et al., 1972), which produces predominantly sulfHb, induced less extensive lipid extraction. FerrylHb, produced by H_2O_2 oxidation of metHb, induced a comparable degree of lipid extraction. Both ferrylHb and sulfHb preparations contained choleglobin as a minor but significant component (Table 3).

Electron Paramagnetic Resonance (EPR) Studies of Hb−Lipid Mixtures. The association of oxidized Hb with fatty acids was investigated by EPR. 12-Doxylstearic acid is sufficiently soluble to yield an EPR spectrum typical of a fully mobile spin label (Figure 5A). Addition of bovine serum albumin (BSA, a lipid scavenger similar in size to Hb) to a solution of 12-doxylstearic acid generated an immobilized spectrum, consistent with formation of a slowly tumbling protein−lipid complex (Figure 5B). 12-Doxylstearic acid incubated with HbO_2 or metHb showed no evidence of immobilization (Figure 5C,D). The spectrum of 12-doxylstearic acid with H_2O_2-treated Hb is a combination of bound and free spin components (Figure 5E). Similar results were seen with ascorbate- and H_2S-treated Hb (Figure 5F,G). FerrylHb destroyed the spin label (Figure 5H). Using the spectra of free 12-doxylstearic acid and its BSA complex as standards, the contribution of free and Hb-bound spin labels was determined for the spectrum of each sample (Table 4). The fraction of immobilized label correlated positively with oxidation conditions that generate choleglobin and sulfHb.

DISCUSSION

The phospholipid organization of RBC may be studied by incorporation of synthetic lipids that distribute according to headgroup structure. PS generates stomatocytes by preferentially localizing in the inner monolayer; distribution of PS may then be monitored by examining cell shape by light microscopy (Daleke & Huestis, 1985). In a recent study, erythrocyte shape changes were used to examine the effects of oxidative stress on maintenance of membrane phospholipid asymmetry in RBC (Brunauer et al., 1994). Treatment with H_2O_2 induced alterations in RBC morphology; normal discocytes became echinocytic while stomatocytes generated by incubation with foreign PS reverted toward discoid morphology. Within the framework of the bilayer couple model, these shape changes are consistent with movement of inner monolayer lipids to the outer monolayer. However, H_2O_2 treatment did not result in an increased exposure of PS on the cell surface. Instead, ^{3}H-DLPS was demonstrated to be associated with the cytosolic fraction of oxidized RBC, suggesting that H_2O_2 induces alterations in the organization of RBC membrane phospholipids by promoting extraction of inner monolayer components into the cytosol.

These results implicate a cytosolic component as the extracting agent in oxidized RBC. A likely candidate is hemoglobin, an oxidation-sensitive protein abundant in RBC cytosol. The results of the present study indicate that oxidation of Hb is a requirement for both H_2O_2-induced shape change and PS redistribution. Carbon monoxide treatment of erythrocytes inhibits oxidation of Hb by H_2O_2. Carbon monoxide also prevents the shape reversion of RBC made stomatocytic by DLPS (Figure 1); in addition, extraction of ^{3}H-DLPS into the cytosol is inhibited significantly in cells that have been treated with CO followed by H_2O_2 (Table 1). These findings demonstrate a correlation between Hb oxidation and cytosolic extraction of membrane components, suggesting that Hb derivatives solubilize phospholipids. This conclusion is strengthened by the results of chromatographic fractionation of cytosol from ^{3}H-DLPS and H_2O_2-treated RBC; radiolabel and Hb coelute during both gel filtration (Figure 2A) and ion exchange chromatography (Figure 2B), indicating that ^{3}H-DLPS is bound to Hb or one of its oxidation products.

The reaction between Hb and H_2O_2 has been studied *in vitro* (Giulivi & Davies, 1990; Winterbourn, 1990) and in intact cells (van den Berg et al., 1992; Weiss, 1982). The predominant reaction product is metHb, with choleglobin a minor product. FerrylHb is the intermediate in the formation of both products, but it is rapidly reduced to metHb or degraded to choleglobin. The Hb oxidation product correlated with lipid extraction was characterized using several oxidants that have better defined products. Nitrite induces rapid metHb formation but does not alter the shape of DLPS stomatocytes or cause cytosolic extraction of ^{3}H-DLPS. Therefore, metHb, the most abundant protein in H_2O_2-treated RBC, is not a lipid solubilizer.

Table 3: Cation Exchange Chromatography of Different Hemoglobin Species Incubated with Lipid Vesicles[a]

treatment	% Hb-bound DLPS	% oxyHb	% metHb	% ferrylHb	% sulfHb	% choleglobin
control	7.5	98	0	2	0	0
1.5 equiv of $K_3Fe(CN)_6$	3.4	2	95	0	-	2
2 equiv of H_2O_2	51.2	27	39	10		23
2.5 equiv of ascorbate	52.7	40	21	4		35
2.5 equiv of H_2S	28.3	71	14		7	8
1.5 equiv of $K_3Fe(CN)_6$, 4 equiv of H_2O_2	29.6	34	33	25		9

[a] Hemoglobin (3 mM) was oxidized with ferricyanide, H_2O_2, ascorbate, H_2S, or ferricyanide and H_2O_2. Oxidant quantities correspond to heme equivalents. The Hb samples were then incubated with DMPC/^{3}H-DLPS vesicles and separated on short cation exchange columns as described in Materials and Methods.

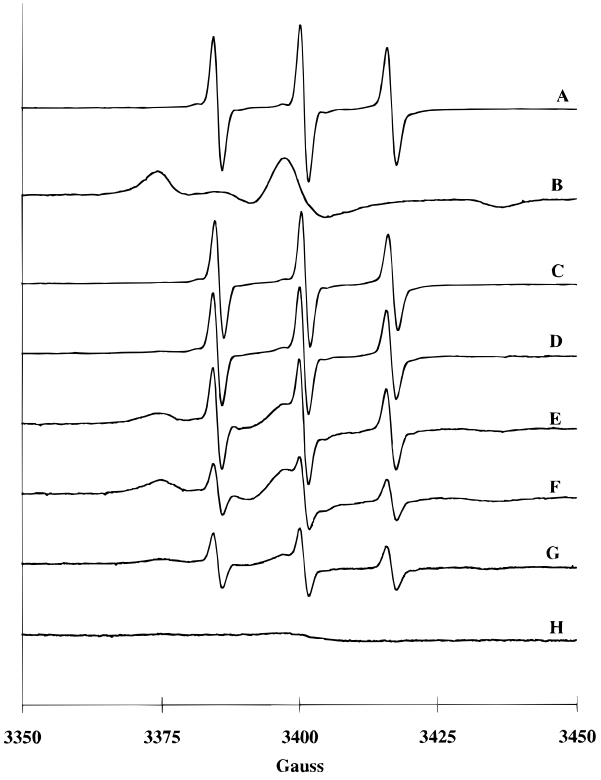

FIGURE 5: EPR spectra of 12-doxylstearic acid with Hb oxidation products. 12-Doxylstearic acid (0.5 mM) was incubated (A) alone or (B) with 6% w/v BSA. 12-Doxylstearic acid (0.5 mM) was also incubated with 2 mM Hb pretreated with the following reagents: (C) control (no treatment), (D) $K_3Fe(CN)_6$, (E) H_2O_2, (F) ascorbate, (G) H_2S, or (H) $K_3Fe(CN)_6$ followed by H_2O_2. After 30 min of incubation at room temperature, samples were analyzed by EPR spectroscopy.

Table 4: Doxylstearic Acid Binding to Hb Species[a]

	% of spin label	
sample	free	Hb bound
HbO_2	93.3	6.7
metHb	99.5	0.5
Hb + 1 equiv of H_2O_2	37.8	62.2
Hb + 5 equiv of ascorbate	23.2	76.8
Hb + 5 equiv of H_2S	45.0	55.0

[a] EPR spectra (Figure 4) were analyzed with software provided by the Laboratory of Molecular Biophysics, NIH. Double integrals of the ranges 3370−3380 and 3380−3390 G were calculated for each spectrum. The free 12-doxylstearic acid (Figure 5A) and BSA + 12-doxylstearic acid (Figure 5B) spectra were used as standards, and the concentrations of free spin label and Hb-bound spin label were determined for each sample.

While choleglobin cannot be formed in the intact cell without significant byproducts, H_2S treatment produces the related species sulfHb as the major oxidation product. SulfHb and choleglobin, collectively designated the green hemoglobin derivatives, are not well characterized, but are similar in having covalently modified porphyrin rings (Berzofsky et al., 1972; Lemberg & Lakewood, 1941). Generation of sulfHb induces shape reversion of DLPS stomatocytes and cytosolic extraction of ^{3}H-DLPS. In addition, ^{3}H-DLPS extraction correlates with the concentration of choleglobin, while no such correlation exists for ferryl or metHb.

These studies in intact cells implicate heme oxidation products (particularly choleglobin) in H_2O_2-dependent lipid solubilization. However, these Hb products are chemically reactive and could modify other cytosolic components that are responsible for lipid binding. It is conceivable that inhibition of Hb oxidation would also prevent a subsequent series of reactions involving other proteins and eventually leading to cytosolic extraction.

The identity of the lipophilic species was addressed further in model systems. Isolated Hb can be treated with oxidants that are inaccessible to the cytosol of intact RBC. Chromatography of lipid−Hb mixtures verifies that oxidation products of purified Hb form protein−lipid complexes (Table 3). The quantity of lipid extracted from SUV increases significantly upon incubation with oxidized Hb enriched in choleglobin and sulfHb. In contrast, HbO_2 and metHb do not extract phospholipid in model studies. These results correlate well with observations in the intact cell system.

Lipid motion, and thus binding to high molecular weight aggregates, can be monitored by EPR. The EPR spectrum of 12-doxylstearic acid is consistent with a freely tumbling spin label. Incubation with HbO_2 or metHb does not alter this spectrum significantly. However, when the spin labeled fatty acid is incubated with Hb treated with H_2O_2, ascorbate, or H_2S, its spectrum develops a significant immobilized component, consistent with protein binding. Thus, the green hemoglobin derivatives exhibit a general hydophobic affinity not limited to phospholipids (but not found with uncharged amphipaths such as benzyl alcohol and chlorpromazine (Brunauer et al., 1994)).

It should be noted that, like the spin labeled fatty acid, the short acyl chain phospholipids used in the present study are significantly more water soluble than endogenous lipids. If oxidized Hb induces a similar solubilization of native inner monolayer components, the process would be expected to occur on a much slower time scale than the shape changes monitored here. Consistent with that expectation, earlier work showed that cells treated with peroxide but no foreign lipid undergo a slow crenation (Brunauer et al., 1994). Experiments are in progress aimed at detecting any increase in cytosolic lipid in these prolonged (>24 h) incubations. A

further consideration is the oxidative susceptibility of endogenous phospholipids. In the present and previously published work, the rapidly extracted saturated lipids showed no detectable degradation. However, acyl chain oxidation and phospholipase degradation of endogenous lipids would be expected to generate relatively hydrophilic byproducts that might be subject to solubilization. Experiments in progress address this possibility.

The mechanism of Hb oxidation-linked lipid extraction is still unclear. Previously, thin layer chromatographic analysis of cytosol from H_2O_2-treated RBC demonstrated that radiolabeled DLPS is not altered as a consequence of treatment with H_2O_2. Thus, the lipid−Hb association is not due to an alteration in the structural characteristics of the lipid (Brunauer et al., 1994). This earlier work also demonstrated the capacity of organic solvents to remove intact lipid from a Hb−lipid complex, indicating that the association is not covalent. In sulfHb and choleglobin, the porphyrin is covalently modified or cleaved. Alteration in globin structure may expose a hydrophobic locus, in the heme pocket or elsewhere, that is accessible to lipid binding. Membrane phospholipids minimally soluble in aqueous medium may then be extracted via association with a soluble protein possessing a hydrophobic pocket. The present findings also do not address the stoichiometry of the protein−lipid complex or the site of binding. The data reported in Figure 4 show a molar ratio of choleglobin to lipid of approximately 300. This outcome may be influenced by the limitation on the amount of foreign PS that can be introduced into cells. Indeed, the reversion of the cells to discoid and slightly echinocytic forms indicates essentially complete extraction of inner monolayer exogenous lipid (exogenous DLPS is not sequestered quantitatively in the inner monolayer (Daleke & Huestis, 1989)). However, the observed ratio of choleglobin to solubilized lipid may indicate that the active species in the solubilization is a minor product that coincidentally is produced in proportion to choleglobin. Examination of this possibility and determination of binding stoichiometry and locus await development of protocols to purify and stabilize choleglobin.

Choleglobin and sulfHb are produced *in vivo* by oxidative stresses. Choleglobin is a normal product of Hb degradation that is further catabolized to bilirubin (Harris & Kellermeyer, 1970). Weiss (1982) has reported that stimulated neutrophils can convert up to 6% of intraerythrocytic Hb to choleglobin by generating H_2O_2 and superoxide. In addition, ingestion of sulfanilamide, dapsone, phenacetin, or acetaminophen may cause sulfhemoglobinemia (V. F. Fairbanks, personal communication). These drugs are converted by liver microsomes to phenylhydroxylamine, which oxidizes Hb (Bunn & Forget, 1986). Previous reports have shown that H_2O_2 treatment (Pradhan et al., 1990) and aging (Schlegel et al., 1987) of RBC increase the spacing of outer monolayer phospholipids. These workers also demonstrated an enhanced phagocytosis of oxidized cells by macrophages. The extraction of inner monolayer lipids by choleglobin along with other previously described membrane perturbations (Snyder et al., 1985; Chiu & Lubin, 1989) may play a role in cellular senescence and destruction.

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BI952167O