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TITLE PAGE

PRIMAQUINE-INDUCED HEMOLYTIC ANEMIA: ROLE OF MEMBRANE LIPID

PEROXIDATION AND CYTOSKELETAL PROTEIN ALTERATIONS IN THE

HEMOTOXICITY OF 5-HYDROXYPRIMAQUINE

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5-HPQ, 5-hydroxyprimaquine
ROS, reactive oxygen species
DCFDA, 2',7'-dichlorodihydrofluoresein diacetate
NEM, N-ethyl maleimide
HBSS, Hank's buffered saline solution
PBSG, isotonic phosphate-buffered saline with glucose
GC/MS, gas chromatography-mass spectroscopy
CH, cumene hydroperoxide
DTT, dithiothreitol

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ABSTRACT

Primaquine-induced hemolytic anemia is a toxic side effect that is due to premature splenic sequestration of intact erythrocytes. Previous studies have suggested that a phenolic metabolite, 5-hydroxyprimaquine (5-HPQ), mediates primaquine hemotoxicity by generating reactive oxygen species (ROS) within erythrocytes that overwhelm antioxidant defenses. However, the nature of the oxidative stress is not understood, and the molecular targets, whether protein and/or lipid, are unknown. To investigate the mechanism underlying the hemolytic activity of 5-HPQ, we have examined the effect of hemolytic concentrations of 5-HPQ on ROS formation within rat erythrocytes using the cellular ROS probe, 2',7'-dichlorodihydrofluoresein diacetate. In addition, we examined the effect of 5-HPQ on membrane lipids and cytoskeletal proteins. The data indicate that 5-HPQ causes a prolonged, concentration-dependent generation of ROS within erythrocytes. Interestingly, 5-HPQ-generated ROS was not associated with the onset of lipid peroxidation or an alteration in phosphatidylserine asymmetry. Instead, 5-HPQ induced oxidative injury to the erythrocyte cytoskeleton, as evidenced by changes in the normal electrophoretic pattern of membrane ghost proteins. Immunoblotting with an anti-hemoglobin antibody revealed that these changes were due primarily to the formation of disulfide-linked hemoglobin-skeletal protein adducts. The data suggest that cytoskeletal protein damage, rather than membrane lipid peroxidation or loss of phosphatidylserine asymmetry, underlies the process of removal of erythrocytes exposed to 5-HPQ.

INTRODUCTION

Malaria is a life-threatening parasitic disease of epidemic proportions, and it is a major public health concern for developing countries. Each year malarial infection is responsible for an estimated 300-500 million acute illnesses and 2 million deaths in tropical and subtropical regions worldwide (Kain and Keystone, 1998). Primaquine, the prototype 8-aminoquinoline tissue schizontocide, is effective against all four malarial species that infect humans and is the only drug approved for the radical cure of relapsing malaria. As resistance has developed from extensive use of blood schizontocides, such as chloroquine, the importance of primaquine in combating multiple drug resistance has increased, and it is now used in combination with several blood schizontocides for the treatment and prophylaxis of all forms of malaria, including the most lethal malarial species, *Plasmodium falciparum* (Shanks et al., 2001). Primaquine is also effective in the treatment of *Pneumocystis carinii* pneumonia, a common infection in AIDS patients (Toma et al., 1998). The clinical utility of primaquine, however, is restricted by its hemotoxicity, particularly in patients who are genetically deficient in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity (Beutler, 1990).

The hemolytic activity of primaquine has long been known to be due to an intra-erythrocytic oxidative stress that is mediated by redox-active metabolites rather than by the parent drug itself. The oxidatively damaged erythrocytes are thought to be recognized by splenic macrophages as the equivalent of senescent red cells, resulting in their selective removal from the circulation (Rifkind, 1966). 5-Hydroxyprimaquine (5-HPQ) is a putative human metabolite of primaquine that forms a redox pair with its quinoneimine form; continuous cycling of this redox pair is thought to generate reactive oxygen species (ROS) within erythrocytes (Vasquez-Vivar and Augusto, 1992). We have shown that when 5-HPQ is incubated with rat red cells *in vitro*,

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damage occurs within the cells such that they are removed rapidly from the circulation of isologous rats as compared to untreated controls (Bowman et al., 2004). While it is clear that ROS attack on the cytosolic surface of the membrane leads to a signal for removal of damaged erythrocytes from the circulation, neither the nature of the crucial target(s), whether lipid or protein, nor the mechanism of transfer of the signal across the membrane is known.

The mechanism(s) by which senescent or damaged (but intact) erythrocytes are selected for splenic sequestration are not known. Two current hypotheses are: 1) that red cell damage is analogous to the apoptotic response observed in other (nucleated) cell types, resulting in changes to the normal asymmetric distribution of phospholipids (Mandal et al., 2002); and 2) that protein oxidation interferes with normal protein-protein interactions among the cytoskeletal, integral membrane and cell surface proteins that confer the recognition of "self" (Oldenborg et al., 2000; Bruce et al., 2003). The present study examines the role of the postulated truncated apoptotic pathway in the hemolytic activity of 5-HPQ, specifically in regard to potential roles for membrane lipid peroxidation and loss of phospholipid (i.e., phosphatidylserine) asymmetry. We also address the alternate hypothesis, that alterations to skeletal membrane proteins in 5-HPQ-damaged red cells provide a crucial link between internal oxidative stress and external recognition and removal of erythrocytes from the circulation.

The data indicate that under *in vitro* incubation conditions that provoke premature splenic sequestration of red cells *in vivo*, 5-HPQ generates intracellular ROS but does not induce lipid peroxidation or cause loss of phosphatidylserine asymmetry in the plasma membrane of the red cell. In contrast, profound alterations occur to certain proteins of the cytoskeleton in 5-HPQ-treated erythrocytes, and these alterations are due primarily to the formation of disulfide-linked hemoglobin-skeletal protein adducts. Further studies are warranted to determine the specific

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signal for erythrocyte phagocytosis, however, the present data are consistent with the concept

that cytoskeletal protein damage, rather than alterations in the lipid bilayer, underlies the process

of splenic uptake of red cells damaged during the course of primaquine therapy.

MATERIALS AND METHODS

Chemicals and materials. 5-HPQ was synthesized by HBr-catalyzed hydrolysis of 5-methoxyprimaquine as described previously (Bowman et al., 2004). Bovine factor V, bovine factor Xa and bovine prothombin were obtained from Enzyme Research Laboratories (South Bend, IN). Chromogenic substrate S-2238 was purchased from Chromogenix (DiaPharma Group, Inc., Westchester, OH). Alexafluor 647-conjugated annexin V and 2',7'-dichloro-dihydrofluorescein diacetate (DCFDA) were obtained from Molecular Probes, Inc. (Eugene, OR). Calcium ionophore A23187 (calcimycin), N-ethylmaleimide (NEM), cumene hydroperoxide (CH) and rabbit anti-rat hemoglobin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest grade commercially available.

Erythrocytes and incubation conditions. Male Sprague-Dawley rats (75-100 g) were obtained from Harlan Laboratories (Indianapolis, IN) and maintained on food and water *ad libitum*. Animals were acclimated for 1 week to a 12-hr light/dark cycle before their use. Blood was collected from the descending aorta of anesthetized rats into heparinized tubes and washed 3 times with Hanks' Balanced Salt Solution with calcium (HBSS, Invitrogen, Carlsbad, CA) to remove the plasma and buffy coat. The cells were resuspended to a 40% hematocrit in HBSS and used on the same day they were collected. Erythrocytes were washed 3 times with HBSS and resuspended to a 40% hematocrit.

Stock solutions of 5-HPQ in argon-purged water were prepared to deliver the appropriate concentration of 5-HPQ in 10 μl to erythrocyte suspensions (1-2 ml). The erythrocyte suspensions (40% hematocrit) were allowed to incubate for up to 2 hr at 37°C, and then washed once with HBSS prior to biochemical analyses.

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Measurement of ROS formation in erythrocytes. Rat erythrocytes were suspended in isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM D-glucose (PBSG) to a 10% hematocrit. DCFDA (600 μM), dissolved in DMSO, was added to the erythrocyte suspension and allowed to incubate for 15 min at 37°C. Immediately after the addition of various concentrations of 5-HPQ, fluorescence was measured at 2 min intervals for 20 min (excitation 488 nm, emission 529 nm) on a Molecular Devices SprectraMAX Gemeni XS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA).

Preparation of erythrocyte membrane ghosts. Red cell ghosts were prepared from vehicle- and 5-HPQ-treated red cells as described previously with modification (Grossman et al., 1992). Briefly, washed red cells were centrifuged and the packed cells were lysed in 30 ml of ice-cold phosphate buffer (5 mM, pH 8.0). The membrane ghosts were pelleted by centrifugation at 20,000 x g for 10 min. The supernatant was removed by aspiration and the ghosts were repeatedly washed with phosphate buffer until the control cells yielded white ghosts, typically 3-4 washes.

Determination of lipid peroxidation in erythrocytes. Lipid peroxidation was assessed by measuring the content of F_2-isoprostanes in red cell ghosts prepared anaerobically from vehicle- and 5-HPQ-treated red cells as described previously (Bolchoz et al., 2002b). Briefly, the lipids were extracted from membrane ghost suspensions (250 μl) with chloroform/methanol (2:1, v/v). The extracted lipids were then subjected to alkaline hydrolysis to release the esterified F_2-isoprostanes. The work-up procedure for quantifying F_2-isoprostanes by GC/MS is described in detail elsewhere (Morrow and Roberts, 1999). Treatment of red cell suspensions for 1 hr at 37°C with the lipid-soluble peroxide, cumene hydroperoxide (1 mM), was used as a positive control for lipid peroxidation (van den Berg et al., 1992).

NEM and calcium ionophore treatment of erythrocytes. As a positive control for translocation of phosphatidylserine from the inner to the outer leaflet of the lipid bilayer, rat erythrocytyes were treated with NEM to inhibit aminophospholipid translocase, and calcium ionophore A23187 was added to induce membrane lipid scrambling as described previously (Kuypers et al., 1996). Briefly, rat erythrocytes were washed three times with HBSS (with calcium) and reconstituted to 40% hematocrit with HBSS. The red cell suspension (1 ml) was allowed to incubate with 10 mM NEM for 30 min at 37°C, and then washed twice with HBSS. Calcium ionophore A23187 (4 μM) in DMSO was then added and allowed to incubate for 1 hour at 37°C. After incubation, the red cells were washed with 2.5 mM EDTA (1 ml) to remove the calcium, and then washed twice more with *calcium-free* HBSS containing 1% bovine serum albumin (BSA) to remove the ionophore. The cells were then re-suspended in 1 ml of annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), and analyzed for annexin V labeling and prothrombinase activity as described below.

Determination of phosphatidylserine translocation in erythrocytes. Erythrocyte labeling with annexin V was performed according to a modification of a flow cytometric technique (Kuypers et al., 1996). Following incubation with the vehicle, the positive control, or 5-HPQ (2 hr at 37°C), aliquots (50 μl) of the suspensions were removed and washed twice with annexin binding buffer (1 ml). After the last wash, aliquots of the packed red cells (2 μl) were combined with annexin binding buffer (997 μl), and AlexaFluor 647-conjugated annexin-V solution (1 μl) was added to the erythrocyte suspensions to bring the total volume to 1 ml. After 30 min incubation in the dark at room temperature, the samples were washed and re-suspended in annexin binding buffer (1 ml), and analyzed on a Becton Dickinson FACSCalibur analytical flow cytometer (BD Biosciences, San Jose, CA).

Phosphatidylserine translocation was also assessed by measuring the conversion of prothrombin to thrombin using a modification of a method described previously (Kuypers et al., 1996). Briefly, after incubation with the vehicle or 5-HPQ, erythrocyte suspensions were washed once with HBSS, and 1 μl of packed cells was added to 1 ml of Tris buffer (pH 7.4) at 37°C. Bovine factor V (0.33 U/ml) and bovine factor Xa (0.33 U/ml) were added, followed by 0.13 mg of prothrombinase. After 4 min, the reaction was quenched with 15 mM EDTA. The erythrocytes were pelleted by centrifugation and 75 μl of the supernatant was added to 1 ml of chromogenic substrate S-2238 working solution. The increase in absorbance at 405 nm was determined over 1 min at 37°C in a Shimadzu UV-160 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with software for enzyme kinetics. The amount of thrombin formed per unit time was determined by comparison with thrombin standards.

Electrophoretic analysis of membrane cytoskeletal proteins. Red cell ghosts from control and 5-HPQ-treated erythrocytes were washed exhaustively to remove unbound hemoglobin (4 washes). The ghost protein was then solubilized in NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA), with or without 40 mM dithiothreitol (DTT), and heated at 70°C for 10 min. Solubilized proteins (15 μg) were loaded and resolved on 4-12% NuPAGE Bis-Tris gels with MOPS Running Buffer (Invitrogen) at 200 V (constant) for 50 min. Resolved proteins were transferred to PVDF membranes for immunoblot analysis according to the Invitrogen protocol. For SDS-PAGE analysis, solubilized membrane ghosts were resolved on continuous gels under non-reducing conditions as described previously (McMillan et al., 1995), then stained with Gel Code Blue Stain Reagent (Pierce, Rockford, IL) and destained with water.

Rat red cell membrane proteins were identified according to molecular weight (Fairbanks et al., 1971) with the use of molecular weight protein standards (Invitrogen). Blotted proteins were

blocked in TBST (pH 7.5) containing 5% non-fat dry milk and incubated in TBST containing

1% BSA and primary antibody (rabbit anti-rat hemoglobin, 1:10,000 vol/vol). After washing and

incubation with the peroxidase-conjugated secondary antibody (anti-rat IgG), the immunoblots

were developed using ECL detection (Amersham Biosciences, Piscataway, NJ).

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RESULTS

Effect of 5-HPQ on ROS generation in rat erythrocytes. DCFDA is a lipid-soluble probe

that can detect the intracellular formation of several types of ROS, including hydroxyl, peroxyl,

alkoxyl, and nitroxyl free radicals, as well as peroxynitrite (Halliwell and Whiteman, 2004). To

assess the formation of ROS in 5-HPQ-treated rat erythrocytes, erythrocyte suspensions were

pre-incubated with DCFDA for 15 min followed by addition of 5-HPQ. Fluorescence was then

measured at 2 min intervals for 20 min using a fluorescence microplate reader. Preliminary

experiments indicated that the concentration of red cells in the suspensions had to be reduced

from 40% to a 10% hematocrit to avoid significant quenching of the fluorescence signal by the

cells. 5-HPQ concentrations were reduced accordingly (by 4-fold) to maintain the same 5-HPQ-

to-red cell ratio that was used in the other experiments.

As shown in Fig.1, incubation of erythrocytes with DCFDA in the absence of 5-HPQ resulted

in a slight but measurable increase in fluorescence over a 20 min time period, consistent with the

known low-level, steady production of ROS in normal red cells (Jandl et al., 1960). Inclusion of

5-HPQ (5-30 μM) in the erythrocyte suspension caused a marked and concentration-dependent

increase in the generation of fluorescence, indicating enhanced production of ROS by 5-HPQ.

This observation is consistent with previous observations which showed that 5-HPQ has the

ability to redox cycle and to cause oxidative stress in rat erythrocytes, as evidenced by GSH

oxidation and methemoglobin formation (Bowman et al., 2004). No fluorescence signal was

detected in the absence of red cells or in the absence of DCFDA (data not shown).

Effect of 5-HPQ on erythrocyte membrane lipid peroxidation. To determine whether

ROS production by 5-HPQ resulted in lipid peroxidation, rat red cells were incubated for 2 hr at

37°C with concentrations of 5-HPQ corresponding to EC_{10} (10 µM), EC_{50} (40 µM) and EC_{90} (70

μM) concentrations for hemolytic activity (Bowman et al., 2004). The 2-hr incubation time was chosen because this time period was used in an earlier study to document the hemolytic activity of 5-HPQ (Bowman et al., 2004), and it was important to determine the state of erythrocyte lipids and proteins under the same incubation conditions that were used to measure hemolytic activity. The red cells were then washed and lysed in argon-purged hypotonic buffer for the preparation of red cell ghosts, as described previously (Grossman et al., 1992). Esterified isoprostanes were extracted from the ghosts and hydrolyzed, and the total F_2-isoprostane content in the samples was quantified by GC-MS (Morrow and Roberts, 1999). As shown in Fig. 2, F_2-isoprostane content in 5-HPQ-treated incubates was not different from that of control incubates, regardless of the concentration of 5-HPQ. In contrast, treatment of red cells with the positive control, cumene hydroperoxide (CH, 1 mM), resulted in a 3-fold increase in F_2-isoprostane formation. Treatment with this concentration of CH was the lowest that produced a significant elevation in F_2-isprostane formation that was not associated with significant frank lysis of the erythrocytes; concentrations of CH greater than 1 mM caused progressive and complete red cell lysis (data not shown).

Effect of 5-HPQ on red cell membrane phospholipid asymmetry. To determine whether exposure to 5-HPQ resulted in a loss of phosphatidylserine asymmetry, the transmembrane location of phosphatidylserine was assessed by two methods: enhancement of prothrombinase activity, and annexin-V labeling of intact erythrocytes.

Erythrocyte suspensions (40%) were incubated with hemolytic concentrations of 5-HPQ (10 to 70 μM) for 2 hr. At the end of the incubation, the red cells were washed, re-suspended in HBSS and analyzed for prothrombinase activity, which is dependent on cell surface exposure to phosphatidylserine (Bevers et al., 1982). For a positive control, erythrocytes were treated with 10

mM NEM for 30 min, followed by treatment with 4 μM calcium ionophore A23187 in the presence of calcium for 1 hr. As shown in Fig. 3A, thrombin formation in 5-HPQ-treated erythrocytes was not significantly different from the control. In contrast, the positive control, NEM plus ionophore and calcium, induced an 8-fold increase in prothrombinase activity as compared to the control.

The presence of phosphatidylserine in the outer leaflet of the lipid bilayer was confirmed by direct labeling of erythrocytes with fluorescently conjugated annexin V, which binds to acidic phospholipids, particularly phosphatidylserine (Kuypers et al., 1996). As shown in Fig. 3B, annexin-positive erythrocytes from 5-HPQ-suspensions constituted less than 2% of the red cells, which was similar to the vehicle-treated control. In contrast, treatment of red cells with NEM plus ionophore and calcium resulted in labeling of about 45% of the erythrocytes.

Effect of 5-HPQ on rat erythrocyte membrane skeletal proteins. To determine whether 5-HPQ-induced hemolytic activity was associated with alterations to the membrane cytoskeleton, rat erythrocyte ghost proteins from control and 5-HPQ-treated erythrocytes were separated by SDS-PAGE and either stained with Gel Code Blue, or transferred to PVDF membranes and immunostained with antibodies to rat hemoglobin. As shown in Fig. 4A, 5-HPQ caused concentration-dependent changes to the normal electrophoretic pattern of erythrocyte skeletal proteins as compared with that of the vehicle-treated control. 5-HPQ treatment induced the appearance of new protein bands at 16, 32 and 64 kDa, which is consistent with formation of membrane-bound hemoglobin monomers, dimers and trimers, respectively (Grossman et al., 1992). In addition, there was a concentration-dependent loss of resolution of protein bands 1 and 2 (α and ß spectrins), splitting of band 2.1 (ankyrin) and loss of band 4.2. On the other hand, band 4.1 and band 5 (actin) appeared not to be affected significantly by treatment with 5-HPQ.

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Immunoblot analysis of the skeletal proteins from 5-HPQ-treated red cells showed a dramatic increase in the content of membrane-bound hemoglobin (Fig. 4B). Only insignificant amounts of hemoglobin were bound to skeletal proteins at sub-hemolytic concentrations of 5-HPQ (<40 μM), whereas increasing amounts of hemoglobin were bound to a wide variety of proteins at hemolytic concentrations. When 5-HPQ-treated ghosts were treated with DTT (40 mM at 70°C for 1 hr) prior to electrophoresis, binding of hemoglobin to skeletal proteins was reversed (Fig. 5). This observation indicated that hemoglobin was bound covalently to skeletal protein sulfhydryl groups through intermolecular disulfide bonds.

DISCUSSION

Primaquine administration is well known to cause oxidative damage to erythrocytes, resulting in methemoglobinemia and hemolytic anemia. At pharmacologically relevant concentrations, primaquine is not directly toxic to erythrocytes; the toxicity is thought to be due to redox-active metabolites that generate ROS. When formed in sufficient quantity and rate, these ROS are considered to overwhelm cellular defenses and attack cellular macromolecules.

Previous studies showed that the phenolic metabolite, 5-HPQ, has the capacity to redox cycle and generate ROS, suggesting that it could contribute to the hemotoxicity of the parent drug (Vasquez-Vivar and Augusto, 1992). We recently demonstrated the hemolytic potential of 5-HPQ by showing that it is a direct-acting hemolytic agent in rats (Bowman et al., 2004). In these studies, ^{51}Cr-tagged erythrocytes were treated with 5-HPQ *in vitro* then re-administered to rats. The treated cells were rapidly removed from the circulation as compared with saline-treated controls. The concentration-dependence of this response was sharp, with a minimum response at 25 μM, an EC$_{50}$ at 40 μM, and a maximal response at 75 μM. Additionally, hemolytic activity was strongly correlated with GSH depletion and formation of glutathione-protein mixed disulfides. These observations were similar to the pattern of oxidative stress-induced damage observed previously in red cells exposed to other direct-acting, pro-oxidant hemolytic agents, such as dapsone hydroxylamine (Bradshaw et al., 1997). However, the precise intracellular molecular targets of oxidative damage have not been identified, nor has the mechanism whereby internal oxidative damage is translated to external cell-surface marker(s) that enable splenic macrophages to recognize and remove damaged red cells.

Since mature erythrocytes lack organelles, lipids and proteins of the plasma membrane are the most logical targets of intracellular ROS. In regard to lipids, it is known that they are prime

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targets for ROS-initiated lipid peroxidation, which is thought to result in membrane blebbing and cell lysis. Additionally, oxidative stress was shown to alter the asymmetric distribution of phospholipids within the bilayer (Jain, 1984). A functional role for this asymmetry was demonstrated when it was shown that phosphatidylserine externalization resulted in increased susceptibility of erythrocytes to phagocytosis (Zwaal and Schroit, 1997).

More recently, studies on the normal removal of senescent erythrocytes have suggested that they undergo a process analogous to apoptosis. Human erythrocytes treated with *t*-butylhydroperoxide were shown to activate caspase 3 and induce phosphatidylserine externalization (Mandal et al., 2002). Additional studies showed that peroxidation of phosphatidylserine in cells undergoing apoptosis causes a reduced affinity of aminophospholipid translocase for the oxidized phospholipid, resulting in the accumulation of phosphatidylserine in the outer leaflet (Tyurina et al., 2000). Other investigators have found no association between oxidative stress and loss of erythrocyte phospholipid asymmetry (de Jong et al., 1997).

An alternate hypothesis is that erythrocyte proteins are critical targets of intracellular ROS. Previous studies showed that hemoglobin and cytoskeletal proteins of the membrane cytoskeleton are altered in response to pro-oxidant hemolytic agents, which is reflected by the generation of reactive forms of hemoglobin, such as ferrylhemoglobin (Bolchoz et al., 2002a), hemoglobin thiyl radicals (Bradshaw et al., 1995), and by alterations to the mobility of skeletal proteins on non-reducing SDS-PAGE gels (Grossman et al., 1992; McMillan et al., 1995; McMillan et al., 2001). Evidence for the importance of normal protein-protein interactions among cytoskeletal, integral membrane and external cell surface proteins is illustrated by recent studies showing that these interactions are necessary to confer the recognition of "self" to circulating erythrocytes (Oldenborg et al., 2000; Bruce et al., 2002).

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The present studies were undertaken to determine the effect of the primaquine metabolite, 5-HPQ, on the generation of ROS in erythrocytes and on the consequences of ROS generation to erythrocyte membrane lipids and proteins. Given the association between 5-HPQ-induced oxidative stress and its hemolytic activity, we analyzed rat erythrocyte lipids for peroxidation and loss of phosphatidylserine asymmetry, and skeletal proteins for electrophoretic alterations and hemoglobin binding under experimental conditions associated with generation of intracellular ROS.

DCFDA is a fluorescence-based probe developed to detect intracellular production of ROS. DCFDA diffuses passively into cells where it is deacetylated to form 2',7'-dichlorodihydrofluorescein. 2',7'-Dichlorodihydrofluorescein is oxidized to a fluorescent product, 2',7'-dichlorofluorescein, by ROS, thus providing a general assessment of intracellular oxidative stress (Hempel et al., 1999; Halliwell and Whiteman, 2004). The data presented in Fig. 1 show a concentration-dependent increase in DCFDA oxidation in rat erythrocytes treated with 5-HPQ, indicating that ROS formation is associated with the hemolytic response in both a concentration- and time-dependent manner. DCFDA oxidation continued for at least 20 min, which is of interest because 5-HPQ has an extremely short half-life (<1 min) in erythrocyte suspensions (Bowman et al., 2004). Although the reason for this relatively long generation of ROS relative to 5-HPQ stability is not understood, it suggests that some unknown reactive intermediate with a longer half-life is generated in erythrocytes under hemolytic conditions.

To assess whether lipid peroxidation was associated with 5-HPQ-induced hemolytic injury, the formation of F_2-isoprostanes was quantified in erythrocyte ghosts by GC-MS. Treatment with a range of concentrations of 5-HPQ, from sub-hemolytic (10 μM) to maximally hemolytic (70 μM), did not reveal any increase in F_2-isoprostanes content in 5-HPQ-treated erythrocytes as

compared to the control (Fig. 2). In agreement with the lipid peroxidation data, analysis of phosphatidylserine externalization (Fig. 3) supported the concept that erythrocyte membrane lipids are not targets of 5-HPQ-generated ROS, and suggests that phosphatidylserine exposure on the external cell surface does not occur and thus is not a signal for uptake of 5-HPQ-treated erythrocytes into the spleen.

In contrast to membrane lipids, exposure to 5-HPQ caused profound alterations to the erythrocyte protein cytoskeleton (Fig. 4A). Importantly, the concentration dependence for this effect coincided with that of 5-HPQ-induced hemolytic activity. Staining of proteins resolved by SDS-PAGE showed alterations to several proteins, including loss of resolution and/or splitting of protein bands in the spectrin/ankyrin region, loss of band 4.2, and the formation of membrane-bound hemoglobin. The association of hemoglobin with erythrocyte ghosts is noteworthy because this response has been observed previously with dapsone hydroxylamine (McMillan et al., 1995) and divicine (McMillan et al., 2001). The process by which hemoglobin becomes bound to the membrane cytoskeleton is not well understood, but is thought to arise from ROS-induced oxidation of hemoglobin free-sulfhydryl groups to thiyl free radicals (Bradshaw et al., 1995), dissociation of the normal tetramer, and recombination of oxidized monomers via disulfide bond formation into polymeric forms that can be reduced back to monomers by treatment with DTT (Grossman et al., 1992).

Disappearance of band 4.2 was perhaps the most intriguing effect. Band 4.2 has been shown to be a member of the band 3 complex, which includes band 3, band 2.1 (ankyrin) and CD47 (Bruce et al., 2003). Band 3 is an anion transporter protein that is anchored to the underlying cytoskeleton through its interaction with ankyrin. The extracellular domain of CD47 acts as a ligand for inhibitory signal regulatory protein alpha on splenic macrophages (Oldenborg et al.,

2001). As noted above, this interaction is thought to confer self-recognition to normal erythrocytes, preventing their phagocytic removal (Oldenborg et al., 2000). These findings are of relevance to the hemolytic response because other studies have shown that band 4.2 forms a vertical association between CD47 and ankyrin, thereby mediating cell-surface expression of CD47 and its membrane skeleton attachment in human erythrocytes (Dahl et al., 2004). While the fates of band 4.2 and ankyrin are not clear from these experiments, immunoblotting with anti-rat hemoglobin showed that significant amounts of hemoglobin are bound to multiple proteins of the band 3 complex (Fig. 4B). Treatment of erythrocyte ghosts with the disulfide-reducing agent, DTT, indicated that covalent binding of hemoglobin to these proteins occurred through the formation of intermolecular disulfide bonds (Fig. 5).

Our working hypothesis for 5-HPQ-induced hemolytic anemia is shown in Fig. 6. This scheme illustrates the preferential attack of ROS on hemoglobin as a consequence of 5-HPQ redox cycling. As noted above, attack of ROS on hemoglobin may result in the formation of hemoglobin thiyl radicals. Thiyl radicals are highly reactive, but the globin chain lacks sufficient lipophilicity to penetrate into the membrane to initiate lipid peroxidation. Thiyl radicals instead react with free sulfhydryl-groups on skeletal proteins to form disulfide-linked adducts (Jollow and McMillan, 2001). Additional studies are necessary to determine whether thiyl radicals or other reactive species can be detected in response to treatment with 5-HPQ. Additional studies are also warranted to determine the functional importance of hemoglobin adduct formation; however, it is plausible that binding of hemoglobin to the band 3 complex would lead to disturbances in cytoskeletal protein-protein interactions that are transmitted to the external surface of the erythrocyte, providing a signal for macrophage recognition.

In summary, we have shown that a hemolytic metabolite of primaquine, 5-HPQ, is not associated with lipid peroxidation or phosphatidylserine translocation despite the generation of ROS. Rather, the data are consistent with the concept that cytoskeletal protein damage, in the form of disulfide-linked hemoglobin adducts, underlies the removal of damaged erythrocytes. Additional studies are warranted to elucidate the signal that underlies the loss of self-recognition.

ACKNOWLEDGEMENTS

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effect of 5-HPQ on ROS generation in rat erythrocytes. Erythrocyte suspensions (10% hematocrit) were incubated with DCFDA (600 μM) for 15 min. The red cells were then treated with the vehicle (●), 5μM (◆), 15 μM (▲), and 30 μM (■) 5-HPQ. Fluorescence emission intensity was monitored on a fluorescence microplate reader (excitation, 488; emission, 529 nm) at 2 min intervals. The values are means ± S.D. (n = 3).

Fig. 2. Effect of 5-HPQ on F_2-isoprostane formation in rat erythrocytes. Erythrocytes were incubated for 2 hr at 37°C in HBSS containing the indicated concentrations of 5-HPQ. After incubation erythrocyte ghosts were prepared and analyzed for F_2-isoprostane content by GC-MS as described in the *Materials and Methods*. The values are means ± S.D. (n = 3). * Significantly different from control ($p < 0.05$).

Fig. 3. Effect of 5-HPQ on phosphatidylserine asymmetry in rat erythrocytes. Erythrocyte suspensions (40% hematocrit) were incubated with the indicated concentrations of 5-HPQ for 2 hr at 37°C. NEM/Ca^{2+} was used as a positive control. (A) Prothrombinase activity and (B) Alexa Fluor 647-conjugated annexin V labeling were determined as described in *Materials and Methods*. The values are means ± S.D. (n = 3). * Significantly different from control ($p < 0.05$).

Fig. 4. Effect of 5-HPQ on rat erythrocyte membrane skeletal proteins. Red cells were incubated with vehicle (lane 1), 10 μM (lane 2), 25 μM (lane 3), 50 μM (lane 4), 75 μM (lane 5) and 100 μM (lane 6) 5-HPQ for 2 hr at 37°C. The cells were washed, and ghosts were prepared and washed exhaustively to remove unbound hemoglobin (Hb). The ghosts were then solubilized in SDS and subjected to PAGE. (A) Gel Code Blue-stained gel and densitometric scans; (B) immunoblot stained with polyclonal antibodies to rat hemoglobin. Molecular weight markers

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appear along the right side of the gel; the major cytoskeletal proteins are identified along the left side of the gel according to (Fairbanks et al., 1971).

Fig. 5. Effect of DTT on 5-HPQ-induced hemoglobin binding to membrane skeletal proteins. Erythrocytes were incubated with 5-HPQ for 2 hr at 37°C. Ghosts were prepared, washed exhaustively to remove unbound hemoglobin, and each sample was divided into two aliquots. Prior to electrophoresis, one aliquot was incubated with water and the other with 40 mM DTT for 1 hr at 70°C. The samples were then subjected to SDS-PAGE and blotted onto PVDF membranes. (A) Immunoblot stained with polyclonal antibodies to rat hemoglobin. Vehicle control (lanes 1 and 5), 25 μM (lanes 2 and 6), 50 μM (lanes 3 and 7) and 75 μM (lanes 4 and 8) 5-HPQ. (B) Selected densitometric scans.

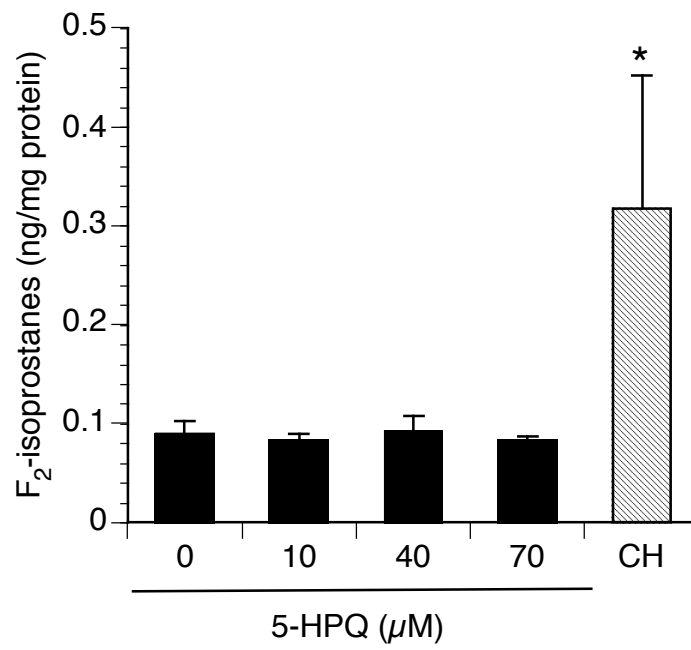**Fig. 6.** Working hypothesis for the mechanism underlying the hemolytic activity of 5-HPQ in rat erythrocytes. See text for description.
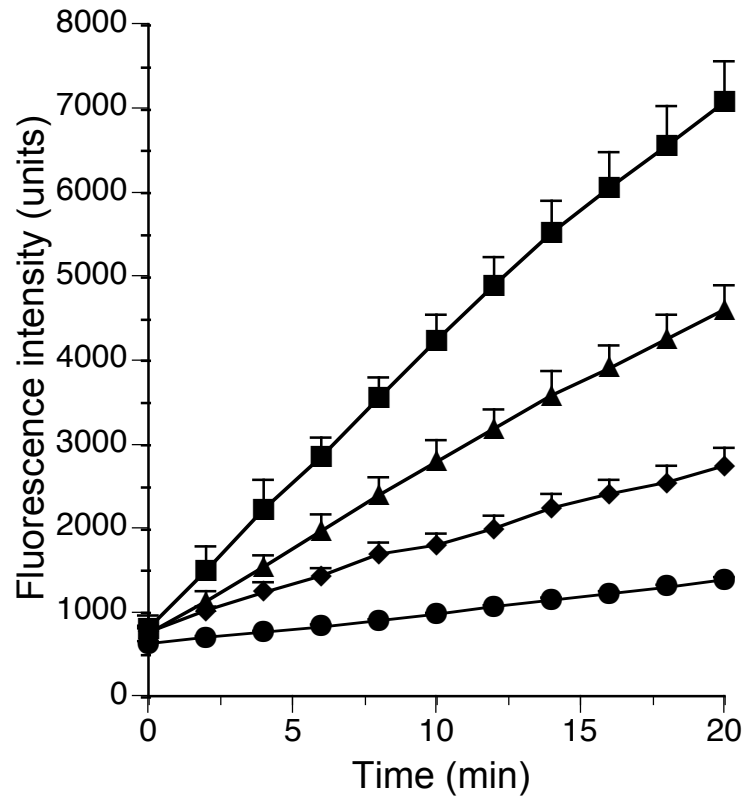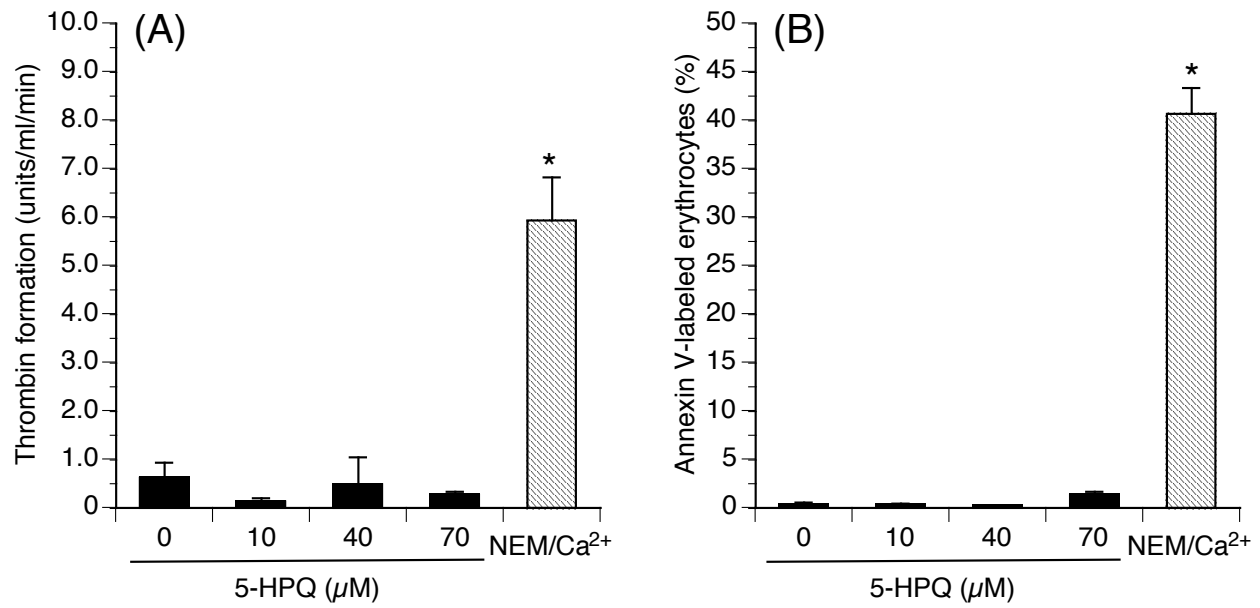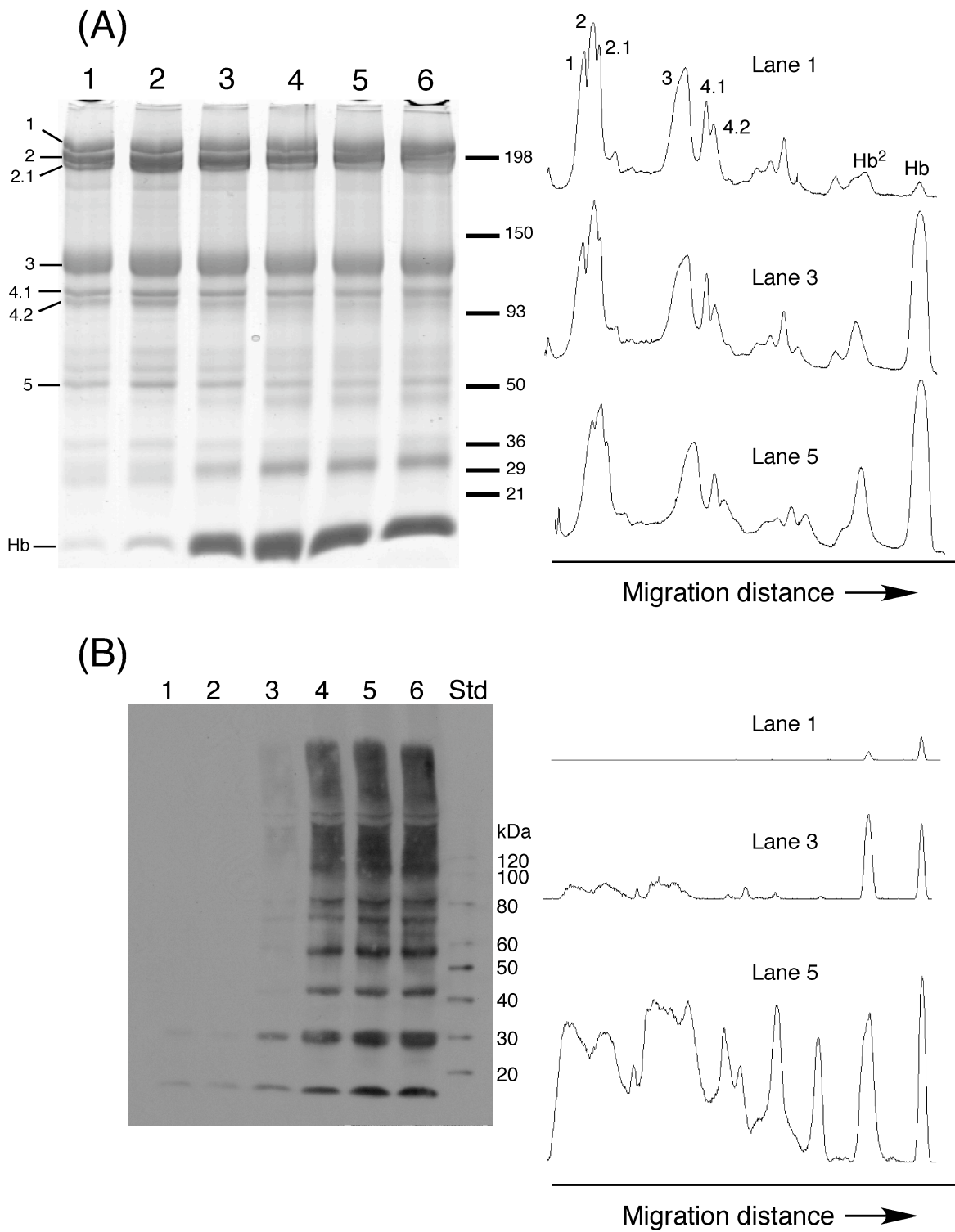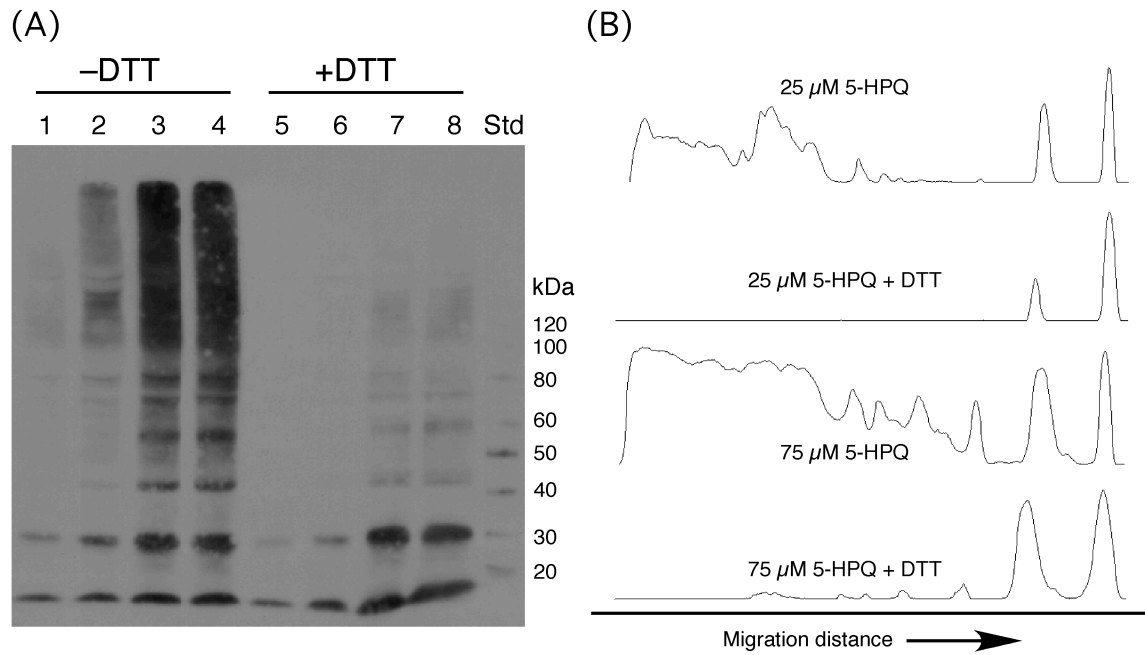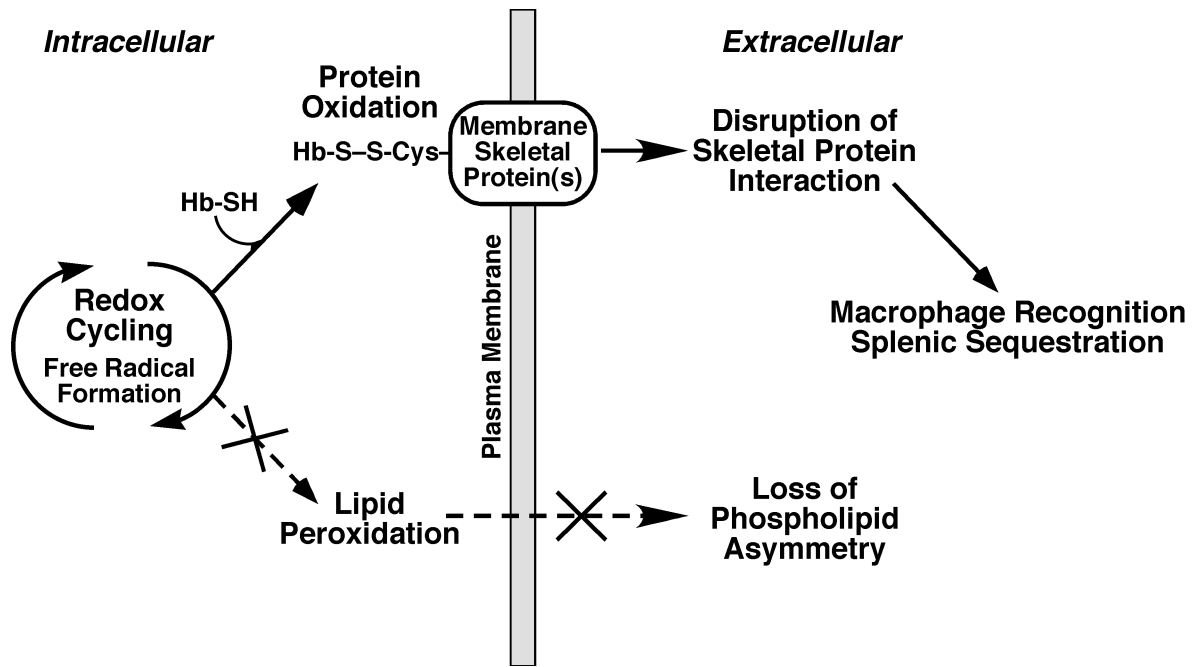
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6