Free Rad. Res., Vol. 27, pp. 55–62
Reprints available directly from the publisher
Photocopying permitted by license only

Hemin-Induced Membrane Sulfhydryl Oxidation: Possible Involvement of Thiyl Radicals

DANIEL T.Y. CHIU*[,a], TSUN-YING HUANG[a], IOU-JIH HUNG[b], JENG-SHU WEI[a], TSAN-ZON LIU[a] and ARNOLD STERN[c]

[a]*School of Medical Technology, Chang Gung College of Medicine & Technology, 259 Wen-Hua 1st Road, Kwei-Shan, Tao-Yuan, Taiwan;* [b]*Dept. of Hematology, Chang Gung Children's Hosp., Kwei-San, Tao-Yuan, Taiwan;* [c]*Dept. of Pharmacology, New York Univ. Med. Ctr., New York, NY, USA*

Accepted by Prof C Rice-Evans

(Received 25 July 1996; In revised form 10 December 1996)

Sublytic levels (μM) of hemin destabilized RBC membrane as indicated by ghost fragmentation pattern using a laser viscodiffractometer. Furthermore, electron microscopic study shows that 5μM of hemin induced echinocytic transformation whereas higher hemin concentration (40μM) induced spherocytic transformation. In addition, hemin oxidized sulfhydryl groups in a dose dependent fashion and Electron Spin Resonance study suggests that such oxidation may involve a thiyl radical. Moreover, sulfhydryl compounds enhanced hemin-induced lipid peroxidation. Desferroxamine could prevent hemin-induced sulfhydryl oxidation as well as hemin-induced decrease in membrane stability. In contrast, vitamin E could effectively prevent hemin-induced lipid peroxidation but could not prevent hemin-mediated membrane destabilization.

Keywords: Hemin, sulfhydryl oxidation, thiyl radical, red cell membrane, lipid peroxidation

Abbreviations: DFO, desferroxamine; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DI, deformability index; ESR, electron spin resonance spectroscopy; GSH, glutathione; RBC, red blood cell; SEM, scanning electron microscopy; TBARS, thiobarbituric acid reactive substances

INTRODUCTION

Hemin (or hematin) is by no means a stranger to the scientific or medical communities. Hemin has been used for the treatment of acute porphyria.[1] More interestingly, hemin has major effects on many cellular processes. Hemin affects the biosynthesis of proteins,[2,3] activates platelets and affects hemostasis.[4] In addition, hemin can induce cell differentiation[5,6] and stimulate gene expression.[7] It has been proposed that the formation of intersubunit disulfide in heme-regulated eIF-2a kinase induced by hemin could be a model to explain the effects of hemin on protein synthesis.[2]

Hemin, an essential cell constituent as well as a product of hemoglobin denaturation,[8] has also been shown to be deleterious to the red cell (RBC) membrane. At micromolar concentrations, hemin induces potassium leak, decreases osmotic fragility, causes RBC swelling, and eventually

*Corresponding author. Tel.: 886-328-9540. FAX: 886-3-328-9540. E-mail: dtychiu @ cguaplo.cgu.edu.tw.

lyses RBCs.[9,10] Hemin has also been shown to inhibit a number of RBC enzymes.[11] In addition, hemin mediates the dissociation of RBC membrane skeletal proteins by altering the conformation of protein 4.1 and weakening spectrin dimer-dimer association and spectrin-protein 4.1 association.[12] The hemolytic effect of hemin can be prevented by glutathione[13] and desferroxamine(DFO), which is an iron chelator that has been shown to bind to hemin via the iron moiety.[14] These latter findings strongly implicate that an oxidative mechanism contributes to the hemolytic effect of hemin. The major objective of the current study is to further delineate the deleterious effects of hemin on human RBCs and to elucidate the underlying oxidative mechanism of hemin-induced alterations in human RBCs.

MATERIALS AND METHODS

Materials and blood procurement.. After study subjects gave informed consent, heparinized blood specimens were obtained. All blood samples were used within 24 hours of collection. Before any experimental manipulations, red cells were washed 3 times in phosphate buffered saline(PBS: 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl), pH 7.4 and the buffy coat aspirated each time. DFO was from Ciba-Geigy (Summit, NJ, USA). Phospholipids, hemin, hydrogen peroxide and many other biochemicals were from Sigma (St. Louis, MO, U.S.A.). Ghosts of hemin-treated or control erythrocytes were prepared by the method of Dodge et al..[15]

Sulfhydryl group determinations.. RBC membrane thiol oxidation by hemin was quantitated by thiol-disulfide column chromatography.[16] Thiol-disulfide chromatography (thiol-activated agarose gel containing 2-pyridyl-disulfide groups) can separate those proteins with reduced sulfhydryl group(s) and those without. In our experiments, an increase in filtrate protein concentration should reflect an increased in thiol oxidation. Quantitation of sulfhydryl group in a cysteine solution was carried out by the method of Ellman.[17]

Lipid analysis.. Extraction of lipids and quantitation of phospholipid as inorganic phosphate were performed as reported before.[18] The susceptibility of RBCs to lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay.[19]

Measurements of membrane mechanical stability.. A viscodiffractometer is commonly used to measure RBC deformability as previously described by Clark et al..[20] This same instrument can be employed to measure the rate of membrane fragmentation as described by Monhandas et al..[21] Briefly, resealed ghoshts were subjected to constant high shear stress (575 dynes/cm^2). Under such condition, the deformability signal progressively declined as the deformable cells were transformed into less deformable, more spherical cell fragments. The deformability signal for normal ghost preparations decayed over a narrowly defined time period and this decay was accompanied by the production of cell fragments.[21] Abnormal membrane preparations with reduced membrane stability would show a reduction in the time required for fragmentation to take place as well as an increase in the rate of decline in deformability.[21]

Scanning electron microscopy (SEM).. SEM was performed similar to previously described.[22] Cells were dried in a BALZERS CPE-030 critical point dryer and coated with gold using a JEOL JFC 1100E ion coater, then examined and photographed in a ABT DS-130S scanning electron microscope at 10 kv.

Electron Spin Resonance Spectroscopy (ESR).. ESR spectrum of a reaction mixture containing hemin and reduced glutathione in the presence of a spin trap, DMPO, was obtained by a Varian E109 instrument. The conditions for obtaining the ESR spectrum were: microwave power, 10 mW; modulation amplitude, 1G; time constant, 0.128s; and scan rate 50G/min.

RESULTS

Destabilization of the Red Cell Membrane by hemin.. Since hemin had been shown to mediate the dissociation of RBC membrane skeletal proteins,[12] the effect of hemin on RBC membrane stability was examined using a laser viscodiffractometer. Untreated ghost were stable for approximately 80 seconds under our experimental condition and started to fragment soon after that (Fig. 1, curve A). At micro molar concentrations, hemin was shown to drastically reduce the mechanical stability of RBC membranes as indicated by the dose-dependent increase of ghost fragmentation (Fig. 1, curves B and C). Ghosts treated with 10 μM of hemin started to fragment at about 40 seconds under identical condition (curve B) and ghosts treated with 15 μM of hemin was unstable at all starting to fragment at time zero (curve C). Additional evidence to indicate that hemin destabilizes RBC membrane comes from our scanning electron microscopic examination of hemin-treated RBCs. Our data show that low hemin concentration (5μM) induced an echinocytic transformation and a higher hemin concentration (40μM) induced a spherocytic transformation with an apparent spontaneous release of membrane materials (Fig. 2).

Oxidation of sulfhydryl groups by hemin.. To elucidate the underlying mechanism of hemin-induced destabilization of the RBC membrane, we first investigated the effect of hemin on sulfhydryl groups. A dose dependent sulfhydryl oxidation in solution of cysteine with increasing concentrations of hemin was observed (Table I).

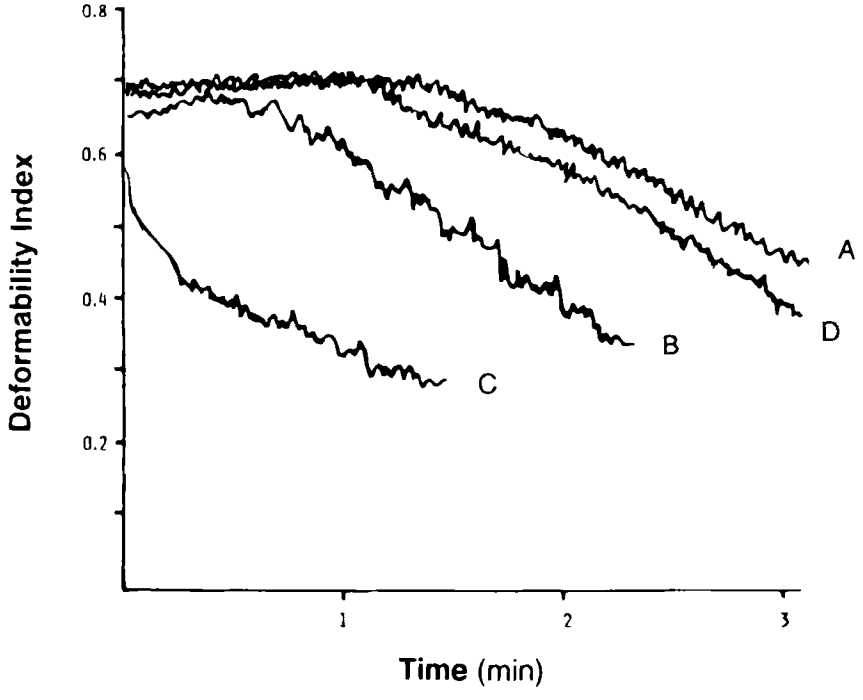

FIGURE 1 *Hemin destabilizes the red cell membrane as measured by a laser visco-diffractometer.* Red cells (5% suspension) were incubated with varying concentrations of hemin in a hypotonic solution and allowed to reseal in an isotonic solution containing Mg^{+2}. Ghost fragmentation patterns were measured after the resealed ghosts were washed 3X with isotonic phosphate buffer. DFO was added in some samples. Vitamin E or DFO alone did not influence the ghost fragmentation pattern of the control(data not shown). Curve A: Control; Curve B: Treated with 10 μM hemin with or without 2 mM of vitamin E; Curve C: Treated with 15 μM hemin; Curve D: Treated with 10 μM hemin in the presence of 0.5 mM DFO.

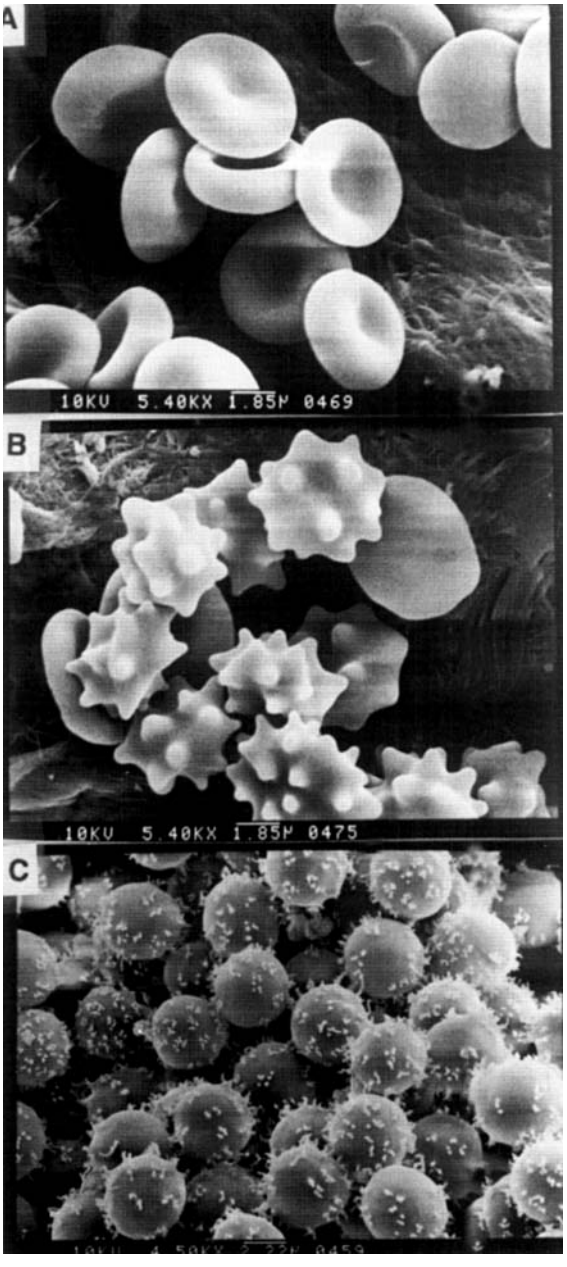

FIGURE 2 *Alteration of RBC morphology by hemin as indicated by SEM.* Control and hemin-treated RBCs were incubated at 37°C water bath for 30 min. Upon completion of incubation, RBCs were fixed with 2% glutaraldehyde and then with 1% OsO_4. Both fixatives were in cacodylate buffer. After fixation, cells were dehydrated with ethanol followed by critical point drying. Cells were then coated with gold and examined by SEM: control RBCs (2-A); RBCs treated with 5 μM hemin (2-B); RBCs treated with 40 μM hemin (2-C).

Hemin was also found to cause an increase of thiol-disulfide in ghost preparations (Table I). These data suggest that hemin is capable of oxidizing protein sulfhydryl groups.

Enhancement of hemin-mediated lipid peroxidation by sulfhydryl compound.. We found that in the presence of sulfhydryl compound (β-mercaptoethanol), hemin could promote TBARS formation without the addition of hydrogen peroxide(Table II). This finding together with the sulfhydryl oxidation data suggest that sulfhydryl groups can reduce hemin from Fe^{+3} to Fe^{+2}. Furthermore, these data also suggest that the reduction of hemin by sulfhydryl groups may involve a thiyl radical intermediate.

Generation of thiyl radical-spin adduct by hemin and glutathione.. When hemin(0.27mM) was incubated with 2.56 mM of reduced glutathione(GSH) in the presence of a spin trapping agent DMPO (0.1 M), in addition to an ESR spectrum typically seen for hydroxyl radical (aN = aH = 14.9G), another spin-adduct was identified with g = 2.0055, a_N = 15.2G and a_H = 16.0G (Fig. 3). This is evident from an apparent broadness of the overall spectrum and from a shoulder appearing on each of the two lines of the upfield segment of the spectrum. In addition, peak to peak measurements reveal differences in splitting constant values. If only hydroxyl radical was present, all splitting constant values would be equal. The ESR parameters obtained for this spin adduct are consistent with a thiyl radical adduct such as DMPO-SG.[23,24] Hence, the ESR data provide strong support to the notion that the oxidation of sulfhydryl groups by hemin may involve a thiyl radical intermediate.

Prevention of Hemin-induced damage by vitamin E or by desferroxamine.. Vitamin E could prevent TBARS formation (Table II) but could not prevent hemin-induced destabilization of the RBC membrane (Fig. 1). Hence, our findings suggest that hemin-induced destabilization of the RBC membrane is not mediated by hemin-promoted

TABLE I Interaction of hemin with sulfhydryl groups in buffer containing cysteine or in fresh ghost samples

Sample	Cysteine Solution (n mole of SH group)	Thiol-disulfide Chromatography (% oxidized)	
Control	201 ± 8.7	8.32 ± 3.61 (n = 13)	
Control + 5 mM hemin	181 ± 7.4	n.d.[a]	
Control + 10 mM hemin	107 ± 6.2	n.d.[a]	
Control + 20 mM hemin	46 ± 3.3	12.1 ± 3.34 (n = 7)	P = 0.0172[b]
Control + 50 mM hemin	11 ± 0.9	17.5 ± 4.08 (n = 7)	P = 0.00003[b]
Control + 20 mM hemin +0.5 mM DFO	189 ± 9.5	8.94 ± 3.66 (n = 4)	

[a]n.d. = not determined
[b]Comparing to control value of 8.32 ± 3.61 (n = 13) by Unpaired Student-T (one tail) test

TABLE II Effects of β-Mercaptoethanol or vitamin E on TBARS formation induced by hemin

Sample	TBARS (nmol/mg lipid phosphorus)
Ghosts	0.343 ± 0.029 (n = 5)
Ghosts + 10 μM hemin	0.925 ± 0.066 (n = 5)
Ghosts + 10 μM hemin + 1mM β-mercaptoethanol(air)	2.941 ± 0.201 (n = 4)
Ghosts + 10 μM hemin + 1mM β-mercaptoethanol(N_2)	1.793 ± 0.181 (n = 4)
Ghosts + 10 μM hemin + 1mM H_2O_2	19.377 ± 1.549 (n = 5)
Ghosts + 10 μM hemin + 1mM H_2O_2 + 2 mM Vit. E	1.876 ± 0.203 (n = 4)

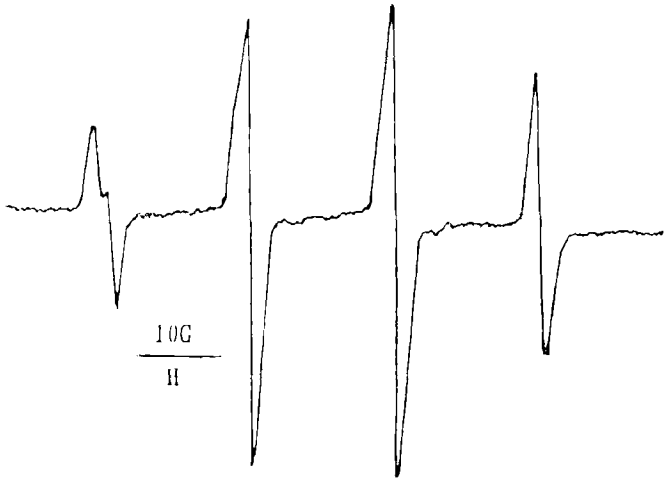

FIGURE 3 *ESR spectrum of free radicals generated from reaction of GSH with hemin.* The reaction of GSH (2.56mmol/L) and hemin (0.27mmol/L) in the presence of radical trapping agent, DMPO(0.1mol/L), produced an ESR spectrum composed of two radical adducts, namely, DMPO-OH($a_N = a_H = 14.9G$) and DMPO-SG($a_N = 15.2G$, $a_H = 16.0G$). This is evident from an apparent broadness of the overall spectrum and from a shoulder appearing on each of the two lines of the upfield segment of the spectrum. In addition, peak to peak measurements reveal differences in splitting constant values. If only hydroxyl radical was present, all splitting constant values would be equal.

lipid peroxidation. On the other hand, the interaction of hemin with sulfhydryl groups either in a cysteine solution or in ghost preparations could be inhibited by desferrioxamine (Table I). Moreover, DFO could also prevent hemin-induced destabilization of the RBC membrane (Fig. 1). These findings support the notion that the interaction of hemin with membrane protein sulfhydryl groups could contribute to the hemin-induced destabilization of the RBC membrane.[14]

DISCUSSION

The ghost fragmentation experiments(Fig. 1) provide new evidence that hemin is able to markedly reduce membrane stability even at the micro molar level. The intracellular Hb concentration is approximately 5 mM and it has been estimated that 3% of Hb in the circulating RBCs would be oxidized daily.[25] Although most of the oxidized hemoglobin would be reduced back to the divalent ferrous form by methemoglobin reductase, small amount of the oxidized hemoglobin can undergo further oxidation and release its heme.[26] Increased amount of heme has been reported in the membrane of several pathologic RBCs such as sickle RBCs,[27] thalassemic RBCs[28] and Glucose-6-phosphate dehydrogenase-deficient RBCs.[29] The hemin concentrations used in our experiments are compatible with the heme concentrations reported in those pathologic RBCs. In the EM study, echinocytic transformation can be observed in intact RBCs treated with 5 μM hemin (Fig. 2B) and spherocytic transformation with an apparent spontaneous releasing of membrane materials in RBCs treated with a higher (40 μM) hemin concentration (Fig. 2C). These findings confirm the observations that hemin has a strong effect on cell membranes.

We have noted that even in the absence of exogenous hydrogen peroxide, hemin can promote lipid peroxidation as measured by TBARS (Table II). More interestingly, the production of TBARS is enhanced by sulfhydryl compound (Table II). These findings suggest that sulfhydryl compound can reduce hemin from Fe^{+3} to Fe^{+2} thus promoting the superoxide radical generating cycle. The fact that hemin can oxidize sulfhydryl groups (Table I) provides additional support to our speculation that sulfhydryl groups can reduce hemin from the ferric state to the ferrous state. Furthermore, since we found that sulfhydryl groups could promote lipid peroxidation in the presence of hemin but in the absence of hydrogen peroxide (Table II), these data suggest that the oxidation of sulfhydryl group by hemin may involve a thiyl radical as an intermediate. Our ESR study provides additional support to our interpretation that the oxidation of sulfhydryl group by hemin may involve a thiyl radical as an intermediate. In this study, hemin can induce the formation of a thiyl radical spin adduct in a solution containing glutathione and DMPO (Fig. 3)

The experiments with inhibitors to prevent sulfhydryl oxidation or lipid peroxidation induced by hemin provide further clarification of the biochemical events in hemin induced membrane alterations. With DFO, both oxidation of membrane protein sulfhydryl groups and hemin-mediated destabilization of the RBC membrane were prevented. In contrast, with vitamin E although hemin-induced lipid peroxidation was prevented, hemin-induced destabilization of the RBC membrane was not. This strongly suggests that destabilization of the RBC membrane by hemin is not mediated via lipid peroxidation, but by the interaction of hemin with membrane protein sulfhydryl groups.

The oxidation of sulfhydryl groups by hemin could be a common mechanism of many hemin effects. Certain enzyme activities have been known to be modulated by redox control involving thiol disulfide exchange such as guanylate cylcase[30] and adenylate clyclase.[31] Gene expression can also be modulated by a similar mechanism involving oxidation and reduction of protein sulfhydryl groups.[32] Hence, hemin-induced membrane sulfhydryl oxidation may

play a role in certain cellular functions such as protein synthesis and gene expression in erythroid cells associated with erythropoiesis.

Acknowledgements

We thank professors W.J. Xin and B.L. Zhao of Academia Sinica(China) for their expert advise in our ESR study and Dr N Mohandas of Lawrence Berkeley Laboratory(USA) for his expert advise in our study of membrane stability. We also thank Dr. J Lee of our Institute for his expert advise in our electron microscopic study. This project is supported by grants from Chang Gung College of Medicine & Technology (CMRP389 and NMRP366) and by grants from the National Science Council of Taiwan (NSC82-0412-B182-025, NSC83-0412-B182-054-M02 and NSC83-0412-B182-034) as well as grants from the National Institute of Health of the United States (HL 36255, ES 03425).

References

[1] H. L. Bonkowsky, D. P. Tschudy, A. Collins, I. Bossenmaier, R. Cardinal and C. J. Watson (1971). Repression of the overproduction of prophyrin precursor in acute intermitten porphyria by intravenous infusions of hematin. *Proceeding of National Academy of Sciences USA*, **68**, 2725–2729.

[2] J. Yang, I. M. London and J. J. Chen (1992). Effects of hemin and porphyrin compounds on intersubunit disulfide formation of heme-regulated eIF-2α kinase and the regulation of protein synthesis in trticulocyte lysates. *Journal of Biological Chemistry*, **267**, 20519–20524.

[3] M. Gross, A. Olin, S. Hessefort and S. Bender (1994). Control of protein synthesis by hemin. *Journal of Biological Chemistry*, **269**, 22738–22748.

[4] S. M. Neely, D. V. Gardner, N. Reynolds and C. H. Tsao (1984). Mechanism and characteristics of platelet activation by hematin. *British Journal of Haematology*, **58**, 305–316.

[5] S. Sassa (1988). Heme stimulation of cellular growth and differentiation. *Seminar in Hematology*, **25**, 312–320.

[6] O. Nakajima, H. Kagechika, M. K. Shudo, Y. Hashimoto and S. Iwasaki (1995). Possible involvement of retinoid-like cofactor in serum in hemin/proto-porphyrin-IX-induced differentiation of human leukemia K562 cells. *Biochemistry Biophysics Research Communicate*, **206**, 1003–1010.

[7] J. F. Palma, X. Gao, C. H. Lin, S. Wu and W. B. Solomon (1994). Iron protoporphyrin IX (hemin) but not tin or zinc protoporphyrin IX can stimulate gene expression in K562 cells from enhancer elements containing binding sites for NF-E2. *Blood*, **84**, 1288–1297.

[8] D. T. Y. Chiu, J. van den Berg, F. A. Kuyper, I. J. Hung, J.-S. Wei and T. Z. Liu (1996). Correlation of membrane lipid peroxidation with oxidation of hemoglobin variants: possibly related to the rates of hemin release. *Free Radical Biology and Medicine*, **21**, 89–95.

[9] C. D. Fitch, R. Chevli, P. Kanjananggulpan, P. Dutta, K. Chevli and A. C. Chou (1983). Intracellular ferriprotoporphyrin IX is a lytic agent. *Blood*, **62**, 1165–1168.

[10] T. H. Schmitt, W. A. Frezzatti Jr. and S. Schrier (1993). Hemin-induced lipid membrane disorders and increased permeability: a molecular model for the mechanism of cell lysis. *Archives of Biochemistry and Biophysics*, **307**, 96–103.

[11] C. R. Zerez and J. W. Hseih, K. R. Tanaka (1987). Inhibition of red blood cell enzymes by hemin: A mechanism for hemolysis in hemoglobinopathies. *Transactions of the Association of American Physicians*, **100**, 329–338.

[12] S. C. Liu, S. Zhai, J. Lawler and J. Palek (1985). Hemin-mediated dissociation of erythrocyte membrane skeletal proteins. *Journal of Biological Chemistry*, **260**, 12234–12239.

[13] Y. Shviro and N. Shaklai (1987). Glutathione as a scavenger of free hemin. A mechanism of preventing red cell membrane damage. *Biochemical Pharmacology*, **36**, 3801–3807.

[14] S. G. Sullivan, E. Baysal and A. Stern (1992). Inhibition of hemin-induced hemolysis by desferrioxamine: Binding of hemin to red cell membranes and effects of alteration of membrane sulfhydryl groups. *Biochimica et Biophysica Acta*, **1104**, 38–44.

[15] J. T. Dodge, C. D. Mitchell and D. J. Hanahan (1963). The preparation and chemical characteristics of hemoglobin-free ghost of human erythrocytes. *Archives of Biochemistry and Biophysics*, **100**, 119–130.

[16] B. H. Rank, J. Carlson and R. P. Hebbel (1985). Abnormal redox status of membrane-protein thiols in sickle erythrocytes. *Journal of Clinical Investigation*, **75**, 1531–1537.

[17] G. L. Ellman (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, **82**, 70–73.

[18] B. Lubin, D. Chiu, J. Bastacky, B. Roelofsen and L. L. M. Van Deenen (1981). Abnormalities in membrane phospholipid organization in sickled erythrocytes. *Journal of Clinical Investigation*, **67**, 1643–1649.

[19] D. Chiu, B. Lubin and S. B. Shohet (1979). Erythrocyte membrane lipid reorganization during the sickling process. *British Journal of Haematology*, **41**, 223–234.

[20] M. R. Clark, N. Mohandas and S. B. Shohet (1983). Osmotic gradient ektacytometry: comprehencive characterizaiton of red cell volume and surface maintainence. *Blood*, **61**, 889–910.

[21] N. Mohandas, M. R. Clark, B. H. Heath, M. Rossi and S. B. Shohet (1982). A Technique to detect reduced mechanical stability of red cell membranes: relevance to elliptocytic disorders. *Blood*, **59**, 768–774.

[22] D. T. Y. Chiu, K. M. Lai, C. M. Xu, S. S. S. Liour, J. Lee and T. Z. Liu (1993). Direct alteration of erythrocyte membrane properties by 1-chloro-2,4-dinitrobenzene without oxidant challenge. *Experimental Hematology*, **21**, 114–118.

[23] R. P. Mason and D. N. R. Rao (1990). Thiyl free radical metabolites of thiol drugs, glutathione, and proteins. *Methods in Enzymology*, **186**, 318–329.

[24] R. Goldman, D. A. Stoyanovsky, B. W. Day and V. E. Kagan (1995). Reduction of phenoxyl radicals by thioredoxin results in selective oxidation of its SH-groups to disulfides. An antioxidant function of thioredoxin. *Biochemistry*, **34**, 4765–4772.

[25] D. Chiu, B. Lubin and S. B. Shohet (1982). Peroxidative reactions in red cell biology. In: Pryor, W. A. eds. *Free radicals in biology*. Vol. V. Academic Press, New York, pp. 115–160.
[26] H. F. Bunn and J. H. Jandl (1967). Exchange of heme along hemoglobins and between hemoglobin and albumin. *J. Biol. Chem.*, **243**, 465–475.
[27] S. A. Kuross, B. H. Rank and R. P. Hebbel (1988). Excess heme in sickle erythrocyte inside-out membranes: Possible role in thioloxidation. *Blood*, **71**, 876–882.
[28] N. Shaklai, Y. Sviro, E. Rabizadeh and I. Kirschner-Zilber (1985). Accumulation and drainage of hemin in the red cell membrane. *Biochim. Biophys. Acta.*, **821**, 355–366.
[29] S. K. Janney, J. H. Joist and C. D. Fitch (1986). Excess release of ferriheme in G6PD-deficient erythrocytes: Possible cause of hemolysis and resistance to malaria. *Blood*, **67**, 331–333.
[30] X. B. Wu, B. Brune, F. von Appen and V. Ullrick (1992). Reversible activation of soluble guanylate cyclase by oxidizing agents. *Archives of Biochemistry and Biophysics*, **294**, 75–82.
[31] M. Wright and G. I. Drummond (1983). Inactivation of the beta-adrenergic receptor in skeletal muscle by dithiols. *Biochemical Pharmacology*, **32**, 509–515.
[32] G. Storz, L. A. Tartaglia and B. N. Ames (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science*, **248**, 189–194.