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Lipid peroxidation in hemoglobin-containing liposomes. Effects of membrane phospholipid composition and cholesterol content

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The effects of phospholipid-oxidation state and vesicle composition on lipid peroxidation in hemolysate-containing liposomes (hemosomes) were studied by the thiobarbituric acid assay. Liposomes (hemosomes) were prepared from egg phosphatidylcholine (PC) with either low ($PC^{0.08}$) or high ($PC^{0.66}$) oxidation indices reflecting low and high conjugated diene/lipid hydroperoxy contents. Thiobarbituric acid reactivity was negligible over 6 h at 38°C in buffer-containing (control) liposomes prepared from $PC^{0.08}$, whereas it was slightly increased in those prepared from $PC^{0.66}$. Encapsulated hemolysate had no effect in $PC^{0.08}$ liposomes, but significantly increased thiobarbituric acid reactivity in those prepared from $PC^{0.66}$. Inclusion of either phosphatidylethanolamine or phosphatidylinositol in the membrane further increased lipid peroxidation in hemosomes prepared from $PC^{0.66}$, whereas phosphatidic acid and phosphatidylserine were inhibitory. Inclusion of cholesterol in the membrane had no effect in $PC^{0.66}$ hemosomes, but significantly inhibited lipid peroxidation in the presence of phosphatidylethanolamine or phosphatidylinositol. The effects of phosphatidic acid and cholesterol were dose-dependent. Co-incorporation of cholesterol and phosphatidic acid or phosphatidylserine in the membrane resulted in almost complete elimination of hemoglobin (Hb)-induced lipid peroxidation. Lysophosphatidic acid had similar effect as phosphatidic acid, whereas lysophosphatidylserine exerted inhibition only in the presence of phosphatidylethanolamine. The rate of lipid peroxidation showed no correlation with the amount of encapsulated Hb, neither with the oxidation indices nor the polyunsaturated fatty acid contents of negatively charged phospholipids. The above findings suggest a possible role for the high cholesterol content and preferential localization of phosphatidylserine in the inner bilayer leaflet of erythrocyte membrane in protecting against Hb-induced lipid peroxidation in the membrane.

Introduction

In recent years, there has been considerable interest in the role of lipid peroxidation in some hemolytic anemias, where increased oxidation of hemoglobin (Hb) is known to occur [1–3]. A recent study [4] showed that the oxidative interaction between Hb and the cell membrane, which can be a key step in the hemolytic process, can be conveniently studied in a liposome model of the erythrocyte (hemosomes). Hemosomes consist of

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Abbreviations: A_{532}, absorbance at 532 nm; Hb, hemoglobin; PC, phosphatidylcholine; $PC^{0.08}$, phosphatidylcholine with an oxidation index of 0.08; $PC^{0.66}$, phosphatidylcholine with an oxidation index of 0.66; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

Hb sandwiched between the membranes of multilamellar liposomes, and are also a subject of interest as a potential blood substitute [5–9]. It has been shown that encapsulated purified Hb induces massive formation of thiobarbituric acid-reactive substances in egg phosphatidylcholine (PC)/cholesterol liposomes, while whole hemolysate is without effect [4]. It was concluded that antioxidants and enzymes in the hemolysate protect against lipid peroxidation [4]. Using the same model, a subsequent study showed that oxidation of Hb encapsulated as hemolysate was increased if negatively charged phospholipids, particularly phosphatidic acid were included with PC in the membrane [10]. This increase of Hb oxidation was inhibited by co-incorporation of cholesterol [10]. In the present study, we extend these findings to examine the effects of cholesterol, phosphatidylethanolamine and negatively charged phospholipids on the Hb-induced lipid peroxidation in hemosomes.

Materials and Methods

Materials

Two egg PC preparations were used differing in their oxidation indices [11]; PC with low (0.08) oxidation index ($PC^{0.08}$) was prepared as described in Ref. 12, and was used within 3 weeks after the preparation. On the basis that unsaturated lipids stored dried tend to autooxidize [13], egg PC with high (0.66) oxidation index ($PC^{0.66}$) was prepared similarly, but was stored lyophilized in closed glass ampoulles at −20°C for 15 months. Both PC preparations were pure by TLC standards. Wheat germ phosphatidylinositol, egg phosphatidylethanolamine, egg phosphatidic acid and bovine brain phosphatidylserine were obtained from Lipid Products (South Nutfield, U.K.). Phosphatidylinositol was pure by TLC using two different developing systems [14], phosphatidylethanolamine, phosphatidic acid and phosphatidylserine were purified by preparative TLC on silica gel N-HR (Polygram®, F.R.G.), using standard developing solvents [14]. The respective lyso-derivatives were also pooled. The oxidation indices of phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid and phosphatidylserine were 0.10, 0.10, 0.08 and 0.13, respectively. Phospholipids were stored in $CHCl_3/CH_3OH$ (2 : 1, v/v), at −20°C. Other chemicals were of analytical grade from Reanal (Budapest, Hungary).

Methods

The polyunsaturated fatty acid contents of the applied phospholipids were determined by gas-liquid chromatography after transesterification using 14% boron trifluoride in methanol (Sigma Chem. Co., St. Louis, MO), as described in Ref. 15. Analyses were done on a Hewlett-Packard gas chromatograph equipped with a flame ionization detector. The GC-oven was maintained at 225°C, and a 2-m glass column packed with 10% Silar 5CP on 80/100 gas-chrom Q11 was used.

The hemolysate was prepared as previously described [4,10], it was stored at −20°C and used within 1 week of preparation. Freshly prepared and 1-week-old hemolysate gave identical results. The Hb concentration and met-Hb level in the hemolysate, determined as in Ref. 10, were 29–34 g/100 ml, and 2–5%, respectively.

Hemosomes were prepared as in Ref. 4, by shaking of 0.5 ml of hemolysate with 5 mg of lipid (mixtures) dried onto the sides of glass flasks. Hemosomes were separated from unencapsulated Hb by centrifugation in 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) (buffer) at 4°C, three times for 20 min at $20000 \times g$. After the final washing, hemosomes were suspended in 0.8 ml of buffer and incubated with shaking in open tubes at 38°C.

The amount of encapsulated Hb relative to liposomal lipids was determined by measuring Hb-iron and phospholipid phosphorus in ashed aliquots of washed hemosomes. Ashing was performed in magnesium nitrate [16], iron was measured with the *o*-phenanthroline method [17] using a Galenopharm S.A. diagnostic kit (Catalogue No. 1090-04, Switzerland), and phospholipid phosphorus was determined as in Ref. 16.

Lipid peroxidation was measured as previously described [4] with the following modifications: 0.1-ml aliquots were mixed with 0.05 ml of 28% (w/v) trichloroacetic acid/0.1 M sodium metaarsenite solution, and 0.1 ml of the supernatant was diluted in 1 ml of 1% thiobarbituric acid in 50 mM NaOH. After heating at 100°C for 15 min, lipids were extracted with $CHCl_3/CH_3OH$ (2 : 1,

v/v), and A_{532} of the aqueous phase was measured. Thiobarbituric acid reactivity was expressed as malondialdehyde equivalents, using tetraethoxypropane for calibration.

Results

Fig. 1A shows that thiobarbituric acid reactivity, a marker of lipid peroxidation [18] did not change over 6-h incubation at 38°C in buffer-containing (control) liposomes prepared from PC with low oxidation index ($PC^{0.08}$). When PC with high oxidation index ($PC^{0.66}$) was used, thiobarbituric acid reactivity increased during incubation, and this increase was greater in liposomes consisting of equimolar $PC^{0.66}$ and phosphatidylethanolamine ($PC^{0.66}$/PE, 1 : 1). Encapsulated hemolysate in $PC^{0.08}$ liposomes had no effect on lipid peroxidation, but in those prepared from $PC^{0.66}$, and particularly in liposomes made of $PC^{0.66}$/PE (1 : 1), thiobarbituric acid reactivity was significantly increased. Since stimulation of lipid peroxidation by hemolysate was apparent only in $PC^{0.66}$-containing liposomes, all subsequent experiments were carried out with $PC^{0.66}$, in the following referred to as 'PC' (without superscript).

Fig. 1B shows that incorporation of cholesterol in the liposome membrane at 50 mol% (PC/cholesterol, 1 : 1 mol ratio) did not affect the rate of lipid peroxidation. Inclusion of negatively charged phospholipids in the membrane at 25 mol% markedly influenced lipid peroxidation, but depending on the phospholipid species, in alternate directions. Phosphatidic acid (PC/phosphatidic acid, 3 : 1 mol ratio) and phosphatidylserine (PC/PS, 3 : 1) significantly decreased, whereas phosphatidylinositol (PC/PI, 3 : 1) increased thiobarbituric acid reactivity. Replacing PC by PC/PE (1 : 1), the pattern and degree of the effects of negatively charged phospholipids did not change, however, cholesterol significantly inhibited lipid peroxidation (Fig. 1C). The most remarkable finding is illustrated in Fig. 1D; irrespective of the presence of phosphatidylethanolamine, co-incorporation of cholesterol with phosphatidylinositol significantly inhibited, and with phosphatidic acid or phosphatidylserine, almost completely abolished Hb-induced lipid peroxidation.

Fig. 2 shows the concentration dependence of the inhibitory action of phosphatidic acid and cholesterol. Phosphatidic acid in PC hemosomes did not affect lipid peroxidation at 14 mol%,

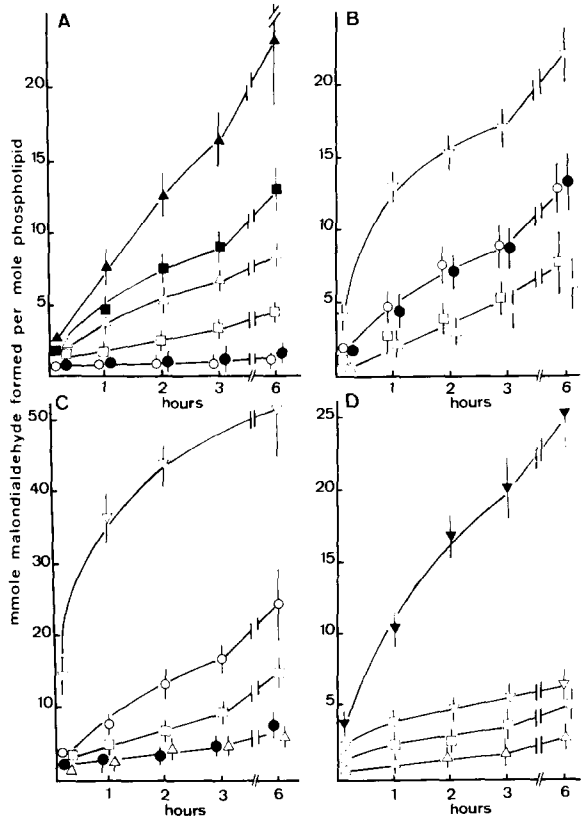

Fig. 1. Rates of malondialdehyde formation at 38°C in liposomes and hemosomes of different composition. (A) Buffer-containing liposomes (open symbols) and hemosomes (filled symbols) were prepared from $PC^{0.08}$ (○), $PC^{0.66}$ (□), or from $PC^{0.66}$/PE (1 : 1 mole ratio) (△). For further details see Materials and Methods. Means ± S.E. for four experiments with liposomes and 8–11 experiments with hemosomes are presented. (B) Hemosomes were prepared from $PC^{0.66}$ (= PC) and other constituents with the following mole ratios: PC/cholesterol, 1 : 1 (●); PC/phosphatidic acid (3 : 1) (▲); PC/PS (3 : 1) (■); PC/PI (3 : 1) (▽). The values for PC (○) are transposed from panel A. All other conditions are the same as in A. 4–11 experiments. (C) Identical experiments with those in B, except that PC/PE (1 : 1) was used instead of pure PC. The values for PC/PE (○) are transposed from A. 4–6 experiments. (D) Hemosomes were prepared from PC/phosphatidic acid/cholesterol (3 : 1 : 4) (▲); PC/PS/cholesterol (3 : 1 : 4) (■); PC/PI/cholesterol (3 : 1 : 4) (▽) and PC/PE/PI/cholesterol (3 : 3 : 2 : 8) (▼). PC/PE/phosphatidic acid/cholesterol (3 : 3 : 2 : 8) and PC/PE/PS/cholesterol (3 : 3 : 2 : 8) hemosomes gave identical results with those prepared with PE (△, □). 6–11 experiments.

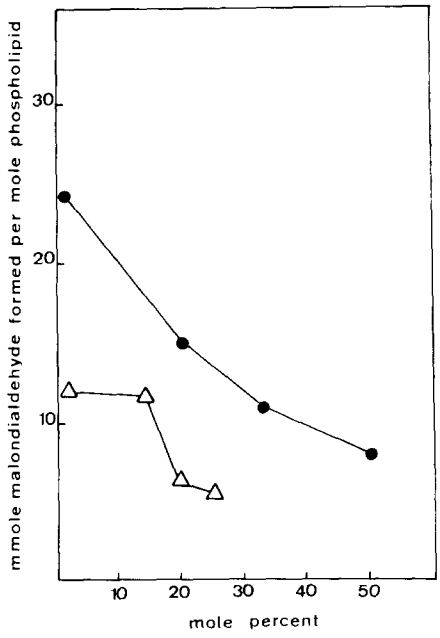

Fig. 2. Concentration dependence of the lipid peroxidation inhibiting actions of phosphatidic acid and cholesterol. PC/phosphatidic acid (△) or PC/PE/cholesterol (●) hemosomes were prepared with mole ratios of phosphatidic acid and cholesterol (related to total lipids) given on the abscissa. Thiobarbituric acid reactivity was determined after 4-h incubation. Other details are the same as for Fig. 1. Typical experiments out of 3–4.

TABLE I

THE EFFECTS OF LYSOPHOSPHATIDIC ACID AND LYSOPHOSPHATIDYLSERINE ON LIPID PEROXIDATION IN HEMOLYSATE-CONTAINING LIPOSOMES

Experimental details are as for Fig. 1. Hemosomes were prepared from $PC^{0.66}$ and were incubated for 4 h at 38°C. PS-lysoPS, a mixture of PS and lysoPS in 1:0.4 (w/w) ratio. PA, phosphatidic acid.

Composition of hemosomes		Malondialdehyde/phospholipid (mmol/mol)	
lipids	mole ratio	mean ± S.D.	*n*
PC		8.9 ± 2.7	8
PC/PA	3:1	4.8 ± 2.9	4
PC/lysoPA	3:1	4.3 ± 2.3	4
PC/PS	3:1	5.5 ± 1.8	9
PC/lysoPS	3:1	8.5 ± 2.1	5
PC/PS-lysoPS	3:1	8.8 ± 2.5	4
PC/PE	1:1	17.8 ± 3.5	4
PC/PE/PS	3:3:2	9.4 ± 2.5	9
PC/PE/lysoPS	3:3:2	9.6 ± 2.8	4

whereas at 20 mol% its inhibitory potential was close to that afforded by 25 mol%. Thus, only in a narrow concentration range (14–20 mol%) is the inhibitory action of phosphatidic acid dependent on its membrane concentration. In contrast, increasing concentrations of cholesterol in the 5–50 mol% range gradually decreased lipid peroxidation in PC/PE (1:1) hemosomes.

Table I compares the effects of phosphatidic acid and phosphatidylserine with their lyso-derivatives. Lysophosphatidic acid in PC-hemosomes exerted similar inhibition on lipid peroxidation as phosphatidic acid. The inhibitory effect of lysophosphatidylserine was, however, apparent only in PC/PE (1:1) hemosomes. A mixture of PS/lysoPS (1:0.4, w/w) had the same effect as lysophosphatidylserine.

Table II shows that the Hb-iron content relative to phospholipid phosphorus was slightly increased in PC/PE (1:1) hemosomes as compared to that in pure PC ones, with significant further increase caused by cholesterol and phosphatidic acid. Additive effects between cholesterol and phosphatidic acid in increasing Hb-encapsulation was not apparent. The Hb/lipid weight ratios paralleled the above changes.

Table III shows the polyunsaturated fatty acid contents of the phospholipids used. The fatty acid

TABLE II

ENCAPSULATION OF HEMOGLOBIN IN LIPOSOMES OF DIFFERENT COMPOSITION

From washed hemosome suspensions, Hb-iron and phospholipid phosphorus were determined as described in Materials and Methods. The values were converted to protein and lipid weights to obtain the Hb/lipid weight ratios. The data apply to hemosomes prepared from $PC^{0.66}$. PA, phosphatidic acid.

Hemosome composition		Fe/P (mol/mol·10^{-3})		Hb/lipid (g/g)	
lipids	mole ratio	mean ± S.D.	*n*	mean ± S.D.	*n*
PC		34.3 ± 10.1	9	0.66 ± 0.19	9
PC/PE	1:1	54.5 ± 14.7	6	1.10 ± 0.24	6
PC/PE/cholesterol	1:1:2	116.4 ± 32.1	7	1.61 ± 0.52	7
PC/PE/PA	3:3:2	107.6 ± 24.0	6	2.14 ± 0.69	6
PC/PE/PA/cholesterol	3:3:2:8	98.1 ± 34.5	5	1.48 ± 0.51	5

TABLE III

THE POLYUNSATURATED FATTY ACID CONTENTS OF PHOSPHOLIPIDS USED TO PREPARE HEMOSOMES

Gas-chromatographic analysis was carried out as described in Materials and Methods. Means ± S.D. of the values from 3–6 analyses of each sample are presented. Σ denotes the sum of the polyunsaturated fatty acid percentages, thus $\Sigma/100-\Sigma$ is the ratio of polyunsaturated to saturated (plus monounsaturated) fatty acids. The amounts of 20:0–1 and > 20:0–1 added to the polyunsaturated fraction were negligible. PA, phosphatidic acid.

	Percent of total fatty acids					Ratio of polyunsaturated to saturated fatty acids: $\Sigma/100-\Sigma$
	18:2	18:3	20:0–3 [a]	20:4	> 20: X [b]	
PC	14.5 ± 1.7	1.4 ± 0.4	5.7 ± 1.2	11.7 ± 0.9	7.5 ± 2.3	0.68
PA	14.8 ± 1.4	0.7 ± 0.5	1.4 ± 0.7	11.6 ± 3.2	14.3 ± 6.5	0.74
PE	9.2 ± 1.6	0.7 ± 0.1	1.3 ± 0.7	11.2 ± 1.1	34.3 ± 6.9	1.31
PI	11.8 ± 1.5	1.9 ± 0.7	1.5 ± 0.6	1.2 ± 0.6	18.6 ± 8.9	0.53
PS	9.9 ± 4.2	3.9 ± 0.7	22.7 ± 1.8	4.3 ± 1.0	31.2 ± 4.3	2.57

[a] The areas of peaks located between 18:3 and 20:4 were summed.
[b] The areas of peaks located after 20:4 were summed.

patterns of different phospholipids varied significantly, but neither the levels of C18:2, 18:3, 20:0–3, 20:4 or > 20: X fatty acids, nor the ratios of polyunsaturated to saturated (plus monounsaturated) fatty acids could be consistently correlated with the effects of the parent phospholipids on lipid peroxidation. The polyunsaturated fatty acid profiles support, and in part complete, the data in Refs. 19, 20 and 21.

Discussion

In an attempt to extend earlier information on Hb-induced lipid peroxidation in Hb-containing liposomes [4], this study focused on the role of membrane constitution in the process. The effects of negatively charged phospholipids and cholesterol were tested both alone and in combinations, starting out from the observation that these constituents significantly affect the oxidation rate of Hb enclosed in liposomes as hemolysate [10]. The PC sources used to prepare liposomes had either low or high oxidation indices reflecting low and high amounts of conjugated dienes and lipid hydroperoxides in PC [11,22]. In liposomes prepared from PC with low conjugated diene/lipid hydroperoxy content, encapsulated hemolysate did not elicit lipid peroxidation. This is in keeping with earlier findings demonstrating the strong protection against Hb-induced lipid peroxidation afforded by whole hemolysate [4]. In hemosomes prepared from PC with high conjugated diene/lipid hydroperoxy content, however, the protective system in the lysate was insufficient to prevent lipid peroxidation. It is likely that under these conditions the system is loaded with excess of highly oxidising intermediates deriving from the reaction of preformed lipid hydroperoxides with the heme-iron complex liberated from, or bound in oxy- and/or met-Hb [23–25,10]. These radicals may either directly initiate lipid peroxidation or exhaust natural antioxidants, triggering indirectly the lipoperoxidative cascade.

The effects of different phospholipid additives depended on the molecular species. Phosphatidylethanolamine and phosphatidylinositol increased lipid peroxidation, whereas phosphatidic acid and phosphatidylserine decreased it. Consequently, it is difficult to explain the effects of phosphatidylinositol, phosphatidic acid and phosphatidylserine with their anionic character. The conjugated diene/lipid hydroperoxy contents of the above additives were not significantly different, and neither the amount of liposome-encapsulated Hb, nor the polyunsaturated fatty acid content of the phospholipids showed direct relationship with the rate of lipid peroxidation. Phosphatidylinositol, for example has relatively low polyunsaturated fatty acid content and yet promotes lipid peroxidation, whereas phosphatidylserine with the

highest amount of polyunsaturated fatty acids among the studied phospholipids inhibits it. The finding that lysophosphatidylserine did not inhibit lipid peroxidation (although it has obviously less polyunsaturated fatty acids than phosphatidylserine) also argue against the critical role of polyunsaturated fatty acid content. Conceivably, the observed changes are related to diverse steric effects of different phospholipid headgroups and acyl moieties on membrane structure. Similar conclusions were drawn by Mooibroek et al. [26], from their experiments with irradiated micelles and liposomes of different lipid composition. Detailed studies of the structure-function relationship in this respect will probably give more precise indications of the molecular mechanism.

The findings with cholesterol are in keeping with some other observations on the suppression of lipid peroxidation by cholesterol in liposomes [27,28]. The results clearly show that under the conditions studied, the presence of another phospholipid species in addition to PC is also required for the inhibitory action of cholesterol to take place. Phosphatidylethanolamine and negatively charged phospholipids equally served in this role. The most drastic inhibition of Hb-induced lipid peroxidation was obtained with cholesterol plus phosphatidic acid or phosphatidylserine. In such hemosomes, almost complete suppression of thiobarbituric acid reactivity took place, presumably as a result of additive inhibitory effects. There are several possible mechanisms by which Hb-induced lipid peroxidation might be reduced by cholesterol; (i) the tighter packing of the fatty acid chains in the presence of cholesterol [29] may present a steric barrier to the access of Hb and/or heme detached from globin [10] to lipid hydroperoxides; (ii) the decreased acyl-chain mobility related to the tighter packing may reduce the reactivity of polyunsaturated fatty acid hydroperoxides and/or polyunsaturated fatty acids towards Hb or oxygen; (iii) chemical trapping of oxygen upon conversion of cholesterol into non-radial oxide-derivatives [30] may decrease the amount of oxygen participating in the peroxidation of polyunsaturated fatty acids; (iv) slower oxidation of Hb to met-Hb and/or reduced heme loss from globin in the presence of membrane cholesterol [10] may restrain the formation of oxygen radicals and/or porphyrin radicals [2,3,24]. The validity and relative contributions of the above proposals remain to be clarified, along with the molecular mechanism of the interactions that convert cholesterol to become inhibitory on Hb-induced lipid peroxidation.

Our findings may help understanding an aspect of the structure-function relationship in the red cell membrane. The concentrations of PC, phosphatidylethanolamine and phosphatidylserine in this membrane are 29, 28 and 14% of the total phospholipids, respectively [31]. Further known features of the erythrocyte membrane are the high cholesterol/phospholipid mole ratio (near 1 : 1) [32], and the predominant localization of both phosphatidylethanolamine and phosphatidylserine in the inner bilayer leaflet [32,33]. The data presented in this study suggest that an important role of this composition and special molecular topology can be to decrease the susceptibility of the membrane to the peroxidative stress that arise from the interaction between the membrane and Hb. From the parallelism between Hb oxidation and lipid peroxidation [4] and from the revealed inhibition of Hb oxidation by membrane cholesterol in hemosomes [10], it also follows that a function of cholesterol interacting with negatively charged phospholipids and/or phosphatidylethanolamine in the inner bilayer shell may be the protection of Hb from oxidation and denaturation. The following observations support this proposal; the membrane phospholipid asymmetry is lost in sickled red cells (in sickle cell anemia) with concurrent shift of phosphatidylethanolamine and phosphatidylserine to the outer bilayer leaflet [3,33]. At the same time, these cells show a remarkable (about 8-fold) increase of susceptibility to peroxidation in vitro [3], and their membrane contains a 5-times higher amount of denatured Hb than the normal red cells [34].

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