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Phenylhydrazine-induced changes in erythrocyte membrane surface lipid packing

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Phenylhydrazine-induced oxidative damage in red cells results in increased binding of merocyanine 540, a fluorescence probe sensitive to changes in lipid packing. Fluorescence polarization studies with diphenylhexatriene did not reveal major changes in order parameters both in intact red cells and lysates treated with phenylhydrazine. These fluorescence studies indicate that major changes are observed in membrane lipids. Analytical studies of membrane phospholipids revealed a significant decrease in phosphatidylethanolamine. The results of the fluorescence and lipid studies, taken in association with our previously reported findings on spectrin and other cytoskeletal protein degradation in red cells exposed to phenylhydrazine, suggests that degradation of cytoskeleton membrane proteins is also responsible for changes in the lipid bilayer surface of the red cell membrane.

Introduction

Phenylhydrazine serves as a useful probe of oxidative damage in red cells. It reacts with hemoglobin via a redox reaction to form superoxide, hydrogen peroxide, free radical intermediates and oxidised products of hemoglobin [1]. Phenylhydrazine causes changes in lipids, which is manifest as either chromolipid formation and/or lipid peroxidation and changes in the major cytoskeletal proteins, which is manifest as degradation and polymerization [2–4].

Spectrin can cross-link hemoglobin by disulfide exchange in rabbit red cells exposed to phenylhydrazine during prolonged incubations [4]. Recent observations by SDS-PAGE and an immunoblot technique employing IgG antibodies to spectrin on a gel of the major cytoskeletal proteins showed that phenylhydrazine caused spectrin degradation that was not associated with the formation of significant amounts of high molecular weight material [3].

Intact red cell membrane phospholipids are asymmetrically distributed in the bilayer [5,6] and the cytoskeleton membrane proteins may play an important role in the genesis of the asymmetry [7–9]. An important contribution in studying the role of spectrin in the maintenance of phase-state asymmetry in red cell membranes comes from studies with merocyanine 540, an impermeant fluorescent dye that has a greater affinity of binding for the fluid-phase rather than the gel-phase of the lipid bilayer [10]. Thus, red cells that were either deficient in spectrin or modified by oxidation of spectrin sulfhydryl groups to form polymers of spectrin, showed the external membrane leaflet

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more disordered compared to normal red cells [11].

In this work, we utilize the fluorescent probes, diphenylhexatriene and merocyanine 540 for studying the interaction of phenylhydrazine in intact red cells. Our objectives are to determine the effects of phenylhydrazine on red cell membrane lipid packing. We have measured the phospholipid content of the intact red cell membrane and have related these changes found in the lipids to our fluorescence measurements.

Materials and Methods

Treatment of red cells with phenylhydrazine

Human blood was drawn daily into a test tube containing a small amount of 3.8% sodium citrate solution. Plasma and buffy coat were removed by centrifugation and the red cells were washed three times with 0.9% NaCl. Washed cells were suspended in Krebs-Ringer glycylglycine buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 50 mM glycylglycine at pH 7.4) at 5% (v/v) red cell suspensions. For oxidative treatment red cells were incubated in 10-ml Erlenmeyer flasks containing 1 ml of cell suspension. Flasks were sealed with rubber stoppers and incubated in a shaking water bath at 37°C for 1 h (unless indicated in the text). Phenylhydrazine was always added last, after all other additions to the incubation mixture. The other variable additions were 5 mM glucose and 0.25 mM butylated hydroxytoluene. Butylated hydroxytoluene was added to the incubation mixture as stock solution dissolved in ethanol. The presence of 1% ethanol in a control experiment did not alter the course of the experiment.

Fluorescence studies with diphenylhexatriene

After the oxidative treatment, the red cells were washed three times in isotonic buffer containing 145 mM NaCl, 5 mM NaH_2PO_4 and 5 mM KCl. In the fluorescence studies, 1.0% (v/v) washed red cells were suspended in the above reported isotonic buffer. Diphenylhexatriene dissolved in absolute ethanol was added with rapid mixing to the suspension to give a final concentration of 10–15 μM diphenylhexatriene and 1.0% ethanol. Control cell suspensions were treated with only ethanol. After being shaken at 37°C for 30–60 min in an Erlenmeyer flask, the cells were pelleted by centrifugation, washed four times with the isotonic buffer and suspended finally to 0.05% (v/v) for fluorescence estimations. We have also carried out fluorescence studies on the lysates. 50-μl aliquots of the final washed cell suspensions (1.0% v/v) were loaded as described above and were then added to 950 μl of 5 mM NaH_2PO_4 (pH 7.4), so that the final cell concentration was 0.05% (v/v). In this way the membrane preparations are comparable and have been subjected to similar conditions. Washed membrane suspensions were prepared from the lysates by washing the pelleted membranes twice with 5 mM NaH_2PO_4 (pH 7.4). The fluorescence parameters obtained with the lysates did not differ from the washed membrane suspensions, indicating that hemoglobin, in the lysate suspensions, did not interfere with the fluorescence measurements [12].

Total fluorescence intensity and steady-state fluorescence anisotropy were measured with a Perkin Elmer 650-10S fluorescence spectrophotometer. The sample was excited by vertically polarized light at 357 nm, and the emitted fluorescence originating from the sample at 450 nm was passed through optical filters and a monochromator and was then analyzed into vertical and horizontal polarized components I_v and I_h. The steady-state anisotropy, r_s, is obtained by: $r_s = (I_v - I_h)/(I_v + 2I_h)$. For each measurement of r_s, I_v and I_h a correction was made for the contribution of intrinsic fluorescence and light scattering. We have also estimated the lipid order parameter [13]: where $S = r_\infty/r_0$; r_0 for diphenylhexatriene is assumed to be 0.390;

r_∞ is computed as follows:

when r_s is in the range $0.13 < r_s < 0.28$ $r_\infty = (4/3)r_s - 0.10$;

when $r_s > 0.28$ $r_\infty = r_s$;

when $r_s < 0.13$ the Perrin equation must hold.

Erythrocyte staining with merocyanine 540

Staining with merocyanine 540 was performed as described by Williamson et al. [10]. The dye was dissolved at a concentration of 1 mg/ml in 50% ethanol-water. After the oxidative treatment, red cells were washed three times with saline and suspended at 2.5% (v/v) in isotonic buffer con-

taining 140 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 10 mM Tris (pH 7.4). To 1 ml of the cell suspension was added 10 μl of stock dye solution plus autologus serum (final concentration 2%) and incubated in a shaking water bath for 30 min at ambient temperature. After the incubation, the cell suspensions were washed two times in saline and resuspended in saline. Prior to fluorescence microscopic examinations, the cell suspensions were maintained at 0–4°C. In order to minimize photobleaching and photolysis, all procedures were carried out in the dark. Quenching experiments were performed by dilution of the stained and washed cells on the slide with 0.2 vols. of 1 mg/ml bis[3-(trimethylamino)propylbenzothiazole-(2)-]pentamethinecyanine bromide, diS-C_3N-$(Cl)_3$-(5), (a gift of Dr. A. Waggoner) in saline. After mixing, the cells were immediately viewed by fluorescence microscopy.

Lipid extraction

Phospholipid extraction was carried out by the method of Rose and Oklander [14]. 5 mM EDTA solution was added to 0.25 ml packed washed cells and vortexed. Lysis was completed in about 10 min. The lysates were chilled on ice, and 3.5 ml cold isopropanol was added. The samples were kept on ice for 15 min and then placed at room temperature for 1 h with frequent vortexing. After the isopropanol extraction the samples were placed on ice after addition of 1.6 ml chloroform for 15 min and then placed at room temperature for 1 h. The samples were centrifuged at 1500 × *g* for 10 min. The supernatants were decanted into glass tubes and dried with nitrogen.

Phospholipid analysis by thin-layer chromatography

The dried extracts of red cells were redissolved in methanol/chloroform (1 : 1, v/v) and separated on TLC plates (20 cm × 20 cm, Silica G plates from Analtech Co.) by chloroform/methanol/concentrated ammonia (70 : 30 : 5, v/v) for one-dimensional studies. In two-dimensional TLC studies the samples were separated on Silica H plates (10 cm × 10 cm, Analtech Co.) with chloroform/methanol/concentrated ammonia/water (58.5 : 35.1 : 3 : 5.7, v/v) in the first dimension and chloroform/methanol/acetic acid/water (60 : 30 : 8 : 5.7, v/v) in the second dimension. Between runs, the plates were dried with an air blower for 1 h. The dried extracts of red cells were redissolved in diethyl ether/petroleum ether/glacial acetic acid (100 : 97 : 3, v/v) and run on 10 cm × 20 cm Silica G plates (Analtech Co.).

The TLC plates were air dried, stained with iodine and phospholipids were identified by comparing with the standards. The spots were scraped from plates after staining. The phosphorus determination was carried out as described by Bottcher et al. [15] except that perchloric acid was added directly to the scraped powder.

Results

Diphenylhexatriene fluorescence studies in phenylhydrazine treated red cells

Fig. 1 shows a time-dependent profile on the extent of the diphenylhexatriene incorporation in intact red cells. On the same red cell preparation, for each total fluorescence intensity measurement, we have also estimated the fluorescence anisotropy (Fig. 1). It is evident from these results that at 60 min the incorporation of diphenylhexatriene in red cells is almost complete, though the fluorescence anisotropy values are indepen-

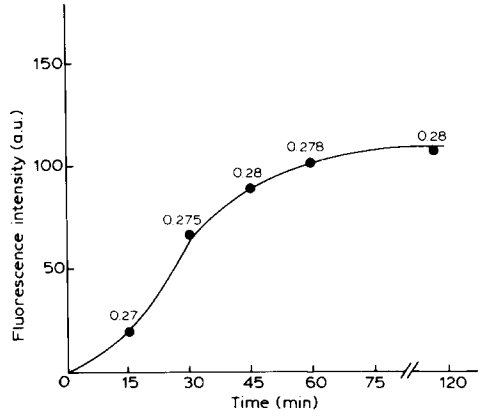

Fig. 1. Time-dependent profile of diphenylhexatriene incorporation in human red blood cell suspensions at 37°C. After the addition of diphenylhexatriene, see Materials and Methods, the red cells were incubated at different times. At the end of the incubation total fluorescence intensity (arbitrary units, a.u.) and fluorescence anisotropy (values above points) of the fluorophore were evaluated. Each time point represents three separate experiments in triplicate. The supernatants of these cell preparations show negligible fluorescence (< 2%).

dent of the time of incorporation. This is apparent because similar values of fluorescence anisotropy occur throughout the time course. In another set of experiments, we compare the total fluorescence intensity in intact red cells and lysates treated with different concentrations of phenylhydrazine (Fig. 2). Phenylhydrazine up to 1 mM did not modify the total fluorescence emission in both intact red cells and lysate suspensions with respect to untreated samples (Fig. 2).

These results indicate that the observed differences in fluorescence intensity between red cells and lysates may be related to a nonradiative energy transfer to heme in the intact red cell [16]. We have reported that oxidative stress induced by phenylhydrazine in red cells increases hemoglobin binding to the membranes in the form of cross-linked polymers [17] which could alter the fluorescence intensity measurements. However, our data do not seem to support this possibility because phenylhydrazine up to 1 mM did not modify the total fluorescence emission in both intact red cells and lysate suspensions with respect to untreated samples (Fig. 2).

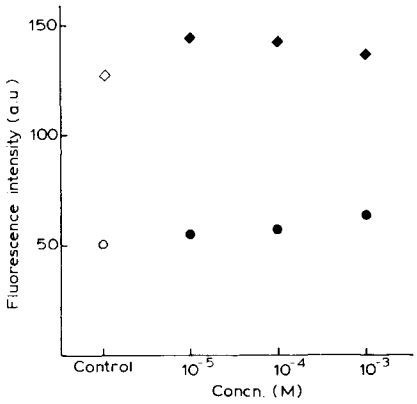

Fig. 2. Phenylhydrazine concentration dependent profile of diphenylhexatriene total fluorescence emission in red blood cells and lysates. Phenylhydrazine-treated erythrocytes (○) were processed for diphenylhexatriene incorporation (Materials and Methods), washed and analyzed. Lysates (◇) corresponding to each cell preparation were prepared for comparison. Each point represents three separate experiments in triplicate for each concentration of phenylhydrazine. The supernatants of these cell preparations show negligible fluorescence (< 2%). Control, open symbol; phenylhydrazine treatment, closed symbol.

The steady-state fluorescence polarization data of the diphenylhexatriene-labeled red cells which are untreated or treated with varying concentrations of phenylhydrazine at 25°C appear in Table I. The order parameter (S_{DPH}) are derived using the method proposed by Van Blitterswijk et al. [13] in which an empirical relationship between the fluorescence anisotropy, r_s, and the limiting fluorescence anisotropy, r_∞, allow us to estimate a lipid structural order parameter directly from steady-state fluorescence polarization measurements. A slight increase of the order parameter value is observed in red cells treated with up to 100 μM phenylhydrazine though a further increase of the concentration to 1 mM does not further increase the order parameter (Table I). In fluorescence studies with diphenylhexatriene, the molecule, under lipid peroxidation conditions, may undergo a loss of fluorescence intensity by a chemical modification of the fluorophore [18]. In our experimental model no lipid peroxidation was detectable (data not shown); all the fluorescence measurements were performed in less than 50 min. We did not observe a reduction of the diphenylhexatriene fluorescence intensity of phenylhydrazine-treated red cells.

Merocyanine 540 staining of phenylhydrazine-treated red cells

Light microscopy studies, utilizing merocyanine 540 fluorescence staining revealed that untreated red cells, in agreement with Schlegel et al. [19], did not show any fluorescence after the incubation with the dye in the presence of fresh autologous serum (data not shown). When the red cells were incubated with different concentrations of phenylhydrazine, we observed incorporation of the dye

TABLE I

FLUORESCENCE ANISOTROPY (r_s) AND LIPID ORDER PARAMETERS (S_{DPH}) AT 25°C OF ERYTHROCYTES TREATED WITH PHENYLHYDRAZINE

Phenylhydrazine (mM)	r_s	S_{DPH}
0	0.275	0.825
0.01	0.281	0.848
0.1	0.305	0.884
1	0.304	0.883

that was proportional to the concentration of phenylhydrazine. As can be seen in Fig. 3 (A, B, C), an increase of the fluorescence on the red cells is directly proportional to increasing concentrations of phenylhydrazine. The presence of butylated hydroxytoluene, a well known free radical scavenger does not effect the merocyanine 540 binding in red cells exposed to phenylhydrazine. To evaluate whether or not the fluorescence was present on the outer leaflet of the intact red cell membrane, intact red cells were exposed to diS-C_3N-$(Cl)_3$-(5) treatment, a water-soluble compound that has an absorption maximum at 590 nm and quenches the fluorescence of merocyanine 540 molecules located in the external leaflet of the membrane, but not internal fluorescence [11]. Fluorescence from phenylhydrazine-treated red cells was immediately quenched by the addition of this compound indicating that merocyanine 540 was still on the outer leaflet of the red cell membrane.

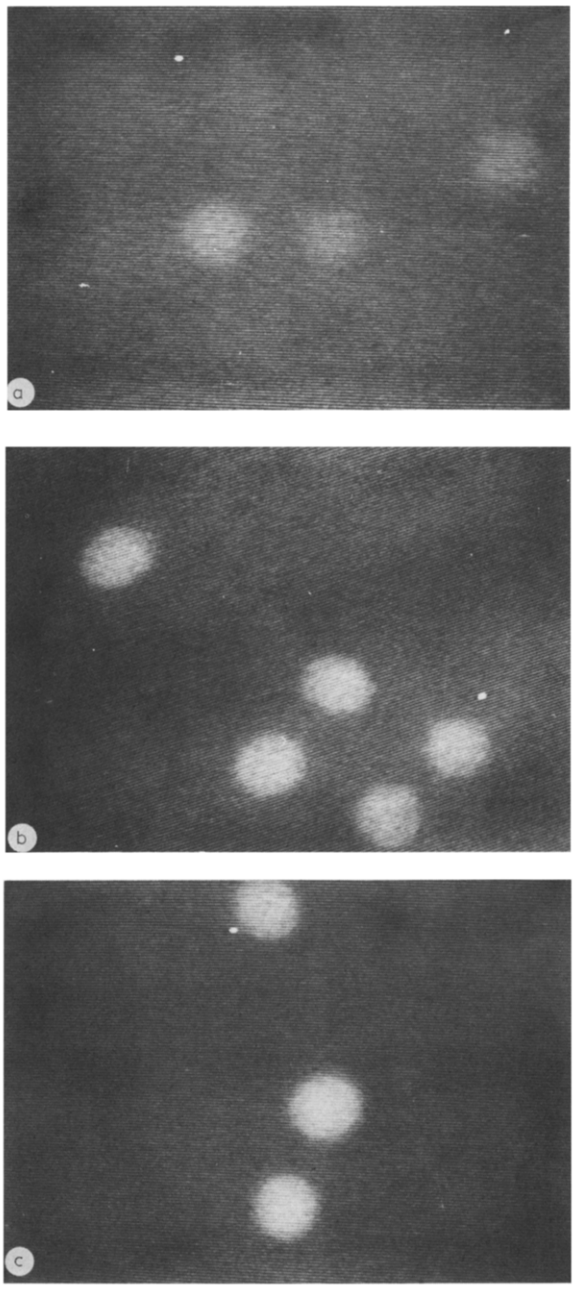

Fig. 3. Merocyanine 540 staining of phenylhydrazine-treated red blood cells. Human erythrocytes were stained with merocyanine 540 after phenylhydrazine treatment at different concentrations: (A) 0.01 mM, (B) 0.1 mM, (C) 1 mM.

The effect of phenylhydrazine on red cell membrane phospholipids

As shown in Table II, the PE content in red cell membranes was decreased markedly when the phenylhydrazine concentration was raised to 10^{-3} M ($P < 0.01$). Other phospholipid components and total phospholipids did not show significant changes after treatment with phenylhydrazine up to 10^{-3} M.

TABLE II

THE EFFECT OF PHENYLHYDRAZINE ON ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS

Values are reported as the mean ± S.E. from five experiments of phospholipid measurements. Blood was drawn daily from different volunteers and treated with phenylhydrazine for 1 h at 37°C. Data were based upon 2 ml of a 5% red cell suspension. Phospholipid values are reported in μg P. Total phospholipid was calculated by adding the various phosphorus components. Values were compared by the paired *t*-test.

Phospholipid	Untreated	Phenylhydrazine (mM)		
		0.01	0.1	1.0
PE	2.04 ± 0.04	1.98 ± 0.05	1.83 ± 0.07	1.68 ± 0.03 **
PC	2.00 ± 0.09	2.02 ± 0.10	2.05 ± 0.10	1.95 ± 0.14
SM	1.97 ± 0.07	1.92 ± 0.08	2.06 ± 0.12	1.84 ± 0.06
PS	0.97 ± 0.13	0.98 ± 0.12	1.04 ± 0.12	0.85 ± 0.08 *
Total P	7.08 ± 0.18	6.90 ± 0.14	7.24 ± 0.40	6.58 ± 0.22 **

* $P < 0.05$.
** $P < 0.01$.

Discussion

The data presented indicate that membrane fluidity in human red cells are influenced by phenylhydrazine treatment. Steady-state measurements of membrane fluidity of phenylhydrazine-treated red cells showed a slight increase of the order parameter S. A fluorescence microscopic study using the fluorescent probe merocyanine 540 indicated that the outer leaflet of the red cell membrane becomes more disordered upon exposure of intact red cells to phenylhydrazine.

Rice-Evans and Hochstein [20] have reported an increase in the microviscosity parameter from 1.56 to 3.24 in human red cell ghosts exposed to phenylhydrazine (0.5 mM) for 60 min at 37°C. This finding is contrary to our observation of a slight increase of the S parameter for red cells exposed to 1 mM phenylhydrazine for 60 min at 37°C. The microviscosity parameter [21] neglects the major contribution to the steady state fluorescence anisotropy (r_s), the limiting long time fluorescence anisotropy (r_∞), determined by the orientational order of the fatty acid chains of the phospholipids [12,22,23]. Recalculating the original fluorescence anisotropy (r_s) data of Rice-Evans and Hochstein for the order parameter S we obtained 0.70 and 0.75 for the untreated and phenylhydrazine-treated red cell ghosts, respectively.

Merocyanine 540 binding to the outer leaflet is strongly enhanced after phenylhydrazine treatment of red cells. This fluorescent dye does not cross intact red cell membranes and the binding properties are influenced by the degree of the lipid packing in the bilayer [10]. We have also demonstrated, by means of quenching experiments, that the localization of the dye was in the outer leaflet of red cell lipid bilayers after the phenylhydrazine experiments. It is generally reported that merocyanine 540 binds preferentially to fluid-phase lipid bilayers [11,24]. However, it should be taken into account that the molecular packing may be responsible for the change in the affinity of the fluid-phase and gel-phase bilayers for his dye [10]. In other words, a loosening of the lipid packing near the headgroup of phospholipids is the principal factor for determining an increase of merocyanine 540 bilayer affinity. In comparison with the diphenylhexatriene data, which did not show any real change in the fluidity of the red cell membrane treated with phenylhydrazine, the merocyanine 540 data indicate that rather than a change in the fluid phase of the lipid bilayer, there is a loosening of the lipid packing at the bilayer surface.

We have demonstrated that phenylhydrazine-treated human red cells induced spectrin degradation, as a major change in the cytoskeleton membrane proteins, possibly through a proteolytic dependent mechanism [3]. Spectrin is the principal protein of the membrane skeleton [25] and among the several properties that have been described [26–28] it seems to also be involved in the maintenance of membrane phospholipid asymmetry [9]. Haest et al. [29] have reported that after treatment of human red cells with the oxidizing agents tetrathionate and diamide, phospholipase A_2 hydrolyzed 30% of PS and 50% of PE accompanied by a 50% decrease of SH-groups and crosslinking of spectrin. Williamson et al. [11] confirmed and extended their study on the role of the spectrin network in the maintenance of the phase-state asymmetry between the two lipid leaflets in red cell membranes. These authors showed that merocyanine 540 stained all those cells in which the spectrin network was altered or missing such as in tetrathionate-treated red cells, spectrin-free vesicles from in vitro aging of mature red cells and spectrin-deficient red cells from mice with the genetic disease, spherocytic anemia. Our merocyanine 540 data is in good agreement with Williamson's data in which spectrin deficiency is followed by an increase of merocyanine 540 binding to red cell membranes, indicating a change in the phase-state of the individual monolayers.

It is known that the fatty acid side chains esterified to PE and to PS are mostly unsaturated [30] making these lipids susceptible to lipid peroxidative processes than its saturated counterparts [31]. The PE content is significantly reduced after the phenylhydrazine treatment at the highest concentration though we fail to detect any thiobarbiturate reaction products in the phenylhydrazine-treated red cells. However, we did not observe a significant degradation of PS in phenylhydrazine-treated red cells.

Membrane phospholipid reorganisation in the red cell membrane occurred in rats exposed to

phenylhydrazine [32]. This is associated with a significantly small degradation of PS. Destabilization of membrane phospholipid reorganisation, a process that could lead to changes in lipid packing, can occur in red cells that have been treated with malondialdehyde, a thiobarbiturate acid reaction product [33]. An increase in hypercoaguability was also observed in the mice red cells treated with phenylhydrazine [32]. The latter observation on hypercoaguability is consistent with our results which suggest that major alterations occur in the red cell bilayer surface.

In contrast to the change in PE, spectrin degradation induced by phenylhydrazine occurs at lower concentrations than observed for the changes in PE [3]. That merocyanine 540 binding occurs at lower concentrations than observed for the changes in PE, indicate that the lipids are undergoing changes which influence the bilayer surface. These results support the concept that changes in the lipid bilayer depends on the presence of a normal spectrin network [11]. Spectrin polymerization may account for the observed change in membrane lipids in certain conditions [11,29]. The absence of spectrin polymerization in phenylhydrazine-induced oxidative damage implies that the array of events leading to changes in lipid bilayer surface may also include degradation of membrane proteins and changes in membrane lipid content.

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