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Inhibition of hemin-induced hemolysis by desferrioxamine: binding of hemin to red cell membranes and the effects of alteration of membrane sulfhydryl groups

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Hemin binds to red cell membranes during hemin-induced hemolysis but the precise mechanism of hemolysis has not been characterized. Desferrioxamine (DFO), an iron chelator, inhibited hemin-induced hemolysis. DFO partially prevented hemin binding to red cell membranes and partially removed previously bound hemin. Glutathione, an intracellular sulfhydryl compound, also inhibited hemin-induced hemolysis but was only about one tenth as potent as DFO. Decrease of membrane sulfhydryl groups by treatment of cells with either *N*-ethylmaleimide (NEM) or diamide (azodicarboxylic acid bis[dimethylamide]) enhanced hemin-induced hemolysis. Enhancement of hemin-induced hemolysis by NEM and diamide and inhibition of hemolysis by DFO were independent with no evidence of synergism or interference between the two processes. Red cell membranes were saturated with hemin at approximately 75 nmol per mg protein. DFO decreased the hemin saturation level to 25 nmol per mg protein. In the presence of DFO, hemin was bound as the DFO-hemin complex since membranes preferentially removed DFO-hemin complexes from mixtures of complexed and free hemin while free DFO was not bound by the membranes. Access to the inner surface of the membrane was required for binding of the DFO-hemin complex since DFO completely prevented hemin binding in intact cells but not in cells undergoing hemolysis or red cell ghosts. Approximately $50 \cdot 10^6$ molecules of hemin were bound to the membrane of one red cell following hemin-induced hemolysis.

Introduction

Hemin, a potent hemolytic agent, is a product of oxidized hemoglobin [1]. Increased hemin binding is observed in red cell hemoglobinopathies such as thalessemia [2] and sickle cell disease [3]. On binding to red cell membranes it causes K^+ leak, osmotic fragility and hemolysis [4]. Binding of hemin to red cell membranes occurs in the lipid and protein domains [2], but the relevance of these findings to the hemolytic process is not clear.

Hemin may induce an oxidative effect in red cell membranes, as hemin serves as a potent catalyst of 'peroxide-induced' lipid peroxidation [5]. Thiol oxidation in sickle cells has been correlated with excess hemin in their membranes [3]. Excess non-heme iron is found in sickle cell membranes and may contribute to membrane damage in these cells [6]. How and to what extent the peroxidative effects induced by hemin relate to the hemolytic process requires further investigation.

Glutathione, an intracellular sulfhydryl compound, has been shown to be capable of binding to hemin and inhibiting hemin-induced hemolysis [7]. Desferrioxamine (DFO), an iron chelator, has been shown to bind to hemin via the iron moiety and inhibit hemolysis with greater potency than glutathione [8]. DFO was able to partially prevent hemin binding to ghost membranes and partially remove hemin previously bound to membranes [8].

Membrane protein sulfhydryl groups might bind hemin similarly to glutathione. Such binding might either have a protective effect on the membrane or enhance hemin-induced hemolysis. To study the effects of membrane sulfhydryl groups on hemin-induced hemolysis, cells were pretreated with either *N*-ethyl-

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maleimide (NEM) or diamide (azodicarboxylic acid bis[dimethylamide]). NEM oxidizes and binds covalently to membrane sulfhydryl groups while diamide crosslinks sulfhydryl groups. The effects of DFO on hemolysis and binding of hemin to red cell membranes were studied in normal, NEM-treated and diamide-treated red cells.

Materials and Methods

Chemicals

DFO was purchased from Ciba-Geigy (Summit, NJ). Hemin (Bovine, Type I (ferriheme chloride)), glutathione, diamide and NEM were purchased from Sigma (St. Louis, MO).

Solutions of hemin were prepared as described in Baysal et al. [8]. Solutions of hemin and DFO-hemin mixtures were prepared in phosphate-buffered saline (139 mM NaCl, 10 mM Na_2HPO_4/ NaH_2PO_4, pH 7.4). The pH of hemin solutions was always brought to pH 7.4 and precise hemin concentrations were calculated using the absorbance at 385 nm ($E_{mM} = 48$) [9].

Red cell preparation

Freshly drawn human blood was centrifuged at 2500 $\times g$ for 5 min at 25°C. After the removal of plasma and white buffy coat, the cells were washed three times with 0.9% NaCl. Cell suspensions were prepared at 20% in phosphate-buffered saline. Some cell suspensions were then incubated in either NEM (1, 5 or 10 mM) or diamide (1, 5 or 10 mM) for 30 min at 37°C after which they were washed three times with 0.9% NaCl and resuspended in phosphate-buffered saline.

Hemoglobin-free red cell ghosts were prepared by hypotonic lysis (1 : 40) essentially according to Dodge et al. [10] except using 5 mM Tris buffer (pH 7.4). After the final wash, white ghosts were suspended in phosphate-buffered saline. Ghost membranes were kept on ice and used within 24 h of preparation.

Assays of metabolic capacity and sulfhydryl content

Glucose consumption of 25% red cell suspensions 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) containing 5 mM glucose was measured by comparing the glucose content before and after incubation at 37°C for 4 h using the Glucose Diagnostic Assay Kit from Sigma. Hexokinase activity, glutathione content and ghost protein sulfhydryl content were measured as described by Beutler [11]. Protein was measured using the technique of Lowry et al. [12].

Hemolysis of red blood cells by hemin

Red blood cell suspensions (0.5%) in phosphate buffered saline were incubated with hemin or mixtures of hemin and either DFO or glutathione for 30 min at 37°C. Hemolysates were centrifuged at 24 000 $\times g$ for 3 min and hemoglobin in the supernatant was determined by the absorbance at 540 nm after subtracting the small background absorbance due to hemin [8]. 100% hemolysis values were obtained by lysing cells in water.

Binding of hemin to ghosts

Binding of hemin to red cell membranes was measured as described by Baysal et al. [8] using the method of Fish [13]. In some experiments red cell ghosts were incubated with hemin followed by assay for hemin binding. In other experiments suspensions of whole red cells were incubated with hemin followed by harvesting of red cell ghosts and assaying for hemin binding.

Results

The effect of membrane sulfhydryl groups on hemin-induced hemolysis was studied by comparing control red cells with cells preincubated with NEM or diamide. Table I shows the characterization of the three cell types with regard to glucose consumption, hexokinase activity, glutathione content and membrane protein sulfhydryl groups. Hexokinase activity and total glucose consumption were effectively inhibited by incubation of 20% suspensions of red cells with 1 mM NEM and 1 mM diamide. Glutathione was completely oxidized by 1 mM NEM and 5 mM diamide. Membrane protein sulfhydryl groups were decreased by 39% by 1 mM NEM, 63% by 10 mM NEM and 19% by 1 mM diamide. Higher diamide concentrations resulted in binding of hemoglobin to membranes.

Hemin-induced hemolysis was studied in these three cell types (Fig. 1). Preincubation with both NEM and diamide increased the cell's susceptibility to hemin-induced hemolysis with NEM showing more potency at a given preincubation concentration. DFO was able to inhibit hemin-induced hemolysis in all three cell types studied. Enhancement of hemin-induced hemolysis by NEM and diamide was still apparent, superimposed over the inhibitory effect of DFO with no evidence of synergism or interference between the two processes, that is, the absolute increase or decrease in degree of hemolysis caused by NEM, diamide and DFO was simply additive. Glutathione inhibited hemolysis with a similar pattern to DFO with all three cell types but with one tenth the potency of DFO. In control and NEM-treated cells, similar levels of inhibition of hemolysis were observed with 100 μM DFO and 1000 μM glutathione. Glutathione was more efficient in inhibiting hemolysis in diamide-treated cells than in NEM-treated cells.

DFO was an effective inhibitor of hemolysis throughout the range of hemin concentrations that

TABLE I

Characterization of red cells preincubated with NEM or diamide

Red cells were preincubated with NEM or diamide and assayed for glucose consumption, hexokinase activity, glutathione content and membrane protein sulfhydryl content as described in Materials and Methods.

Cell preparation	Glucose consumption (μmol/h per ml RBC)	Hexokinase (% of control)	Glutathione (mM)	Membrane protein sulfhydryl groups (nmol/mg protein)
Control	0.76	100	1.87	59.6
1 mM NEM	0.00	< 2	0.00	36.5
5 mM NEM	0.00	< 2	0.00	22.5
10 mM NEM	0.00	< 2	0.00	21.8
1 mM diamide	0.03	< 2	0.12	48.0
5 mM diamide	0.07	< 2	0.04	– [a]
10 mM diamide	0.00	< 2	0.00	– [a]

[a] The membrane protein sulfhydryl assay could not be carried out at 5 mM or 10 mM diamide due to interference by hemoglobin crosslinked to the membrane.

resulted in 0% to 100% hemolysis. For example, in a 0.5% suspension of red cells, 100 μM DFO decreased hemolysis with 25 μM hemin from 50% to 18%, while 200 μM DFO decreased hemolysis with 50 μM hemin from 100% to 50%.

The binding of hemin to red cell membranes was studied using ghosts prepared from control red cells and cells pretreated with either NEM or diamide (Table II). Hemin binding to cell membranes was similar in ghosts prepared from all three cell types. In each cell type, DFO decreased the binding of hemin to ghosts. If hemin was allowed to bind to ghosts before addition of DFO, subsequent addition of DFO removed hemin with the remaining hemin bound only slightly higher than ghosts in which DFO had blocked hemin binding. Glutathione at the same concentration as DFO did not prevent binding of hemin or remove hemin when added after binding.

Fig. 2 shows the dependence of hemin binding to membranes on hemin concentration. Saturation of membranes occurred at approx. 75 to 100 nmol hemin per mg protein. DFO had little effect on hemin binding up to 25 nmol hemin per mg protein, above which DFO prevented further hemin binding.

Table III shows the effect of DFO in decreasing the saturation point of hemin binding to ghosts from control cells and cells pretreated with NEM or diamide. NEM slightly increased the hemin bound at saturation. Regardless of cell type and saturation binding in the absence of DFO, in the presence of DFO saturation binding was decreased to about 25 nmol hemin per mg protein.

The binding of hemin to ghosts was compared to the binding of hemin to membranes during whole cell hemolysis experiments. In hemolysis experiments resulting in 65% hemolysis (0.5% red cell suspensions, 50

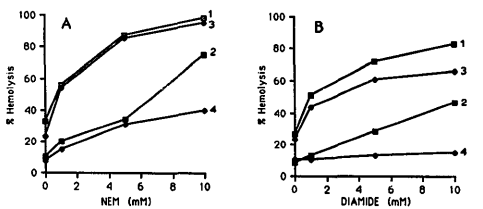

Fig. 1. Effects of DFO and glutathione on hemin-induced hemolysis in normal and NEM- and diamide-treated red cells. Hemolysis of 0.5% suspensions of red cells was determined after a 30 min incubation at 37°C in the presence of 25 μM hemin (□, 1), hemin plus 100 μM DFO (■, 2), hemin plus 100 μM glutathione (◆, 3), or hemin plus 1000 μM glutathione (○, 4). (A) Control cells and cells pretreated with 1, 5 or 10 mM NEM. (B) Control cells and cells pretreated with 1, 5 or 10 mM diamide. The results of representative experiments are shown. Pretreatment of red cells and measurement of hemolysis are described in Materials and Methods.

TABLE II

Effects of DFO and glutathione on hemin binding to red cell ghosts

Ghosts were prepared from untreated red cells or cells preincubated with 10 mM NEM or 10 mM diamide. Suspensions of ghosts (0.5 mg protein/ml or 1 mg protein/ml) were incubated with 50 μM hemin for 30 min at 37°C and hemin binding was determined as described in Materials and Methods. DFO or glutathione was added to suspensions of ghosts either before (15 min at 37°C) of after (30 min at 37°C after washing out excess hemin) incubation with hemin. Results of representative experiments are shown.

Cell preparations	Additions	Addition of DFO before hemin binding [a]		Addition of glutathione before hemin binding [a]		Addition of DFO after hemin binding [b]		Addition of glutathione after hemin binding [b]	
		nmol hemin per mg protein	Percent inhibition of binding	nmol hemin per mg protein	Percent inhibition of binding	nmol hemin per mg protein	Percent inhibition of binding	nmol hemin per mg protein	Percent inhibition of binding
Control	None	23.2		14.5		39.0		40.0	
Control	1 mM DFO	15.5	33			29.0	26		
Control	1 mM glutathione			17.5	0			37.0	8
10 mM NEM	None	25.9		16.0		50.0		51.9	
10 mM NEM	1 mM DFO	18.7	28			32.4	36		
10 mM NEM	1 mM glutathione			16.0	0			49.1	5
10 mM diamide	None	25.5		20.0		47.4		50.9	
10 mM diamide	1 mM DFO	21.4	16			30.2	36		
10 mM diamide	1 mM glutathione			20.0	0			48.3	5

[a] Experiments carried out with ghost suspensions at 1.0 mg protein/ml.

[b] Experiments carried out with ghost suspensions at 0.5 mg protein/ml. Note that hemin binding at 0.5 mg protein/ml is approximately twice that of ghost suspensions at 1.0 mg protein/ml.

μM hemin), hemin bound to cell membrane totaled 35 nmol hemin per mg protein. When hemolysis experiments were carried out in the presence of 2 mM DFO, hemolysis was prevented and harvested membranes contained only 3 nmol hemin per mg protein. DFO was relatively efficient in preventing binding of hemin to membranes of whole cells (3 nmol hemin per mg protein) when compared to levels of hemin binding in the presence of DFO in experiments with ghosts (12 to 30 nmol hemin per mg protein) (Table II, Table III, Fig. 1).

Visible spectra of solutions containing mixtures of hemin and DFO were observed with or without exposure to ghost membrane suspensions (Fig. 3). Under conditions of the experiment in Fig. 3, exposure to

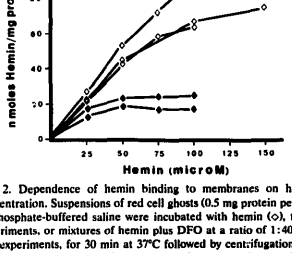

Fig. 2. Dependence of hemin binding to membranes on hemin concentration. Suspensions of red cell ghosts (0.5 mg protein per ml) in phosphate-buffered saline were incubated with hemin (◇), three experiments, or mixtures of hemin plus DFO at a ratio of 1:40 (◆), two experiments, for 30 min at 37°C followed by centrifugation and two washes. Hemin bound per mg membrane protein was determined as described in Materials and Methods.

TABLE III

Effect of DFO on saturation binding of hemin to red cell ghosts

Ghosts were prepared from untreated red cells or cells preincubated with 10 mM NEM or 10 mM diamide. Suspensions of ghosts at 0.5 mg protein/ml were incubated with 75 μM hemin for 30 min at 37°C and hemin binding was determined as described in Materials and Methods. These conditions were found to result in maximum or saturation binding of hemin to red cell ghosts. DFO was added to the hemin stock solution at a 40:1 ratio of DFO:hemin and brought to pH 7.4 before addition to ghost suspensions. The results of three independent experiments are shown.

Cell preparations	Additions	Hemin binding (nmol hemin/mg protein)		
		Expt. 1	Expt. 2	Expt. 3
Control	75 μM hemin	79.8	70.2	76.0
Control	75 μM hemin +3 mM DFO	13.5	12.5	17.3
10 mM NEM	75 μM hemin	100.0	84.6	93.3
10 mM NEM	75 μM hemin +3 mM DFO	17.7	19.2	22.1
10 mM diamide	75 μM hemin	–	71.8	79.0
10 mM diamide	75 μM hemin +3 mM DFO	–	21.8	20.2

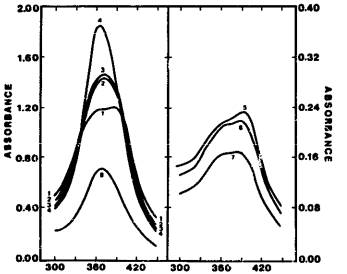

Fig. 3. Visible spectra of solutions containing hemin and DFO with and without exposure to ghost membrane suspensions. Hemin solutions with or without DFO or red cell ghosts were incubated for 30 min at 37°C followed by centrifugation and measurement of the visible spectra of the supernatant. Curves: 1, 50 μM hemin; 2, 50 μM hemin plus 25 μM DFO; 3, 50 μM hemin plus 50 μM DFO; 4, 50 μM hemin plus 2000 μM DFO. Curves 5, 6, 7 and 8: These spectra represent mixtures 1 through 4, respectively, with the addition of red cell ghost suspensions (1 mg protein per ml). Note the 5-fold difference in the Absorbance scale between the left and right panel of the figure.

membranes removed about 80% of the hemin in solution but did not affect the spectra of remaining hemin (spectra 1 and 5). The spectra of a mixture of hemin : DFO with a ratio of 2 : 1 showed the presence of a percentage of the DFO-hemin complex by the shift of the peak at 385 nm to 370 nm (spectrum 2) (see Ref. 8). Exposure of this mixture to membranes resulted in removal of this DFO-hemin complex leaving only pure hemin in solution (spectrum 6). A mixture of hemin : DFO at 1 : 40 showed the spectra of the DFO-hemin complex with shift of the absorbance maximum to 364 nm (spectrum 4). Exposure to membranes removed about 70% of the complex leaving behind remaining complex without qualitative change in the spectrum (spectrum 8). When a solution of DFO without hemin was exposed to membranes and later hemin was added to the solution, it was observed that membranes removed no DFO from the solution (data not shown).

Discussion

Treatment of red cells with NEM or diamide increased susceptibility to hemin-induced hemolysis. The concentrations of NEM and diamide used were sufficient to completely inhibit hexokinase activity (a sulfhydryl protein) thereby completely inhibiting glucose consumption. Intracellular reduced glutathione was completely oxidized by NEM and diamide. Membrane protein sulfhydryl groups were significantly blocked by NEM and crosslinked by diamide. After intracellular glutathione was completely oxidized by NEM or diamide, increased concentrations of NEM or diamide resulted in further decreases in membrane protein sulfhydryl groups and increased susceptibility to hemolysis. These findings imply that intracellular glutathione and membrane protein sulfhydryl groups protect against hemin-induced hemolysis.

Sulfhydryl compounds might inhibit hemin-induced hemolysis by direct binding of hemin thereby preventing hemin from binding to sites where it initiates hemolytic damage or by protecting against hemin-induced oxidative damage. Chiu and Lubin [14] have suggested that the initial step in hemin-induced membrane damage involves the oxidation of membrane protein sulfhydryl groups and lipids. Solar et al. [15] have provided evidence that hemin can promote peroxidation and crosslinking of red cell cytoskeletal proteins in the presence of hydrogen peroxide, *t*-butyl hydroperoxide and phenylhydrazine. No peroxidation or crosslinking was observed in the absence of hydrogen peroxide or other oxidative agents [15]. In the model of hemin-induced hemolysis presented here, an oxidative process was not observed since sub-hemolytic concentrations of hemin did not increase the flux of glucose through glycolysis or the hexose monophosphate shunt or induce the formation of methemoglobin (data not shown). Glucose metabolism is unlikely to be of importance since hemolysis occurs very quickly after exposure to hemin (80% after 15 min, data not shown, see also Ref. 16).

DFO [8] and glutathione [7] both protect against hemin-induced hemolysis although DFO is about 10-times more potent. Both DFO and glutathione were able to inhibit hemin-induced hemolysis in control, NEM- and diamide-treated cells. Glutathione was more effective as an inhibitor in diamide-treated cells since glutathione probably partially reduced crosslinked membrane sulfhydryl groups. Enhancement of hemin-induced hemolysis by NEM and diamide and inhibition of lysis by DFO and glutathione are independent with no evidence of synergism or interference between these two processes.

Since DFO binds to hemin, the question arises whether DFO prevents hemolysis by preventing binding of hemin to membranes or whether the DFO-hemin complex itself binds to membranes but has no intrinsic hemolytic activity. Inhibition of hemin binding to membranes by DFO was similar in control, NEM-treated and diamide-treated cells, consistent with the independence of the mechanism of DFO inhibition and NEM and diamide enhancement of hemolysis. DFO similarly prevented binding of hemin or removed bound hemin in membranes from all three types of cells.

Although DFO prevented hemolysis at hemin concentrations causing various percentages of hemolysis, DFO had little effect on binding of hemin to membranes up to 25 nmol per mg protein. Above this level, DFO was able to prevent further hemin binding in all three cell types. In the presence of DFO, hemin was bound as the hemin-DFO complex as shown by ability of membranes to remove the DFO-hemin complex from a mixture of the complex and free hemin. Uncomplexed DFO is not removed from solution by membranes. The site of binding of the DFO-hemin complex might be on the inside of the membrane since in the absence of hemolysis, DFO prevented nearly 100% of the binding of hemin to membranes, whereas 35 nmol per mg protein were bound when the complex had access to the inside of the membrane during hemolysis. Alternatively, the process of hemolysis might itself enhance the binding in red cells or some other aspect of ghost structure might enable greater binding in the presence of DFO.

Although membrane protein sulfhydryl groups offer protection against hemin-induced hemolysis, these groups do not appear to be involved in the mechanism of hemolysis since neither blocking with NEM nor crosslinking with diamide has a major effect on binding of hemin, binding of the DFO-hemin complex or inhibition of hemolysis by DFO. Chronic binding of hemin to membrane sulfhydryl groups may provide a focus for membrane damage in vivo.

The experiments presented in this paper all demonstrate the acute effect of hemin on whole cells and isolated membranes. The process of hemolysis by hemin in vivo may involve both acute and chronic effects of hemin. Acute effects of hemin on red cells include potassium loss and inability of the membrane to maintain ion gradients [4] and inhibition of the (Ca^{2+} + Mg^{2+})-ATPase [17]. Chronic effects of hemin binding to membranes may be important in damage to sickle cells [3] and in β-thalassemia [2]. Oxidative processes may be induced by hemin in sickle cells [18] and also in other pathological red cells as in glucose-6-phosphate dehydrogenase deficiency and thalessemia. Oxidation of membrane protein sulfhydryl groups has been observed in sickle cells [19] and this process may be enhanced by membrane bound heme [3]. Lipid peroxidation can be induced by hemin in red cell membranes [5] but has not been observed in normal intact cells. Zerez [20] has found that hemin inhibits five red cell cytosolic enzymes: pyrimidine 5'-nucleotidase, pyrimidinenucleosidemonophosphate kinase, adenylate kinase, 6-phosphogluconate dehydrogenase, and aldolase. Although glucose metabolism does not appear to be important in acute hemin-induced hemolysis, chronic alterations in metabolism may be important in hemin toxicity in vivo.

Hemolysis and saturation of hemin binding to red cell membranes occurred at about 75 nmol hemin per mg membrane protein (see also Ref. 16). This concentration represents approximately $50 \cdot 10^6$ molecules of hemin per red cell. To put this number in perspective, the number of molecules per cell of ATP is $55 \cdot 10^6$; glutathione, $120 \cdot 10^6$; 2,3-diphosphoglycerate, $180 \cdot 10^6$; and lactate, $130 \cdot 10^6$ [21]. Red cell hemoglobin contains approximately $800 \cdot 10^6$ hemin molecules per red cell. If hemolysis occurs at $50 \cdot 10^6$ molecules of hemin per cell, the mechanism of hemin-induced hemolysis must account for this number. Theories of hemin-induced hemolysis have invoked binding of hemin to actin [22], spectrin [23] and cytoskeletal protein 4.1 [24–26]. The number of polypeptide chains per cell of actin, spectrin and cytoskeletal protein 4.1 is about $0.4 \cdot 10^6$ each [27]. Considering the high number of hemin molecules required for hemolysis, the interaction of hemin with membrane lipids [2,28,29] might be more significant to the mechanism of hemin-induced hemolysis than the binding of hemin to specific cytoskeletal or lipid bilayer proteins.

Acknowledgements

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