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MODULATION OF IRON-MEDIATED OXIDANT DAMAGE IN ERYTHROCYTES BY CELLULAR ENERGY LEVELS

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In this work we have investigated the effects of iron-induced oxidative stress on erythrocytes and their membranes, the importance of haemoglobin oxidation and of the maintenance of the metabolic properties of the cells. The results show that by maintaining the energy requirements of the erythrocyte, methaemoglobin production is minimised under conditions of iron-stress. However, in this situation, the membranes of the erythrocytes become more susceptible to the oxidative damage and increased lipid peroxidation ensues.

KEY WORDS: Iron, haemoglobin, peroxidation, membrane, desferrioxamine.

INTRODUCTION

Repeated blood transfusion in patients with chronic refractory anaemias is one important cause of body iron overload, or transfusional siderosis. This is most commonly seen in children with beta-thalassaemia major. The ways in which iron may cause tissue and cellular damage in such pathological conditions are complex. It is now considered that such toxicity may arise from the excess availability of plasma iron[1] and from the accumulation of breakdown products of oxidised haemoglobin in the form of haemichromes on the inner surface of the thalassaemic erythrocyte.[2]

The toxicity of iron arises from its ability to catalyse the formation of oxygen-derived free radical species which can interact with cellular membranes and cytoplasmic constituents.[3,4] Such interactions, *per se*, can affect the structural and functional integrity of the cells and their membranes, by, for example, initiating lipid peroxidation, the propagation of which can produce toxic compounds which can further damage cellular function.

Our previous study[5] has shown that exposing both normal and thalassaemic erythrocytes to extracellular iron-mediated oxidative stress *in vitro* increased methaemoglobin formation and decreased intracellular glutathione levels. These processes were substantially inhibited by the iron (III) chelator, desferrioxamine. Investigations were also undertaken on the effects of direct exposure of haemoglobin in red cell haemolysates to identical iron-stress systems. The results have shown[5] that under these conditions haemoglobin oxidation, as measured by methaemoglobin production, is less extensive than in the intact erythrocyte system and suggests a role for the

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erythrocyte membrane in enhancing iron-catalysed oxidative damage, possibly through the formation of lipid hydroperoxides and their reactive intermediates.

Recent work from this laboratory[6,7] has also shown that desferrioxamine alone has an oxidising effect on haemoglobin in red cell haemolysates in the absence of iron-stress. The rate of methaemoglobin production is very significantly faster in haemolysates in the presence of desferrioxamine alone than with the iron-stress system. Ascorbate suppresses the oxidising effect of desferrioxamine on haemoglobin under these conditions.[7]

The purpose of the present study is to investigate further the effects of extracellular iron stress on normal erythrocytes and their membranes and the significance of the maintenance of the energy requirements of the cell.

MATERIALS AND METHODS

Fresh human erythrocytes were obtained from normal, healthy donors and used immediately. After centrifugation, the plasma and buffy coat were removed and the erythrocytes washed three times with iso-osmotic phosphate buffer, pH 7.4. Erythrocytes at a 5% suspension in phosphate buffer were incubated at 37°C for various time intervals up to 24 hours. The experimental systems consisted of iron (II) sulphate (100 μM), ascorbic acid (1 mM), hydrogen peroxide (200 μM), desferrioxamine mesylate or its iron (III)-chelated complex, ferrioxamine (CIBA-Geigy) (400 μM). Some incubations additionally contained substrates for maintaining the metabolic state of the erythrocytes, according to Lutz *et al.*[8] and appropriate controls were incorporated into the various assays.

Methaemoglobin formation was measured by the decrease in absorbance at 620 nm after the addition of cyanide.[9] Total haemoglobin was estimated as cyanmethaemoglobin using Drabkin's reagent.[10]

The degree of haemolysis was estimated spectrophotometrically at 575 nm using the method of Brownlee *et al.*[11] The binding of haemoglobin to the membrane was assessed fluoriometrically after the conversion of haem to porphyrin.[12] Cellular reduced glutathione levels were estimated spectrophotometrically[13] at 412 nm with 5,5′-dithiobis-(2-nitrobenzoic acid). Assays for ATP were performed using the test-combination kits from Boehringer.

Membrane lipid peroxidation was assayed by the interaction of thiobarbituric acid with breakdown products of lipid hydroperoxides, under acid conditions, and the pink chromophore was assayed spectrophotometrically at 532 nm;[14] and by the spectrofluorimetric assay of fluorescent chromolipids formed by Schiff's base interactions.[15] Lipids were extracted from control and treated samples,[16] quantitated on the basis of phospholipid phosphorous[17] and the fluorescence characteristics of the cross-linked lipids were analysed on a Perkin-Elmer MPF 44B spectrofluorimeter using an excitation wavelength of 350 nm and emission spectra were recorded between 400–500 nm.

RESULTS

Normal erythrocytes are exposed to extracellular iron-catalysed oxidative stress in the form of incubation with iron (II) sulphate (100 μM)/ascorbate (1 mM)/hydrogen

peroxide (200 μM) in the presence and absence of the iron (III) chelator, desferrioxamine.

The effects of such extracellular iron stress on the oxidation state of the haemoglobin after incubation for 5 h, 10 h and 24 h are shown in Table I. In control erythrocytes incubated in buffer, haemoglobin oxidation becomes significant after 10 h, such that 16% methaemoglobin is formed after 24 h. After incorporation of the iron/ascorbate/hydrogen peroxide system, the degree of haemoglobin oxidation increases to 8% at 5 h, 20% at 10 h, to 93% at 24 h. The addition of desferrioxamine immediately prior to the initiation of iron stress very significantly reduces methaemoglobin production, almost totally at the shorter incubation time, and by about 50% after more extensive periods of incubation. Maintaining the cellular energy requirements during incubation in the systems described above significantly affects methaemoglobin production (Table I). Haemoglobin oxidation is not apparent up to 10 h incubation; at 24 h, methaemoglobin production is evident only in the iron-stress system but is totally inhibited in the iron-stress system containing desferrioxamine and in the control erythrocytes. Erythrocyte haemolysis was also monitored in these systems; the extent of lysis did not exceed 10% in any of the treated systems.

Extensive haemoglobin oxidation is usually accompanied by the formation of further breakdown products of methaemoglobin which may bind to the membrane. Membrane-bound haem compounds are measured after incubation of the erythrocytes in the system described above for 5 h and 24 h (Table I). The data show that the pattern of membrane-bound haemichrome formation parallels that of haemoglobin oxidation, as expected, and that after 24 h incubation, increased haemichrome binding is observed in the membranes prepared from erythrocytes under extracellular iron-stress.[18] In the desferrioxamine-containing iron-stress systems, the increase is significant but considerably less extensive. No significant haemichrome formation occurs after 5 h incubation, which again reflects the methaemoglobin formation.

TABLE I

The relationship between haemoglobin oxidation, cellular energy production and membrane-bound haemichrome formation

	% Methaemoglobin			Membrane-bound haem compounds (nmoles/mg protein)	
	5 h	10 h	24 h	5 h	24 h
Control erythrocytes	0	0	16 ± 1	0.07	0.14
Iron II (0.1 mM)/hydrogen peroxide (0.2 mM)/ascorbate (1 mM)	8 ± 5	20 ± 7	93 ± 7	0.07	0.42
Desferrioxamine (0.4 mM)/iron II/hydrogen peroxide/ascorbate	0	2 ± 0.2	44 ± 7	0.07	0.26
		$n = 6$		$n = 2$	
ATP levels maintained					
Control erythrocytes	0	0	1 ± 1		
Iron II/hydrogen peroxide/ascorbate	0	1 ± 1	16 ± 4		
Desferrioxamine/iron II/hydrogen peroxide/ascorbate	0	0	1 ± 1		
		$n = 4$			

Maintaining the ATP levels totally suppresses any measurable haemichrome formation above that in the control erythrocytes.

The effects of extracellular iron stress on the cellular reduced glutathione levels and the ATP levels as a function of time of incubation, are shown in Table II. The decline in ATP levels on incubation is unaffected by the presence of the iron (II)/ascorbate/hydrogen peroxide system whether desferrioxamine is present or not. However, the response of the reduced glutathione levels is a dramatic decrease on iron stress, compared with the incubated control, which is significantly but only partially alleviated when the iron chelator is additionally incorporated in the incubation medium.

Investigations of damage to the cellular membranes after exposure of the erythrocytes to iron-mediated oxygen radical production were approached by monitoring membrane lipid peroxidation in terms of the formation of thiobarbituric acid-reactive (TBAR) products (nmoles/10^{10} cells) and the formation of fluorescent chromolipids both in treated erythrocytes in which methaemoglobin formation progressed, and those in which it was suppressed by maintaining the ATP levels during the incubation. The results are shown in Table III. After the 5 h incubation of the erythrocytes with iron/ascorbate/hydrogen peroxide there is a small increase in lipid peroxidation which is not affected by the presence of the iron chelator, desferrioxamine. More extensive lipid peroxidation takes place after 24 h incubation but a similar trend is observed with incorporation of desferrioxamine. No fluorescent chromolipids are formed under these conditions. In the ATP-maintained incubation system, lipid peroxidation was significantly increased in all treated samples at both time intervals.

DISCUSSION

Exposing erythrocytes extracellularly to such iron-stress leads to intracellular damage in the form of haemoglobin oxidation and membrane-bound haemichrome production, both of which are partially suppressed, but not totally prevented, by the chelation of iron by desferrioxamine, and modifications to the membrane components in the form of enhanced lipid peroxidation which is not affected significantly by the presence of desferrioxamine in the incubation.

TABLE II
Reduced glutathione levels and ATP levels of erythrocytes under conditions of extracellular iron stress

	Reduced glutathione (mg/l cells)	ATP levels (% decrease)	
	5 h	5 h	24 h
Control erythrocytes	361 ± 48	39 ± 2	84 ± 1
Iron II (0.1 mM)/hydrogen peroxide (0.2 mM)/ascorbate (1 mM)	29 ± 10	40 ± 8	84 ± 3
Desferrioxamine (0.4 mM)/ iron II/hydrogen peroxide/ ascorbate	110 ± 11	42 ± 1	85 ± 1
	$n = 6$	$n = 3$	

TABLE III
Peroxidative damage in the membranes of erythrocytes exposed to iron-mediated oxidative stress and its dependence on the metabolic state of the cell

	Increase in TBAR-products over control (nmol/10^{10} cells)	
	5 h	24 h
Iron II (0.1 mM)/hydrogen peroxide (0.2 mM)/ascorbate (1 mM)	6.2 ± 1 $n = 4$	9.4 ± 3 $n = 9$
Desferrioxamine (0.4 mM)/ iron II/hydrogen peroxide/ ascorbate	4.0 ± 2 $n = 4$	7.0 ± 2 $n = 7$
ATP levels maintained Iron II/hydrogen peroxide/ascorbate	22.2 ± 3	41.3 ± 8
Desferrioxamine/iron II/ hydrogen peroxide/ascorbate	24.2 ± 5	21.7 ± 3
	$n = 3$	

By maintaining the energy requirements of the erythrocyte, methaemoglobin production is minimised under conditions of iron stress, but the membranes become more vulnerable to oxidative damage and increased lipid peroxidation ensues. This may imply that methaemoglobin has a role in decreasing the susceptibility of the membranes of erythrocytes to iron-mediated oxidative stress possibly by its potential ability to scavenge propagating oxygen species in the membrane. Interestingly, in contrast to our data, other workers in their studies on purified oxy haemoglobin in haemosomes prepared with phospholipids with unsaturated fatty acyl chains have suggested that an inter-relationship exists between haemoglobin oxidation and lipid peroxidation, with oxidation of either compound stimulating oxidation of the other.[19]

Some workers have implicated a correlation between increased susceptibility to oxidative stress and erythrocyte haemolysis,[20–22] whilst other work has suggested a sequence of events involving increased membrane-bound denatured haemoglobin species, membrane damage, loss of cellular deformability and decreased erythrocyte survival.[23]

Our studies show that erythrocytes under iron-mediated oxidative stress appear to be in a state of balance between the oxidation state of the haemoglobin and the extent of membrane damage, which is enhanced by the metabolic state of the cell. This is consistent with earlier studies on model systems for oxidative damage in erythrocytes involving non iron-mediated stress both from our laboratory[24] and that of Stern *et al.*[25]

The results reported here have important implications for thalassaemic erythrocytes which may be exposed to excess plasma iron levels, in which excessive membrane-bound iron in the form of haemichromes is a characteristic feature[2,26] and in which cellular ATP levels are diminished.[26]

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