Biochem. J. (1987) 244, 191-196 (Printed in Great Britain)

Iron-mediated oxidative stress in erythrocytes

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Erythrocytes subjected extracellularly to iron-mediated oxidant stress undergo haemoglobin oxidation and membrane damage, which can be modulated by maintaining the energy requirements of the cells. The results presented here suggest that a balance exists between the oxidation state of the haemoglobin and the oxidative deterioration of the membrane lipids, which is dependent on the metabolic state of the erythrocytes. These findings have important implications for thalassaemic erythrocytes that may be exposed to excess plasma iron levels, in which excessive membrane-bound iron in the form of haemichromes is a characteristic feature and in which cellular ATP levels are lowered.

INTRODUCTION

The elucidation of the mechanism of iron-mediated oxidative effects on erythrocytes and their membranes is essential for the understanding of the role of such processes in pathological conditions of iron overload. In thalassaemia, the sources of excess storage iron are transfused red cells and increased gastrointestinal iron absorption [1]. In severe iron overload, the plasma transferrin becomes saturated [2] and the unbound iron may be an important factor in free radical damage to cells [3] and tissues [4]. Furthermore, iron in the form of breakdown products of oxidized haemoglobin accumulates in the interior of thalassaemic erythrocytes as membrane-bound haemichromes [5].

The toxicity of iron arises from its ability to catalyse the formation of oxygen-derived free radical species that can interact with cellular membranes and cytoplasmic constituents. Such interactions, *per se*, can not only potentially modify the functional integrity of the cells and their membranes but also generate toxic products that further modify various cellular functions.

Our previous study [6] 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 [6] were also undertaken on the effects of direct exposure of haemoglobin in red cell haemolysates to identical iron-stress systems. The results have shown that, under these conditions, haemoglobin oxidation, as measured by methaemoglobin production, is less extensive than in the intact erythrocyte system and suggest a role for the erythrocyte membrane in enhancing iron-catalysed oxidative damage, possibly through the formation of lipid hydroperoxides and their reactive intermediates.

Recent work from this laboratory [7,8] has also shown that desferrioxamine alone has an oxidizing 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. The oxidizing effect of desferrioxamine on haemoglobin under these conditions is suppressed when ascorbate is also present in these incubations [8].

The purpose of the present investigation is to determine the effects on the erythrocyte membrane of iron-induced oxidative stress, the importance of the oxidation state of haemoglobin and 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 h. The experimental systems consisted of iron(II) sulphate (100 μM), ascorbic acid (1 mM), H_2O_2 (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, incorporating adenine (0.54 mM), inosine (12.7 mM) and glucose (2 g/l), according to Lutz *et al.* [9] and appropriate controls were incorporated into the various incubations and assays. Samples were fixed at a 5% concentration in glutaraldehyde in cacodylate buffer, dehydrated and critical-point dried. Samples were prepared for scanning electron microscopy according to Rice-Evans *et al.* [10] and micrographs were taken on a Philips 501 scanning electron microscope.

Methaemoglobin formation was measured by the decrease in absorbance at 620 nm after the addition of cyanide [11]. Total haemoglobin was estimated as cyanmethaemoglobin by using Drabkin's reagent [12]. The degree of haemolysis was estimated spectrophotometrically at 575 nm by the method of Brownlee *et al.* [13]. The binding of haemoglobin to the membrane was assessed fluorimetrically after the conversion of haem to porphyrin [14]. Cellular reduced glutathione levels were estimated spectrophotometrically [15] at 412 nm with 5,5'-dithiobis-(2-nitrobenzoic acid) with GSH (Sigma) as standard. Assays for ATP were performed by using the test-combination kits from Boehringer.

Membrane lipid peroxidation was assayed by the following methods: (1) a modification of the method of Kumar *et al.* [16] involving the interaction of thiobarbituric acid with breakdown products of lipid

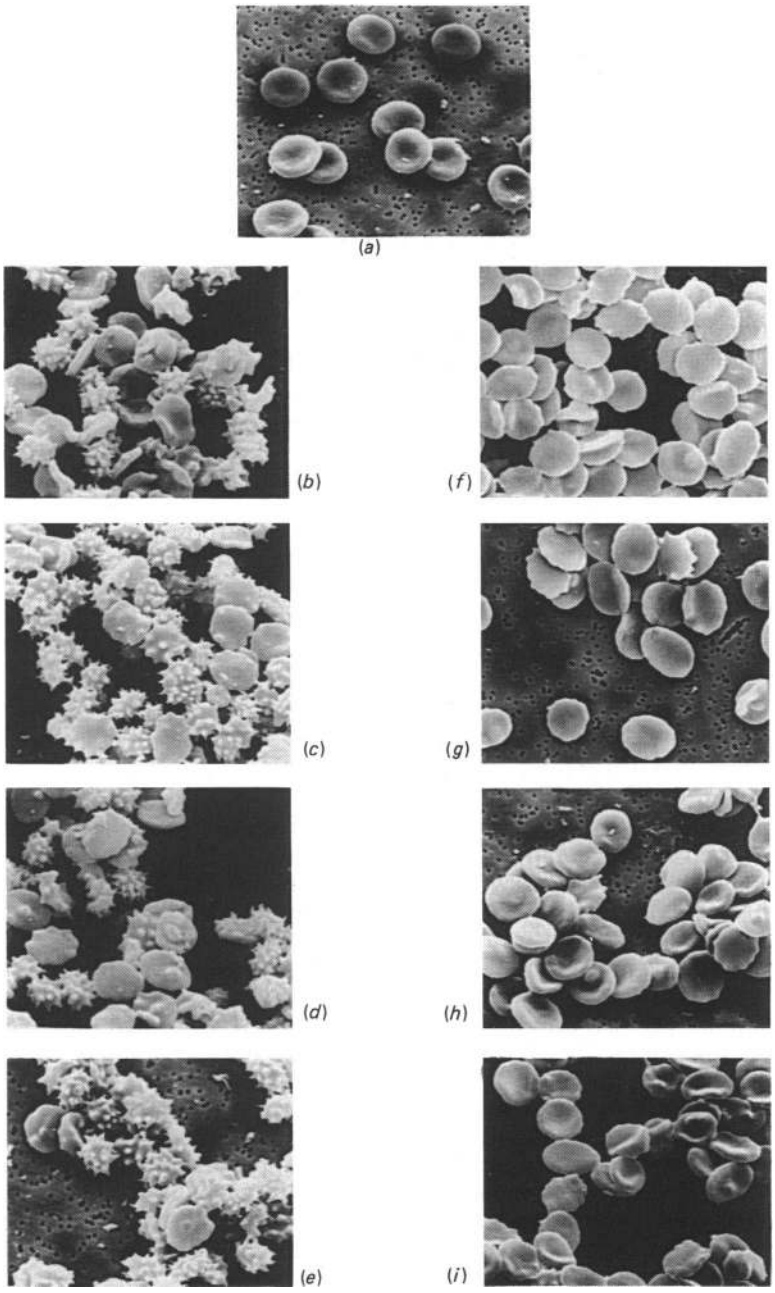

Fig. 1. Morphological effects of maintaining the ATP levels of normal human erythrocytes incubated with iron(II) (100 μM)/ascorbate (1 mM)/H_2O_2 (200 μM), in the presence and absence of desferrioxamine or ferrioxamine (400 μM) for 15 h

(*a*) Control erythrocytes, no incubation; (*b*,*f*) control erythrocytes incubated without and with substrates for maintaining the ATP levels, respectively; (*c*,*g*) erythrocytes + iron(II)/ascorbate/H_2O_2, incubated without and with substrates for maintaining the ATP levels, respectively; (*d*,*h*) as (*c*,*g*), but + desferrioxamine; (*e*,*i*), as (*c*,*g*), but + ferrioxamine.

hydroperoxides, under acid conditions, and the pink chromophore was assayed spectrophotometrically at 532 nm; (ii) the spectrofluorimetric assay [17] of chromolipids formed by Schiff's base interactions of secondary breakdown products of lipid peroxidation with appropriate groups on the phospholipid sidechains: lipids were extracted from control and treated samples [18] 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 and 500 nm. Lipids were quantified on the basis of phospholipid phosphorus [19].

Membranes were prepared by hypo-osmotic haemolysis at pH 7.4 by using standard procedures [20] and suspended in iso-osmotic phosphate buffer at a final membrane protein concentration of 1.5 mg/ml [21]. Haemoglobin-free membranes were exposed to the same experimental treatments as described above for the intact erythrocytes.

Membrane protein changes were analysed by electrophoresis on polyacrylamide gels, performed following

standard procedures [22] using 5% gels. Proteins were visualized after staining with Kenacid Blue (BDH).

RESULTS

Normal erythrocytes (Fig. 1*a*) were exposed to extracellular iron-catalysed oxidative stress in the form of incubation with iron(II) sulphate (100 μM)/ascorbate (1 mM)/H_2O_2 (200 μM) in the presence and absence of the iron(III) chelator, desferrioxamine, or the chelated iron(III) complex, ferrioxamine. Gross morphological changes to the echinocyte conformation were observed in all incubated samples; typical results after 10 h incubation are shown in Figs. 1(*b*)–1(*e*). Under these conditions ATP levels declined to 25%. Maintaining cellular energy requirements during the treatments significantly allays the gross structural modification of the erythrocytes under condition of iron stress as well as when desferrioxamine is additionally present in the incubation (Figs. 1*f*–1*i*).

The effects of such extracellular iron stress on the oxidation state of the haemoglobin after incubation for 5, 10 and 24 h are shown in Fig. 2. 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/H_2O_2 system, the degree of haemoglobin oxidation increases to 7% at 5 h, 22% at 10 h and 93% at 24 h. The addition of desferrioxamine immediately prior to the initiation of iron stress very significantly decreases methaemoglobin production, almost totally at the shorter incubation time, and by about 50% after more extensive periods of incubation. Alternatively, the presence of ferrioxamine, the iron chelator with its iron-binding site occupied by iron(III), does not alter the response of the system from that in its absence. Fig. 2 also illustrates the effects of maintaining the cellular energy requirements during incubation in the systems described above. Haemoglobin oxidation is not apparent up to 10 h incubation; at 24 h, methaemoglobin production is evident only in the iron stress system and that incorporating ferrioxamine to the extent of approx. 15%, but is totally inhibited in the iron stress system containing desferrioxamine and in the control erythrocytes.

Extensive haemoglobin oxidation is usually accompanied by the formation of further breakdown products of methaemoglobin that 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 in the absence and presence of substituents for maintaining the energy requirements of the cell (Table 1). 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, in the presence and absence of the iron-complexed chelator [23]. In the desferrioxamine-containing iron stress systems, the increase is significant but considerably less extensive. No significant haemichrome formation occurs up to 5 h incubation, which again reflects the methaemoglobin formation. Maintaining the ATP levels totally suppresses any measurable haemichrome formation above that in the control erythrocytes. Erythrocyte haemolysis was monitored as haemoglobin oxidation and haemichrome formation progressed under the influence of iron stress and prolonged incubation times. The extent of lysis of the red cells did not exceed 10% in any of the treatments at the various incubation times. The effects on ATP and GSH levels for each treatment and time point are indicated in Table 2. The decline in ATP levels on

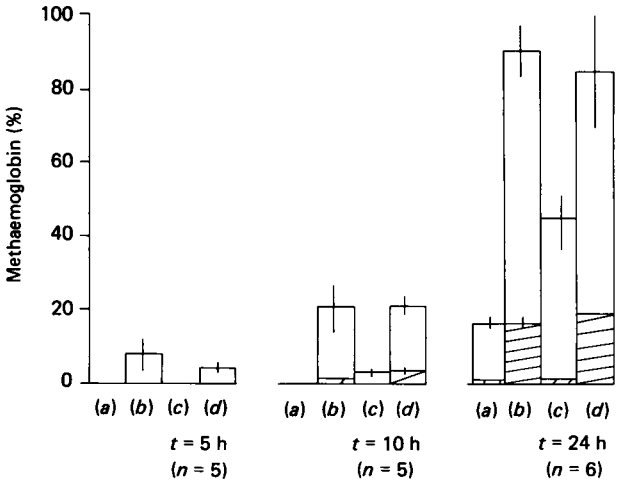

Fig. 2. Effects of the metabolic state of the erythrocyte on haemoglobin oxidation under conditions of iron stress

(*a*) Control erythrocytes; (*b*) iron(II) (100 μM)/ascorbate (1 mM)/H_2O_2 (200 μM); (*c*) as (*b*) but +desferrioxamine (400 μM); (*d*) as (*b*) but +ferrioxamine (400 μM). Hatched bars indicate the presence of substrates for maintaining ATP levels.

Table 1. Effect of iron stress on membrane-bound haem compounds

Erythrocytes were incubated for the indicated length of time with iron(II) (100 μM), ascorbate (1 mM) and H_2O_2 (200 μM) in the presence or absence of desferrioxamine or ferrioxamine (400 μM each) or ATP-maintaining additions (see the text) at 37 °C. Zero-time values of membrane-bound haem were 0 under all conditions. Values are means for two experiments.

Erythrocyte treatment	Membrane-bound haem (nmol/mg of protein) at: 5 h	24 h
ATP not maintained		
Control	0.07	0.14
Iron(II)/ascorbate/H_2O_2	0.07	0.42
Iron(II)/ascorbate/H_2O_2 +desferrioxamine	0.07	0.26
Iron(II)/ascorbate/H_2O_2 +ferrioxamine	0.08	0.42
ATP maintained		
Control	0	0
Iron(II)/ascorbate/H_2O_2	0	0.12
Iron(II)/ascorbate/H_2O_2 +desferrioxamine	0	0.10
Iron(II)/ascorbate/H_2O_2 +ferrioxamine	0	0.12

Table 2. GSH and ATP levels of erythrocytes under conditions of extracellular iron stress

For concentrations of additions see legend to Table 1. Control values at time zero were: GSH, 690±55 mg/l; ATP, 32 mg/100 ml.

Erythrocyte treatment	Decrease (%) of: GSH ($n = 6$) (5 h)	ATP ($n = 3$) 5 h	ATP ($n = 3$) 24 h
Control	48±6	39±2	84±1
Iron(II)/ascorbate/H_2O_2	96±2	40±8	84±3
Iron(II)/ascorbate/H_2O_2 + desferrioxamine	84±2	42±1	85±1
Iron(II)/ascorbate/H_2O_2 + ferrioxamine	96±1	48±3	81±1

Table 3. Peroxidative damage in the membranes of erythrocytes exposed to iron-mediated oxidative stress and its dependence on the metabolic state of the cell

For details of treatments see the text and legend to Table 1. Abbreviation: n.d., not determined.

Erythrocyte treatment	Increase in thiobarbituric acid-reactive products: 5 h	24 h
ATP not maintained [$n = 4$ (5 h) or 7(24 h)]	(nmol/10^{10} cells)	
Iron(II)/ascorbate/H_2O_2	6.2±1.0	9.4±3.0
Iron(II)/ascorbate/H_2O_2 + desferrioxamine	4.0±2.0	7.0±2.0
Iron(II)/ascorbate/H_2O_2 + ferrioxamine	n.d.	n.d.
ATP maintained ($n = 3$)		
Iron(II)/ascorbate/H_2O_2	22.3±3.0	41.3±8.0
Iron(II)/ascorbate/H_2O_2 + desferrioxamine	24.2±5.0	21.7±3.0
Iron(II)/ascorbate/H_2O_2 + ferrioxamine	36.5±5.0	56.1±7.0
Haemoglobin-free membranes ($n = 6$)	(nmol/mg of protein)	
Iron(II)/ascorbate/H_2O_2	11.1±2.0	n.d.
Iron(II)/ascorbate/H_2O_2 + desferrioxamine	1.0±0.5	n.d.
Iron(II)/ascorbate/H_2O_2 ferrioxamine	15.0±2.0	n.d.

incubation is unaffected by the presence of the iron(II)/ascorbate/H_2O_2 system, whether desferrioxamine is present or not. However, the response of the GSH 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

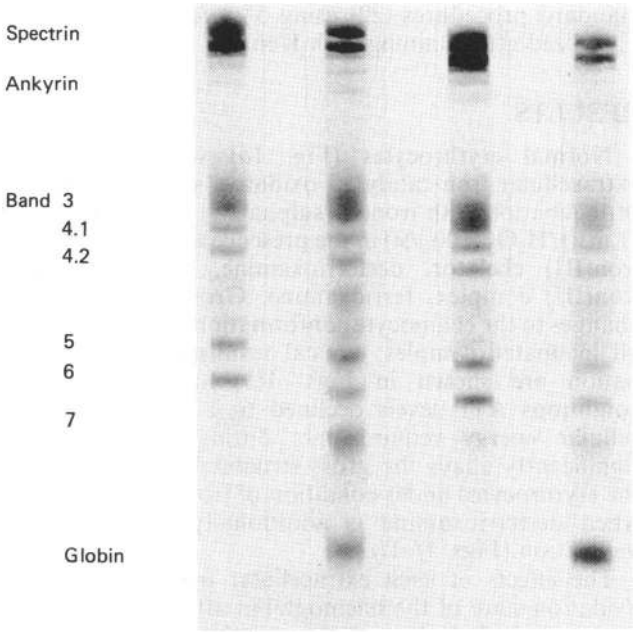

Fig. 3. Polyacrylamide-gel electrophoresis of the membrane proteins of erythrocytes under extracellular iron stress (15 h incubation)

(*a*) Control erythrocytes; (*b*) iron(II) (100 μM)/ascorbate (1 mM)/H_2O_2 (200 μM)-treated erythrocytes; (*c*) desferrioxamine (400 μM)-treated erythrocytes; (*d*) as (*b*) but +desferrioxamine.

formation of thiobarbituric acid-reactive ('TBAR') products (nmol/10^{10} cells) and the formation of fluorescent chromolipids both in treated erythrocytes in which methaemoglobin formation progressed, and in those in which it was suppressed by maintaining the ATP levels during the incubation. Additionally, to clarify further the role of haemoglobin in the susceptibility to iron-induced oxidative damage, haemoglobin-free membranes were treated by incubation for 5 h in identical systems, TBAR products in this case being quantified as nmol/mg of membrane protein. The results are shown in Table 3. After 5 h incubation of the erythrocytes with iron/ascorbate/H_2O_2 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. Lipid peroxidation was considerably more extensive on treatment of haemoglobin-free membranes with the iron/ascorbate/H_2O_2 system (5 h), the increase being totally suppressed by the presence of desferrioxamine. No chromolipid formation was detected in the membrane systems.

The response of the membrane proteins to extracellular oxidative stress mediated by iron was investigated using electrophoresis on polyacrylamide gels (Fig. 3), all samples being run in the presence of dithiothreitol, a reducing agent for disulphide links that may result from

oxidation of protein thiol sidechains during the incubation. The most interesting feature of these gels is the emergence of a broad band between bands 4.2 and 5, the pronounced appearance of band 7 and of membrane-bound haemoglobin in all the incubations containing iron/ascorbate/H_2O_2, although the relative proportions of band 7:haemoglobin are higher in the iron stress system without desferrioxamine than in its presence.

DISCUSSION

In this study, erythrocytes were subjected to extracellular iron-induced oxidative stress in the form of ferrous ions, H_2O_2, an oxidant being essential when iron(II) is involved [24] and the reducing agent ascorbate. The response of the erythrocytes and their membranes to such iron stress, the importance of the oxidation state of the haemoglobin and the metabolic properties of the cells, and the effects of the iron(III) chelator, desferrioxamine, have been investigated.

Exposing erythrocytes extracellularly to such iron stress leads to: (i) 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 (ii) modifications to the membrane components in the form of enhanced lipid peroxidation and the appearance of protein band 7, which are not affected significantly by the presence of desferrioxamine in the incubation.

By maintaining the energy requirements of the erythrocyte, methaemoglobin production is minimized 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.

This is supported by the greater extent of lipid peroxidation in response to iron stress in the haemoglobin-free membrane systems. These observations suggest 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 [10] and from that of Stern *et al.* [25]. Interestingly, in contrast with our data, other workers in their studies on purified oxyhaemoglobin 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 [26].

Some workers have implicated a correlation between increased susceptibility to oxidative stress and erythrocyte haemolysis [27–29], whereas 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 [30]. Our studies here show that morphological damage to the erythrocytes and the onset of echinocytosis are clearly more dependent on the energy status of the cell, and the secondary consequences of ATP loss and GSH oxidation, than on the oxidation state of the haemoglobin or the extent of binding of denatured haemoglobin species to the membrane which result from the iron-induced oxidant stress. Furthermore, our results show no direct correlation between oxidative damage and cellular haemolysis.

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 [5,31] and in which cellular ATP levels are diminished [31].

We gratefully acknowledge financial assistance from the Government of the Turkish Republic of Northern Cyprus and the Peter Samuel Royal Free Fund.

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Received 8 August 1986/9 December 1986; accepted 27 January 1987