Biochem. J. (1992) 285, 295–301 (Printed in Great Britain)

Iron release and membrane damage in erythrocytes exposed to oxidizing agents, phenylhydrazine, divicine and isouramil

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Mouse erythrocytes were incubated with oxidizing agents, phenylhydrazine, divicine and isouramil. With all the oxidants a rapid release of iron in a desferrioxamine (DFO)-chelatable form was seen and it was accompanied by methaemoglobin formation. If the erythrocytes were depleted of GSH by a short preincubation with diethyl maleate, the release of iron was accompanied by lipid peroxidation and, subsequently, haemolysis. GSH depletion by itself did not induce iron release, methaemoglobin formation, lipid peroxidation or haemolysis. Rather, the fate of the cell in which iron is released depended on the intracellular availability of GSH. In addition, iron release was higher in depleted cells than in native ones, suggesting a role for GSH in preventing iron release when oxidative stress is imposed by the oxidants. Iron release preceded lipid peroxidation. The latter was prevented when the erythrocytes were preloaded with DFO in such a way (preincubation with 10 mM-DFO) that the intracellular concentration was equivalent to that of the released iron, but not when the intracellular DFO was lower (preincubation with 0.1 mM-DFO). Extracellular DFO did not affect lipid peroxidation and haemolysis, suggesting again that the observed events occur intracellularly (intracellular chelation of released iron). The relevance of iron release from iron complexes in the mechanisms of cellular damage induced by oxidative stress is discussed.

INTRODUCTION

Iron plays a central role in generating harmful oxygen species by promoting the conversion of superoxide anion and hydrogen peroxide into the very reactive hydroxyl radical through the Haber–Weiss reaction [1–4]. Normally iron is transported and stored in specific proteins (ferritin, transferrin, lactoferrin and haem proteins), which prevents or minimizes its reaction with reduced oxygen derivatives [5,6]. However, in spite of these safegards, increasing evidence suggests that reactive iron becomes available during some disease states. It has been suggested in particular that iron is released from iron stores under conditions in which oxidative stress is involved in both systems *in vitro* [7,8] and isolated perfused organs [9,10]. A fraction of cellular non-haem iron consisting of low-M_r chelatable iron derivatives [11–13] appears to represent the iron species catalytically active in initiating free-radical reaction and lipid peroxidation [14–16]. This fraction is generally considered as decompartmentalized iron, in contrast with iron bound to proteins.

Previous studies [17–19] from our laboratory have shown that allyl alcohol administration to mice produces, along with liver necrosis, a high incidence of haemolysis. It was also observed [17,18] that the incubation of mouse erythrocytes with acrolein, the proximate metabolite of allyl alcohol, produces extremely rapid GSH depletion, which is followed by the appearance of lipid peroxidation and, subsequently, by haemolysis. When the amount of desferrioxamine (DFO)-chelatable iron was measured in the system, a progressive increase in such iron was seen during the incubation [17,18]. On the basis of additional results [19], it seemed likely that iron is released from haemoglobin and that such iron promotes lipid peroxidation in a cell depleted of GSH. A similar pattern of events was also seen [19] in erythrocytes drawn from allyl alcohol-intoxicated mice before the development of haemolysis, and incubated in buffer.

It is well known that phenylhydrazine causes formation of methaemoglobin and Heinz bodies as well as haemolysis both *in vivo* and *in vitro*. Oxidative stress is involved in phenylhydrazine-induced erythrocyte damage [20]. Phenylhydrazine can penetrate to the O_2-binding site of the haemoglobin molecule and react with it. Phenylhydrazine oxidation results in the formation of superoxide and hydrogen peroxide [21–24]. Several reactive intermediates of phenylhydrazine [25–27] are formed as well as complexes of these reactive products with haemoglobin. In the erythrocyte membrane, lipid peroxidation can occur [28] as well as formation of a new antigen which is recognized by autologous IgG [29].

Oxidative reactions have been implicated in erythrocyte damage induced by fava bean consumption in glucose 6-phosphate dehydrogenase-deficient individuals (see ref. [30] for a review). Divicine and isouramil are the pyrimidine aglycones originating respectively from vicine and convicine, the potentially toxic glucosides contained in fava beans (*Vicia faba*). Redox cycling of the hydroquinone forms of divicine and isouramil to the semiquinone radicals produces $O_2^{\cdot-}$ [30]. The latter can oxidize GSH directly or via superoxide dismutase-catalysed formation of H_2O_2 and subsequent activity of GSH peroxidase.

In the present report we show that release of free (DFO-chelatable) iron occurs from iron complexes in phenylhydrazine, divicine or isouramil-treated erythrocytes. Such reactive iron is likely to induce severe cell damage when the antioxidant potential of the erythrocyte is severely compromised. Lipid peroxidation and haemolysis, in fact, accompanied iron release in erythrocytes previously depleted of GSH.

MATERIALS AND METHODS

Desferrioxamine (Desferal, DFO) was kindly supplied by Ciba-Geigy (Basel, Switzerland). Phenylhydrazine hydrochloride was from Carlo Erba (Milan, Italy). Vicine (2,6-diamino-4,5-dihydroxypyrimidinyl β-D-glucopyranoside) and β-glucosidase were obtained from Serva Feinbiochemica G.m.b.H. (Heidelberg, Germany). Divicine (2,6-diamino-4,5-dihydroxypyrimidine) was prepared from vicine by enzymic activity of β-glucosidase, according to the method of Pedersen *et al.* [31] and used immediately. Convicine (2-amino-4,6-dihydroxy-5-pyrimidinyl

Abbreviations used: DFO, desferrioxamine; DEM, diethyl maleate; MDA, malonaldehyde.

Table 1. Release of iron (DFO-chelatable iron), methaemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and haemolysis in erythrocytes incubated with phenylhydrazine (Phz) or preincubated with DEM and then incubated with phenylhydrazine

Phz was added to erythrocytes at 1 mM concentration. When DEM-pretreated cells were used, the erythrocytes were preincubated with 1 mM-DEM for 10 min, recovered by centrifugation and then incubated with (+DEM +Phz) or without (+DEM) Phz, for the indicated times. Results are means ± S.E.M. of three to six experiments. Met-Hb is expressed per haem. All the results, except those for haemolysis, are given as nmol/ml of incubation mixture. It seemed not reasonable to refer the data to ml of packed cells when, in many experiments, haemolysis occurred.

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Haemolysis (%)
Control	0	1.7±0.6	142±19	899±16	–	2.1±0.6
	60	2.6±0.2	108±11	862±33	1.1±0.4	6.2±2.1
+Phz	0	1.8±0.2	136±11	971±48	–	2.5±0.4
	30	17.0±3.2	432±21	774±85	1.9±0.4	6.3±0.9
	60	17.5±2.2	543±21	726±86	2.5±0.5	6.8±0.5
+DEM	0	1.5±0.4	110±11	318±32	–	1.8±0.5
	30	1.6±0.3	140±15	204±54	2.8±0.3	2.3±0.2
	60	2.0±0.5	103±10	192±43	3.9±0.7	11.6±1.5
+DEM+Phz	0	1.3±0.5	122±11	433±74	–	1.9±0.2
	30	21.3±4.1	620±30	202±13	16.9±3.6	26.7±7.6
	60	20.9±3.1	970±90	137±25	71.6±5.8	80.9±7.1

β-D-glucopyranoside) was extracted from fava beans (*Vicia faba*) as described by Blen *et al.* [32]. Briefly ground fresh beans were soaked in 95% (v/v) ethanol at room temperature for 3 days. After filtration, the solution was extracted with ether and the aqueous layer was concentrated under vacuum at 40 °C. Crystallization of convicine started when the solution was kept in the refrigerator, and the crystals, recovered by filtration, were washed with acetic acid and with chloroform. After removal of the solvent, the convicine was stored until use at 0–4 °C. Isouramil (6-amino-2,4,5-trihydroxypyrimidine) was prepared from convicine by enzymic hydrolysis as for divicine.

The solvents used for h.p.l.c. were of h.p.l.c. grade. All other chemicals were of analytical grade.

Male Swiss albino mice (Nossan, Correzzana, Milan, Italy) weighing 25–35 g and maintained on a pellet diet (Nossan) were used. The animals were starved for 16 h before being used.

Blood was withdrawn from the abdominal aorta under ether anaesthesia and heparinized. After centrifugation, the plasma and buffy coat were removed and the erythrocytes washed three times with 0.123 M-NaCl/28 mM-sodium phosphate/potassium phosphate buffer (pH 7.4). Iron contamination was removed from the buffer as previously described [17]. Washed erythrocytes were incubated in the same buffer as a 50% (v/v) suspension. Haemoglobin concentration, measured as in ref. [33] and expressed per haem, was 7000±300 nmol/ml. Phenylhydrazine, divicine and isouramil were added at a concentration of 1 mM.

In the experiments in which erythrocytes were depleted of GSH, the cells were preincubated with 1 mM-diethyl maleate (DEM) for 10 min at 37 °C. DEM was dissolved in a small volume of dimethyl sulphoxide; dimethyl sulphoxide alone had no effect on the parameters examined. At the end of the preincubation, the erythrocytes were recovered by centrifugation and used for the subsequent incubation.

Samples were withdrawn at the indicated times for the determination of free iron (DFO-chelatable), methaemoglobin [34], GSH [35], malonaldehyde (MDA) [36] and haemolysis [17]. With regard to the latter, it should be noted that when there is haemoglobin precipitation as with phenylhydrazine, comparing released haemoglobin with total releasable haemoglobin may not give an accurate estimation of haemolysis if a significant proportion of haemoglobin is denatured. However, it can be assumed that the loss of haemoglobin from the supernatant obtained after centrifuging a sample of the incubation mixture is proportional to the loss of haemoglobin in the sample of the same incubation mixture haemolysed by the addition of a hypo-osmotic solution. Therefore the calculation of percentage haemolysis should not be significantly affected. Furthermore, it should be considered that in the phenylhydrazine experiments with significant haemolysis, the maximal amount of methaemoglobin found did not exceed 14% of total haemoglobin (Table 1).

Free iron was determined as a DFO–iron complex (ferrioxamine), as previously reported [17]. Briefly, the erythrocytes were haemolysed by addition of water (1 vol.) and by freezing (−70 °C)–thawing; the haemolysate was centrifuged and the supernatant was ultrafiltered. Determination of the DFO–iron complex (ferrioxamine) was by h.p.l.c. according to the method of Kruck *et al.* [37] with a number of modifications reported in detail in [17].

In the experiments in which DFO was used to chelate intracellularly the released iron, the erythrocytes were preincubated with 0.1 or 10 mM-DFO for 90 min at 37 °C. At the end of the preincubation, the erythrocytes were washed three times with 30 vol. of buffer. No DFO was present in the supernatant of the last washing, as assessed by h.p.l.c. DFO was determined in the erythrocytes. To this end, 0.5 ml of packed cells was lysed by addition of 1.5 ml of water. The haemolysate was centrifuged (12000 *g*, 20 min) and the supernatant was ultrafiltered (Centriflo CF25, Amicon). $FeSO_4$ (100 μM) was added to the ultrafiltrate, and DFO present in the ultrafiltrate was measured by h.p.l.c. as ferrioxamine, as above. DFO-preloaded erythrocytes were suspended in buffer as above, preincubated with DEM for 10 min and subsequently incubated with phenylhydrazine, divicine or isouramil.

RESULTS

As shown in Table 1, the addition of phenylhydrazine to mouse erythrocytes induced a marked release of iron from iron stores, as documented by the increase in DFO–iron complex measured by h.p.l.c. Concomitant with the release of iron, a progressive production of methaemoglobin was seen. Also, Heinz body formation was observed (not shown). In native erythrocytes phenylhydrazine induced a decrease in the erythrocyte GSH concentration (Table 1, +Phz); such a decrease was 20 and 25% at 30 and 60 min of incubation respectively. Virtually no lipid peroxidation or haemolysis was seen in these erythrocytes.

Since in previous experiments [17–19] carried out with acrolein the release of iron was accompanied by the development of lipid

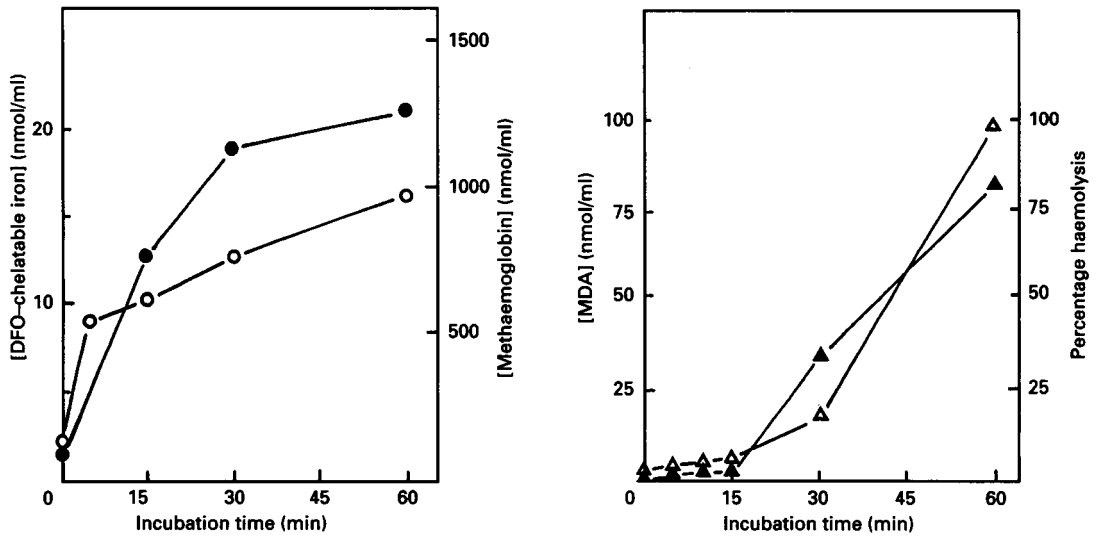

Fig. 1. Time course of iron release (●), methaemoglobin formation (○), lipid peroxidation (MDA formation; ▲) and haemolysis (△) in erythrocytes preincubated with 1 mM-DEM for 10 min and then incubated with 1 mM-phenylhydrazine for the indicated times

Methaemoglobin is expressed per haem. A typical experiment out of three is reported.

Table 2. Release of iron (DFO-chelatable iron), methaemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and haemolysis in erythrocytes preloaded with DFO, preincubated with DEM and then incubated with phenylhydrazine (Phz)

Erythrocytes were incubated with 0.1 or 10 mM-DFO for 90 min, washed and recovered. The intracellular concentration of DFO, after complete removal of extracellular DFO, was 5.0±0.5 and 37.8±4.0 nmol/ml of packed cells, with 0.1 and 10 mM-DFO respectively. DFO-preloaded erythrocytes were preincubated with 1 mM-DEM for 10 min and then incubated with 1 mM-Phz. Results are means±S.E.M. of three to six experiments. Met-Hb is expressed per haem. N.D., not determined.

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Haemolysis (%)
Control	0	2.0±0.2	128±26	977±62	-	2.5±0.2
	60	2.2±0.5	119±17	912±67	0.9±0.3	3.2±0.1
+5 μM-DFO (intracellular concn.)	0	2.2±0.2	132±11	312±15	-	1.8±0.4
+DEM+Phz	30	12.5±0.3	744±72	202±9	3.5±0.6	5.7±0.3
	60	13.1±0.7	1032±46	185±39	15.4±2.7	10.8±0.1
	90	13.6±0.6	N.D.	N.D.	66.9±7.9	43.3±0.5
+38 μM-DFO (intracellular concn.)	0	2.2±0.2	116±20	317±30	-	1.7±0.2
+DEM+Phz	30	20.0±1.1	948±41	203±10	0.2±0.1	4.9±0.1
	60	22.3±1.6	1364±120	143±19	0.1±0.03	6.5±0.3
	90	22.8±2.5	N.D.	N.D.	0.4±0.1	11.6±2.6

peroxidation and haemolysis, and since acrolein caused an immediate and virtually complete loss of erythrocyte GSH, we investigated whether, under conditions of more severe GSH depletion, the addition of phenylhydrazine to erythrocytes resulted in lipid peroxidation and haemolysis. Therefore the erythrocytes were preincubated with DEM for 10 min and then incubated with phenylhydrazine (Table 1, +DEM+Phz). Preincubation with DEM resulted in a severe loss of GSH which further decreased during the incubation with phenylhydrazine. When such DEM-pretreated erythrocytes were incubated with phenylhydrazine, the release of iron was similar to, or even greater than, that seen in non-pretreated cells (Table 1, +Phz). However, the formation of methaemoglobin was higher, lipid peroxidation developed and a nearly complete haemolysis occurred at 60 min. Preincubation with DEM and subsequent incubation without phenylhydrazine did not induce iron release, methaemoglobin formation, lipid peroxidation or haemolysis (Table 1, +DEM). It seems therefore that the release of iron does not depend on the depletion of erythrocyte GSH by itself. Rather, the phenylhydrazine-induced iron release is accompanied by different phenomena depending on the intracellular availability of GSH.

The time-course of iron release, methaemoglobin formation, lipid peroxidation and haemolysis (Fig. 1) shows that in DEM-pretreated erythrocytes the release of iron precedes the appearance of lipid peroxidation. The latter in turn precedes haemolysis (as also observed with acrolein [17]). It is therefore possible that the release of iron initiates a chain of reactions leading ultimately to peroxidation of membrane lipids and to subsequent haemolysis.

Further support for the hypothesis that the release of iron plays a causative role in the subsequent lipid peroxidation and lysis was offered by experiments in which DFO was used to chelate intracellularly the iron released by the action of phenylhydrazine. To this end erythrocytes were preincubated with 0.1 or 10 mM-DFO for 90 min. The cells were then recovered, washed and preincubated with DEM for 10 min. Finally the cells were incubated with phenylhydrazine (Table 2).

Preincubation with 0.1 mM-DFO resulted in an intracellular concentration of DFO of 5.0±0.5 nmol/ml of packed cells

Table 3. Lipid peroxidation (MDA formation) and haemolysis in erythrocytes preincubated with 0.1 mM-DFO and incubated with DEM and phenylhydrazine (Phz)

Erythrocytes were preincubated with 0.1 mM-DFO for 15 min. The intracellular concentration of DFO measured at the end of the preincubation was 1.2 nmol/ml of packed cells. DFO was not removed from the medium and DEM and Phz were added at 1 mM concentrations. Results are means of two experiments.

	Incubation time (min)	MDA (nmol/ml)	Haemolysis (%)
+100 μM-DFO	0	–	3.0±0.1
(extracellular concn.)	10	1.6±0.6	5.4±0.4
+DEM+Phz	30	8.8±0.5	19.0±0.9
	60	58.3±3.9	38.6±2.1

(= 2.5±0.2 nmol/ml of incubation mixture). It has been shown [38,39], in fact, that DFO scarcely penetrates erythrocytes. When these erythrocytes were depleted of GSH by DEM and then incubated with phenylhydrazine, lipid peroxidation and haemolysis developed (Table 2). This could be due to the fact that the amount of iron released (10–15 nmol/ml of incubation mixture) exceeded the amount of intracellular DFO. On the other hand, preincubation of erythrocytes with 10 mM-DFO resulted in an intracellular concentration of DFO of 37.8±4.0 nmol/ml of packed cells (= 18.9±2.0 nmol/ml of incubation mixture). When such DFO-preloaded erythrocytes were depleted of GSH (preincubation with DEM) and then incubated with phenylhydrazine, no lipid peroxidation or haemolysis was seen (Table 2), in spite of the fact that the amount of released iron was even higher than that observed in the previous experiment (Table 1). It therefore is possible to postulate that the amount of DFO available intracellularly is now sufficient to chelate immediately the released iron. Also, Heinz-body formation was inhibited in these erythrocytes (results not shown).

In an additional experiment (Table 3), erythrocytes were preincubated with 100 μM-DFO for 15 min. The intracellular concentration of DFO was 1.2 (mean of two experiments) nmol/ml of packed cells (0.6 nmol/ml of incubation mixture). DFO was not removed from the incubation medium; DEM and phenylhydrazine were subsequently added. The presence of DFO in the incubation medium did not prevent lipid peroxidation and haemolysis. It seems therefore that DFO is not acting from the outside of the cell, and that, only when DFO is present in the intracellular compartment in amounts equivalent to those of released iron, are lipid peroxidation and haemolysis prevented. It should also be noted that in this experiment (Table 3) in which the intracellular concentration of DFO was 1.2 nmol/ml of packed cells, lipid peroxidation and haemolysis were somewhat higher than in the experiment reported in Table 2, in which the intracellular concentration of DFO was 5 nmol/ml of packed cells.

Results similar to those seen with phenylhydrazine were obtained with divicine (Table 4). In native erythrocytes, divicine induced iron release and production of methaemoglobin (Table 4, +divicine), although to a lower extent than phenylhydrazine (Table 1, +Phz). The erythrocyte GSH was minimally affected. No lipid peroxidation or haemolysis occurred. On the other hand, in DEM-pretreated erythrocytes the divicine-induced iron release was followed by lipid peroxidation and haemolysis (Table 4, +DEM +divicine), as observed in the previous experiments with DEM-pretreated erythrocytes incubated with phenylhydrazine. Note also that the release of iron and methaemoglobin formation were higher in DEM-pretreated erythrocytes than in native erythrocytes after incubation with divicine. As seen in the phenylhydrazine experiments, when DFO-preloaded erythrocytes (preincubation with 10 mM-DFO for 90 min) were treated with DEM and then incubated with divicine, lipid peroxidation and haemolysis were prevented (Table 4), in spite of a release of iron and methaemoglobin formation similar to those in non-preloaded cells.

Results virtually identical with those seen with divicine were obtained with isouramil (Table 5). Here again iron release and methaemoglobin formation were higher in DEM-pretreated erythrocytes than in native erythrocytes, which could suggest some role for GSH in the prevention of iron release. Lipid

Table 4. Release of iron (DFO-chelatable iron), methaemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and haemolysis in erythrocytes incubated with divicine, or preincubated with DEM and incubated with divicine, or preloaded with DFO, preincubated with DEM and then incubated with divicine

Erythrocytes were: (i) incubated with 1 mM-divicine (+Divicine); (ii) preincubated with 1 mM-DEM for 10 min and then incubated without divicine (+DEM); (iii) preincubated with 1 mM-DEM for 10 min and then incubated with 1 mM-divicine (+DEM+divicine); (iv) incubated with 10 mM-DFO for 90 min, washed and recovered; the erythrocytes were then preincubated with 1 mM-DEM for 10 min and finally incubated with 1 mM-divicine (+DFO+DEM+divicine). The intracellular concentration of DFO measured at the end of the incubation with DFO, after complete removal of extracellular DFO, was 42.8±6.2 nmol/ml of packed cells. Results are the means±S.E.M. of three to six experiments (two experiments for Met-Hb, GSH, MDA and haemolysis, in controls). Met-Hb is expressed per haem.

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Haemolysis (%)
Control	0	1.9±0.2	103	900	–	2.1
	60	2.8±0.3	110	864	1.4	2.4
+Divicine	0	1.9±0.06	94±8	1083±154	–	2.5±0.2
	30	5.8±0.5	109±6	788±65	1.3±0.1	3.3±0.3
	60	7.2±0.6	108±10	816±54	1.6±0.3	4.4±0.4
+DEM	0	1.8	102	318	–	2.1
	60	2.0	103	192	4.1	5.3
+DEM+divicine	0	1.9±0.06	89	116±48	–	2.5±0.2
	30	15.9±0.8	358±111	102±21	61.0±7.4	67.7±12.2
	60	22.8±1.6	350±89	91±11	87.9±4.6	93.3±3.8
+43 μM-DFO (intracellular concn.)	0	1.8±0.2	94±8	177±65	–	2.3±0.3
+DEM+divicine	30	14.1±1.7	427±111	117±44	0.8±0.2	5.5±1.0
	60	22.5±2.3	341±89	116±51	0.6±0.1	6.3±0.9

Table 5. Release of iron (DFO-chelatable iron), methaemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and haemolysis in erythrocytes incubated with isouramil or preincubated with DEM and incubated with isouramil, or preloaded with DFO, preincubated with DEM and then incubated with isouramil

Erythrocytes were: (i) incubated with 1 mM-isouramil (+Isouramil); (ii) preincubated with 1 mM-DEM for 10 min and then incubated without isouramil (+DEM); (iii) preincubated with 1 mM-DEM per 10 min and then incubated with 1 mM-isouramil (+DEM +isouramil); (iv) incubated with 10 mM-DFO for 90 min, washed and recovered; the erythrocytes were then preincubated with 1 mM-DEM for 10 min and finally incubated with 1 mM-isouramil (+DFO+DEM+isouramil). The intracellular concentration of DFO measured at the end of the incubation with DFO, after complete removal of extracellular DFO, was 41.4±3.8 nmol/ml of packed cells. Results are means±S.E.M. of three to four experiments. Met-Hb is expressed per haem. N.D., not determined.

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Haemolysis (%)
Control	0	1.9±0.2	77±15	1199±36	–	1.9±0.1
	60	2.3±0.3	98±10	1031±42	0.7±0.1	3.4±0.2
+Isouramil	0	1.9±0.2	77±15	1199±36	–	3.4±0.9
	30	5.6±1.4	137±9	805	0.8±0.3	3.6±1
	60	6.5±0.3	128±15	864±57	1.5±0.6	4.7±1.1
+DEM+isouramil	0	2.0±0.2	77±15	305±45	–	3.4±0.9
	30	16.4±2.6	230±59	N.D.	77.6±8.8	77.2±10.8
	60	22.8±3.5	264±62	121±85	98.5±5.5	93.1±3.9
+41 μM-DFO (intracellular concn.)	0	2.1±0.2	77±15	322±34	–	3.4±0.9
+DEM+isouramil	30	14.7±4.0	166±11	N.D.	1.3±0.6	4.8±1.5
	60	20.0±4.7	179±10	208±74	1.1±0.7	6.0±1.0

Table 6. Release of iron (DFO-chelatable iron), methaemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and haemolysis in erythrocytes preincubated with 0.5 mM-DEM and then incubated with divicine or isouramil

The erythrocytes were preincubated with 0.5 mM-DEM for 10 min, recovered by centrifugation and then incubated with 1 mM-divicine or -isouramil. The GSH concentration in native erythrocytes was 1245 and 1127 nmol/ml of incubation mixture in the divicine and isouramil experiment respectively. The results of one experiment are reported. Met-Hb is expressed per haem. N.D., not determined.

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Haemolysis (%)
+0.5 mM-DEM+divicine	0	1.6	102	716	–	1.9
	30	11.0	128	433	15.3	16.5
	60	N.D.	128	350	46.9	59.1
+0.5 mM-DEM+isouramil	0	2.0	102	568	–	3.5
	30	10.4	154	280	10.4	17.8
	60	N.D.	205	356	23.0	21.2

peroxidation and haemolysis, which occurred in GSH-depleted erythrocytes, were prevented in DFO-preloaded cells (preincubation with 10 mM-DFO for 90 min).

Finally, as shown in Table 6, when a less severe depletion of GSH was attained by preincubating erythrocytes with a lower amount of DEM (0.5 mM) than that (1 mM) used in the previous experiments (Tables 4 and 5), the subsequent incubation with divicine or isouramil resulted in less severe lipid peroxidation and haemolysis, which again indicates a strict dependence of lipid peroxidation on the erythrocyte GSH concentration.

DISCUSSION

The present results clearly show that well-known oxidizing agents, such as phenylhydrazine, divicine and isouramil, induce iron release from iron complexes in erythrocytes. The release of iron is accompanied by methaemoglobin formation. Also iron release precedes lipid peroxidation and haemolysis when these phenomena are going to occur. Release of iron in a DFO-chelatable form was also observed on incubation of erythrocytes with acrolein [17,19]. However, since the addition of acrolein resulted in an immediate disappearance of erythrocyte GSH, it was not clear whether iron release was the result of GSH depletion or whether GSH acted to protect iron complexes (haemoglobin) from the interaction with acrolein. The present results clearly show that iron release does not depend on GSH depletion by itself, since incubation of erythrocytes with DEM does not result in any iron release and, on the other hand, significant release can occur with only a small GSH decrease. Rather, the fate of the cell in which iron is released depends on the intracellular availability of GSH. When the GSH concentration is severely decreased (preincubation with DEM), the release of iron induced by the oxidizing agents is followed by lipid peroxidation and haemolysis. When, on the other hand, a sufficient concentration of GSH is still available in the cell, lipid peroxidation and haemolysis are prevented in spite of significant iron release. It should be noted that, especially in the case of divicine and isouramil, the release of iron and the formation of methaemoglobin are higher in GSH-depleted cells than in native cells. In connection with this, it has been reported [40] that GSH in combination with superoxide dismutase can slow divicine and isouramil autoxidation. Thus GSH may play a role in preventing iron release when the cell is challenged with oxidative stress. GSH may also prevent interactions of reactive metabolites with haemoglobin and such interactions may represent important mechanisms of iron release. With divicine and isouramil the release is probably more dependent on the formation of active oxygen species, which can be significantly prevented by GSH-dependent activities. Also, it should be considered that H_2O_2, which can easily be formed in erythrocytes challenged with

phenylhydrazine and other oxidizing agents, can release iron from haemoglobin [41,42]. The higher release of iron in GSH-depleted cells as compared with native ones does not depend on the development of lipid peroxidation, as in the case of the previous experiments [17,19] carried out with acrolein, since a similar release was also seen in DFO-preloaded cells (Tables 2, 4 and 5) in which lipid peroxidation was prevented.

The experiments carried out with DFO seem to indicate that the release of iron by the oxidizing agents considered plays a causative role in the induction of lipid peroxidation and subsequent haemolysis in GSH-depleted cells. In fact, both phenomena were prevented when the erythrocytes were preloaded with DFO in such a way (preincubation with 10 mM-DFO) that the intracellular concentration of DFO was nearly equivalent to that of the released iron, whereas no prevention was seen when such a concentration was much lower than that of reactive iron (preincubation with 0.1 mM-DFO). It seems therefore that in DFO-preloaded erythrocytes intracellular chelation of iron released from haemoglobin occurs. If such iron is completely chelated, no lipid peroxidation occurs even if the cell is virtually devoid of GSH. If some of the released iron remains in a free form, additional radical reactions are likely to occur, finally resulting in membrane disruption.

As shown in the experiment reported in Table 3, the presence of DFO in the incubation medium (with only minor amounts within the cells) did not affect the events induced by the oxidizing agents (lipid peroxidation and haemolysis). This reinforces the view that the observed events occur intracellularly, and rules out the possibility that the oxidizing agents release iron from extracellular haemoglobin (minor spontaneous haemolysis present even at 0 time).

Since it has been reported [43] that DFO can inhibit lipid peroxidation independently of iron chelation, probably by radical scavenging, this possibility must also be considered. However, in view of the correspondence between the amount of iron released and the amount of intracellular DFO, the above-mentioned possibility of intracellular chelation seems to be more likely.

A further comment is needed in relation to these experiments with DFO. It has been shown [38,39] that DFO cannot enter cells by passive diffusion. This has been indirectly confirmed in our experiments in which, after preincubation of erythrocytes with DFO and complete removal of it by several washes, the intracellular concentration of DFO was measured in an ultrafiltered haemolysate. The amount of DFO found, which was minimal with respect to that added externally (5.0–0.4 to 100), could be mainly due to the oil/water partition coefficient of DFO (1:100).

The problem concerned with redistribution of iron in the cell, the so-called 'iron delocalization', has become of increasing interest in cell injury induced by oxidative stress. Several lines of evidence indicate that iron is required for initiation of lipid peroxidation and other radical reactions, but the exact nature of such a requirement is still under investigation [44,45]. There is growing interest in the identification of intracellular sites from which iron can be mobilized. Studies by several groups [46–48] have shown that, in the liver cell, a possible source of iron is ferritin, in which iron can be stored in the ferric form and from which iron can be released on reduction to the ferrous form. Reductants capable of mobilizing iron from ferritin include, among others, the semiquinone radical of adriamicin [49] and the monocationic radical of paraquat [50]. Ferritin iron mobilization will in turn occur directly, via reoxidation at the expense of iron; or indirectly, via reoxidation of radicals at the expense of oxygen and formation of another reductant such as $O_2^{\cdot-}$. In addition to ferritin, a small pool of microsome-bound non-haem iron has been identified as a possible catalyst of lipid peroxidation [51–53]. Recently, loosely bound iron released from haem or non-haem proteins has been shown [7,46,54,55] to account for an important part of the observed stimulatory properties of these proteins towards lipid peroxidation. Also it has been shown [41,42] that, as previously stated, both H_2O_2 and organic hydroperoxides can oxidatively degrade haemoglobin with the release of iron. Such released iron is able to promote hydroxyl radical formation [41,42]. These findings in chemical systems support the view that low-M_r iron complexes are the most likely Fenton catalysts in the living cell. The present and previous [17–19] results show that, in erythrocytes, iron is released from haemoglobin as a result of oxidative stress. With all three drugs considered, superoxide anion, and probably hydroxyl radical, are formed [21,30,56]. In the case of divicine and isouramil, active oxygen species are formed mainly by redox cycling [30]. In the case of phenylhydrazine, oxidase and peroxidase reactions of oxyhaemoglobin and methaemoglobin have been described [22] which lead to formation of both $O_2^{\cdot-}$ and phenylhydrazine radicals (phenylhydrazine and phenyl radicals) [25–27]. These radicals can denature the haemoglobin molecule [20]; iron is probably released from denatured haemoglobin and can induce lipid peroxidation if the cell is depleted of GSH. Lipid peroxidation is always associated with haemolysis. GSH, in turn, when present in sufficient amount, can prevent lipid peroxidation even in the face of substantial iron release, possibly by regenerating α-tocopherol in the membranes [57]. GSH may also have a role in the prevention of iron release by removing H_2O_2, by decreasing autoxidation of redox agents and by interacting with the radicals generated from the drugs.

One point that deserves further consideration is that concerned with the source of released iron. Iron release from haemoglobin is understood to occur via oxidation of the haem group. Yet, release does not correlate well with the extent of oxidation. For example, in DEM-pretreated erythrocytes, isouramil or divicine give relatively little haem oxidation compared with phenylhydrazine, yet just as much iron is released. The reason for such a discrepancy is unknown, as is the different kinetics of iron release with different oxidizing agents. Moreover, a maximum release of about 23 μM was observed regardless of the agent used or extent of oxidation. This corresponds to only a small fraction of haemoglobin, which could suggest the presence of a finite amount of available iron in the erythrocyte. In fact, the incubation of DEM-pretreated erythrocytes with increasing amounts of phenylhydrazine increased the extent of iron release to only a limited amount (36.8 and 35.2 μM with 5 and 10 mM-phenylhydrazine respectively).

In summary, these studies indicate that the phenylhydrazine, divicine- and isouramil-induced erythrocyte damage represents, like that induced by acrolein, a model of iron delocalization. A similar increase in low-M_r intracellular iron chelates (DFO-chelatable iron) has also been observed in kidneys during cold storage and subsequent reperfusion [10] as well as in liver and cerebellum after acute ethanol intoxication [58]. Studies from our laboratory [59] have shown that iron release from iron complexes also occurs in erythrocytes during aging.

In the light of the present results, it is believed that the divicine- and isouramil-induced iron release leads to peroxidative decomposition of membrane lipids in erythrocytes partially depleted of GSH like those affected by glucose 6-phosphate dehydrogenase deficiency.

This research was supported by a grant from the Italian Ministry of University and Scientific Research (Progetto 'Tossicologia Industriale: meccanismo d'azione degli xenobiotici e loro applicazioni'). Additional funds were derived from the Association for International Cancer Research (U.K.). We thank Professor T. F. Slater and Professor M. U. Dianzani for helpful discussions.

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Received 2 September 1991/26 November 1991; accepted 10 December 1991