Volume 319, number 1,2, 40–44 FEBS 12194 March 1993

Iron released from an erythrocyte lysate by oxidative stress is diffusible and in redox active form

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Received 20 January 1993; revised version received 26 January 1993

The incubation of a ghost-free erythrocyte lysate with the oxidizing agent phenylhydrazine resulted in both methemoglobin formation and release of iron in a desferrioxamine (DFO)-chelatable form. The released iron was diffusible, as shown by a dialysis carried out simultaneously with the incubation. When the dialysate was added to erythrocyte ghosts or to microsomes from liver or brain, lipid peroxidation developed in the membranes, indicating that the diffusible iron was in a redox active form. The addition of ATP to the lysate markedly increased both iron diffusion and lipid peroxidation in the membranes subsequently added to the dialysate. The possible implication of these data in some well known pathologies is discussed.

Iron; Oxidative stress; ATP–iron interaction; Membrane damage

1. INTRODUCTION

It is generally accepted that iron plays a central role in oxidative stress, both by generating harmful oxygen species and by promoting lipid peroxidation [1–4]. Since iron is transported and stored in specific proteins that prevent its reaction with reduced oxygen species, the problem concerned with redistribution of iron within the cell appears of major interest in studies of damage induced by oxidative stress. A number of reports have suggested that a low molecular weight iron pool is present in cells, as a transit pool, in which iron is probably chelated to citrate, amino acids and nucleotides [5–7]. The possibility therefore exists that components of this pool represent the iron species catalytically active in initiating free radical reactions and lipid peroxidation under certain conditions.

Previous studies from our laboratory [8] have shown that iron is released in a 'free' (desferrioxamine (DFO) chelatable) form when mouse erythrocytes are incubated with oxidizing agents such as phenylhydrazine, divicine, isouramil and acrolein. Iron is released from hemoglobin [9] and the release is accompanied by methemoglobin formation [8]. When the erythrocytes are severely depleted of glutathione (which occurs directly with acrolein [10] or after a short preincubation with diethylmaleate with the other oxidants [8]), iron release is followed by peroxidation of membrane lipids and cellular lysis. If the cells are properly preloaded with DFO, lipid peroxidation and hemolysis are prevented [8]. It seems therefore that intracellular iron chelation prevents erythrocyte membrane damage, which suggests a primary role for iron in such damage.

In view of the importance of the fact that under conditions of oxidative stress an agent is released which promotes oxidative reactions in the cell, we investigated in the present study whether the released iron is able to diffuse and act at a distance. The diffusion of iron in an active form from the site of its release could represent an important feature to postulate a role for delocalized iron in spreading out the oxidative damage in tissues where the release occurs. The results indicate that iron released from a hemolysate under conditions of oxidative stress diffuses into a dialysis tube in a redox active form capable of initiating oxidative reactions in cellular membranes.

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Abbreviations. NTA, nitrilotriacetate; DFO, desferrioxamine; Tris, tris(hydroxymethyl)methylamine; HPLC, high pressure liquid chromatography; MetHb, methemoglobin; TBARS, thiobarbituric acid-reactive substances.

2. MATERIALS AND METHODS

2.1. *Materials*

Desferrioxamine (Desferal) was kindly supplied by Ciba-Geigy (Basel-Switzerland). Phenylhydrazine was from Merck. The dialysis tube was a section of Visking dialysis tubing 20/32 (Serva, Feinbiochemica, Heidelberg, Germany; diameter 16 mm; wall strength 0 02 mm; average diameter pore 1.5–2.0 nm; exclusion limit 10,000–20,000 mol. wt.). Ultrafiltration membranes were from Amicon (Centriflo, CF 25; exclusion limit 25,000 mol. wt.). ATP was from Sigma Chem. Electrophoresis standards and reagents were from Bio-Rad Laboratories (Richmond, CA). The solvents used for HPLC were of HPLC grade. All the other chemicals were of analytical grade.

2.2. *Preparation of ghost-free erythrocyte lysate*

Male Swiss albino mice (Nossan, Correzzana, Milan) weighing 25–

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35 g and maintained on a pellet diet (Nossan) were used. Blood was withdrawn from the abdominal aorta under ether anaesthesia and heparinized. After centrifugation, the plasma and the buffy coat were removed and the erythrocytes washed three times with buffered physiological solution. Erythrocytes were lysed by addition of 4 vols. of hypotonic (10 mM) Tris-maleate buffer, pH 7.4. The lysate was centrifuged (17,000 × g, 15 min) and the supernatant fraction and the ghosts separately recovered. To restore the physiological osmolarity, 140 mM NaCl was added to the ghost-free lysate. The hemoglobin concentration measured as in [8] and expressed per heme, was 3.04 ± 0.10 μmol/ml.

2.3. *Membrane preparations*

The ghosts recovered from 1 ml of packed cells were washed three times by adding 7.5 ml of Na-phosphate buffer, pH 7.4, according to Dodge [11]. The hemoglobin-free ghosts were washed in 140 mM NaCl, 10 mM Tris-maleate buffer, pH 7.4, and resuspended in the same buffer at a final concentration of 10 mg protein/ml.

Liver or brain microsomes were prepared from 10% (w/v) liver or brain homogenates in 140 mM NaCl, 10 mM Tris-maleate buffer, pH 7.4. The homogenates were centrifuged at 10,000 × g for 15 min and the supernatant fractions were centrifuged at 20,000 × g for 15 min. The post-lysosomal supernatants were centrifuged at 120,000 × g for 60 min. The microsomal pellet from liver or brain was resuspended in the same buffer at a final protein concentration of 10 mg/ml. Protein was determined according to Lowry [12].

2.4. *Incubation procedure*

Twelve ml of ghost-free erythrocyte lysate were incubated in an Erlenmeyer flask into which a dialysis tube containing 4 ml of the same buffer used for the lysate preparation (140 mM NaCl, 10 mM Tris-maleate, pH 7.4) was dipped. The incubation was carried out aerobically for 3 h in the presence of 1 mM phenylhydrazine. At the end of the incubation DFO-chelatable iron was determined in both the lysate and the contents of dialysis tube. Methemoglobin formation in the lysate was also determined [13]. Subsequently, the contents of the dialysis tube (0.9 ml) was added to the membrane (ghosts or microsomes) suspensions (0.1 ml). The mixture was incubated aerobically for 1 h. Thiobarbituric acid-reactive substances (TBARS) formation was measured [14] as an index of lipid peroxidation.

The electrophoretic analysis of ghost proteins was performed on 6% acrylamide gel (SDS-PAGE) according to Laemmli [15].

2.5. *DFO-chelatable iron determination*

DFO-chelatable iron was determined as previously reported [10]. Briefly, at the end of the incubations aliquots of both lysate and contents of dialysis tube were withdrawn; 35 μM DFO was added. The lysate was ultrafiltered by centrifugation in ultrafiltration cones (Centriflo). DFO-chelatable iron was measured in 0.5 ml of both ultrafiltrate and dialysate by HPLC as previously done [10]. Preliminary experiments were performed to ascertain that the addition of DFO to the samples detects all iron present in the solutions even in presence of other chelators such as nitrilotriacetate (NTA) or ATP.

ATP content of both whole erythrocytes and lysate was measured by HPLC according to Debetto and Bianchi [16].

3. RESULTS

When a ghost-free erythrocyte lysate was incubated aerobically with phenylhydrazine, a release of iron was observed (Table I), as shown by the amount of DFO-chelatable iron detectable in the lysate ('outside dialysis tube'). Concomitant with the release of iron, methemoglobin formation was seen. An iron release in whole erythrocyte lysate was also seen with acrolein [9]. In the present experiments ghosts were removed from the lysate to avoid any possible source of lipid peroxidation which could interfere with peroxidation of cellular membranes subsequently added to the dialysate (see below).

When a dialysis tube containing only the buffer was dipped into the lysate from the start of the incubation, part of the released iron could be recovered in the dialysate (Table I, 'inside dialysis tube'). When the contents of the dialysis tube were added to the ghosts, lipid peroxidation developed in the ghosts (Table I). The electrophoretic analysis of ghost proteins showed the appearance of high molecular weight aggregates (not shown), likely produced by cross-linking between proteins and peroxidized lipids.

Table I

Lipid peroxidation (TBARS) in erythrocyte ghosts, liver and brain microsomes exposed to diffusible iron ('free iron') released from a ghost-free erythrocyte lysate by phenylhydrazine (Phz)

Conditions	MetHb (nmol/ml)	'Free iron' (nmol/ml)		TBARS produced by Ghosts (nmol/mg Pt) 60 min	TBARS produced by Liver Microsomes (nmol/mg Pt) 60 min	TBARS produced by Brain Microsomes (nmol/mg Pt) 60 min
	180 min	Outside d.t.	Inside d.t.			
No additions (control)	–	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.9 ± 0.2	1.3 ± 0.4
+ Lysate − Phz (control)	34.1 ± 3.4	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.2	1.3 ± 0.4	1.6 ± 0.2
− Lysate + Phz (control)	–	1.3 ± 0.2	1.3 ± 0.2	1.8 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
+ Lysate + Phz (Exp.)	1126.4 ± 71.9	14.1 ± 1.8	6.0 ± 0.5	6.0 ± 0.6	5.2 ± 0.1	8.2 ± 2.0
+ Lysate + Phz (Exp.) + DFO	1126.4 ± 71.9	14.1 ± 1.8	6.0 ± 0.5	0.7 ± 0.1	1.4 ± 0.4	1.7 ± 0.6

The experimental flask contained, in a final volume of 12 ml, a ghost-free erythrocyte lysate derived from 2.4 ml of packed cells, 140 mM NaCl and 10 mM Tris-maleate buffer, pH 7.4. Phenylhydrazine was added at the concentration of 1 mM. Control flasks were identical to the experimental flasks except that no phenylhydrazine, or no lysate, or neither were added. A dialysis tube containing 4 ml of buffer was introduced into the flask at the start of the incubation. The incubation was carried out at 37°C in air for 3 h. At the end of the incubation the concentrations of DFO-chelatable iron ('free iron') outside and inside the dialysis tube (d.t.) were evaluated. An aliquot of the content of the dialysis tube was added to erythrocyte ghosts or to liver or brain microsomes to have a protein concentration of 1.0 mg/ml. The mixture was incubated for 60 min at 37°C, and TBARS formation was evaluated. Where indicated DFO (35 μM) was added to the content of the dialysis tube before the latter was added to ghosts or microsomes. The values represent the mean ± S.E.M. of four experiments.

Lipid peroxidation was also seen when liver or brain microsomes were used as a target system for the released iron instead of ghosts (Table I). These findings indicate that iron released into the lysate by phenylhydrazine is able to diffuse into a dialysis tube and to induce lipid peroxidation in cellular membranes. The addition of DFO to the dialysate, prior to its incubation with the membranes prevented, as expected, lipid peroxidation (Table I).

Since in the above experiments less than the half of the released iron diffused into the dialysis tube, the possibility to increase iron diffusion was verified by adding iron chelators to the lysate from the beginning of the incubation. As shown in Table II, both DFO and NTA increased the diffusion of iron into the dialysis tube. A small increase was also observed in the amount of free iron detectable in the lysate. These results suggest that the released iron, is more readily transported to other loci when it is chelated to iron chelators. It is possible that even in the absence of externally added iron chelators, iron is transported in small complexes. Table II also shows that when DFO was used as iron chelator no lipid peroxidation was observed in ghosts or microsomes subsequently added to the dialysate. Whereas with NTA lipid peroxidation developed in the membranes, and protein aggregation was also seen. It is known in fact, that as the NTA iron complex, iron is maintained in a redox active form [17,18].

In further experiments we investigated whether some physiological iron chelator acts like NTA in increasing iron diffusion and in maintaining the metal in a redox active form. Since it is known [19] that pyridine nucleotides readily bind iron and since it has been suggested [20,21] that ATP-bound iron is one of the major constituents of the low molecular weight iron pool, the effects of ATP were studied. As shown in Table III, when ATP (1.5 mM) was added to the lysate, an increase was seen both in free iron present in the lysate and in its diffusion into the dialysis tube as compared to the experiments (Table I) carried out in the absence of either ATP or other chelators. The time course of this diffusion is shown in Fig. 1. Moreover, when ghosts or microsomes were incubated with the dialysate (Table III) the extent of lipid peroxidation was much higher than that seen in

Table II

Diffusion of iron ('free iron') released from a ghost-free erythrocyte lysate incubated with phenylhydrazine (Phz) in the presence of nitrilotriacetate (NTA) or desferrioxamine (DFO), and lipid peroxidation in cellular membranes subsequently added to the dialysate

Conditions	'Free iron' (nmol/ml)		TBARS produced by Ghosts (nmol/mg Pt) 60 min	TBARS produced by Liver Microsomes (nmol/mg Pt) 60 min	TBARS produced by Brain Microsomes (nmol/mg Pt) 60 min
	Outside d.t.	Inside d.t.			
Lysate alone	1.1 ± 0.2	0.9 ± 0.2	0.8	1.1	1.0
Lysate + Phz	13.6 ± 3.5	4.1 ± 1.0	5.8	5.4	8.2
Lysate + Phz + 100 μM NTA	18.1 ± 0.7	12.7 ± 0.9	5.0	6.7	8.6
Lysate + 1 mM Phz + 35 μM DFO	19.9 ± 1.1	14.8 ± 2.1	0.7	0.9	1.0

Conditions as in Table I except that NTA or DFO were added to the ghost-free erythrocyte lysate since the beginning of the incubation. The values reported for 'Free iron' represent the mean ± S.E.M. of three experiments. The values reported for lipid peroxidation (TBARS) represent the mean of two experiments.

Table III

Lipid peroxidation (TBARS) in erythrocyte ghosts, liver and brain microsomes exposed to diffusible iron ('free iron') released from a ghost-free erythrocyte lysate by phenylhydrazine (Phz) in presence of exogenous ATP

Conditions	MetHb (nmol/ml)	Free iron (nmol/ml)		TBARS produced by Ghosts (nmol/mg Pt) 60 min	TBARS produced by Liver Microsomes (nmol/mg Pt) 60 min	TBARS produced by Brain Microsomes (nmol/mg Pt) 60 min
	180 min	Outside d.t.	Inside d.t.			
No additions (control)	–	0.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.2	1.9 ± 0.5	1.3 ± 0.7
+ Lysate − Phz (control)	47.8 ± 6.8	0.8 ± 0.2	1.1 ± 0.3	1.5 ± 0.8	1.2 ± 0.8	0.7 ± 0.5
− Lysate + Phz (control)	–	1.3 ± 0.3	1.2 ± 0.2	2.3 ± 0.3	1.8 ± 0.1	0.6 ± 0.1
+ Lysate + Phz (Experim)	1041.1 ± 80.7	26.2 ± 4.3	13 3 ± 4.3	15.8 ± 1.7	13.4 ± 1.6	12.9 ± 1.3
+ Lysate + Phz (Experim) + DFO	1041.1 ± 80.7	26.2 ± 4.4	13.3 ± 2.5	1.0 ± 0.08	0.9 ± 0.08	1.4 ± 0.3

Conditions were as in Table I except that 1.5 mM ATP was added to the ghost-free erythrocyte lysate from the beginning of the incubation. The values reported represent the mean ± S.E.M. of four experiments.

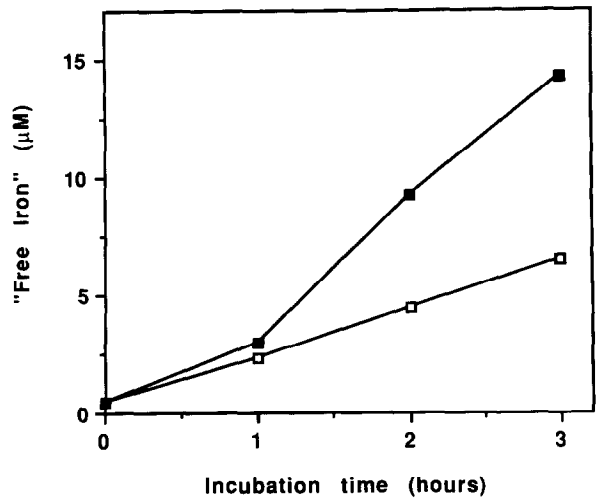

Fig. 1. Time-course of iron diffusion (DFO-chelatable iron) into a dialysis tube dipped in a ghost-free erythrocyte lysate incubated with phenylhydrazine (1 mM), in the absence (□—□) or in the presence (■—■) of exogenous ATP (1.5 mM). The values represent the mean of two experiments.

the experiments carried out in the absence of ATP. Also, lipid peroxidation was higher than in the experiments with NTA, notwithstanding that amount of free iron detectable in the lysate and in the dialysate was quite comparable.

The effectiveness of ATP in maintaining iron in a DFO-chelatable form is shown in Fig. 2. As can be seen, the recovery of iron as a DFO-iron complex from a solution of ferrous ammonium sulphate in the incubation buffer is about 100% in the presence of ATP and only about 40% in the absence of it. Probably the interaction of iron with ATP prevents the formation of insoluble hydroxylated iron forms. In the erythrocyte lysate, in the absence of externally added ATP, there is very likely a loss of released iron and a lower formation of complexes capable to cross the dialysis membrane.

Fig. 3 shows that a prolonged incubation of a ghost-free erythrocyte lysate under aerobic conditions results in the release of iron even in the absence of the oxidizing agent phenylhydrazine. However, the amount of released iron after 48 h of incubation is much lower than that released after 3 h in the presence of phenylhydrazine. No iron release was seen when the lysate was incubated in anaerobic conditions, suggesting again the involvement of oxidative stress in the release of iron.

4. DISCUSSION

Consistent with our previous report [8], it is shown here that when an erythrocyte lysate is subjected to an oxidative stress such as that induced by phenylhydrazine, iron is released in a 'free' form which is DFO-chelatable. Such iron is capable of crossing a dialysis membrane and can be recovered in the dialysate. Moreover, the diffused iron is in a redox active form as shown by its ability to initiate lipid peroxidation in cellular membranes, such as ghosts or microsomes from liver and brain. The accomplishment of a compartmentalization of the two systems – one represented by the lysate plus phenylhydrazine as iron releasing system, and the other represented by ghosts or microsomes as target system – clearly shows that iron, once released from hemoglobin is capable of inducing pathological effects at distant loci. Thus iron released from iron complexes may act as a multiplying factor as to the damage induced by oxidative stress. Not only can it act on the membranes of the cells in which it is released (as previously reported in [8]), but it may also diffuse from these cells and initiate oxidative reactions in membranes of surrounding cells. In an in vivo situation, it can be envisaged that iron released from hemoglobin oxidation after hemorrhagic accidents, induces oxidative alterations in surrounding tissues, thus amplifying the damage due to the hemorrhagic events. It is known that hemoglobin potentiates tissue injuries when, for any reason, cells are exposed to free hemoglobin [22–24].

Another interesting result in the present report is that physiological iron chelators such as ATP can increase iron diffusion and can maintain iron in a redox active form capable of initiating oxidative reactions at a distance. When the lysate was incubated with ATP, in fact, lipid peroxidation in the membranes subsequently added to the dialysate was increased and the increase was roughly proportional to the increase in iron diffusion. With NTA on the other hand, the diffusion of iron was comparable to that seen with ATP, but the level of lipid peroxidation was much lower. It did not increase even when different amounts of NTA (25–50 μM) were

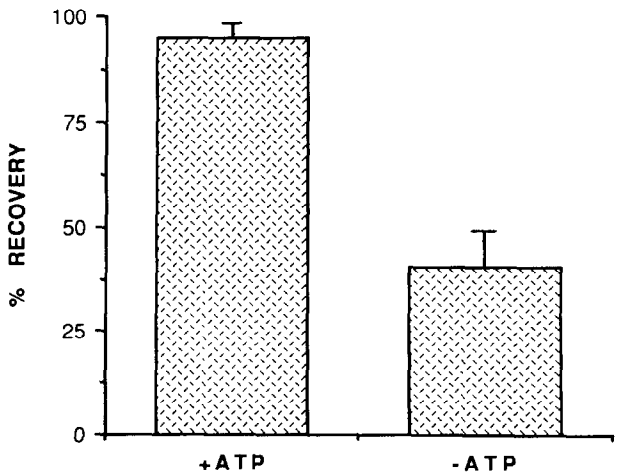

Fig. 2. Percent recovery of iron from a buffer solution in absence or in presence of 2 mM ATP. Ferrous ammonium sulphate was dissolved in 0.01 M Tris-maleate buffer, pH 7.4, at the concentration of 20 μM. The solution was kept at 37°C in air for 3 h. After addition of 35 μM DFO, iron concentration was determined as Fe–DFO complex by HPLC. The values represent the means ± S.E.M. of three experiments.

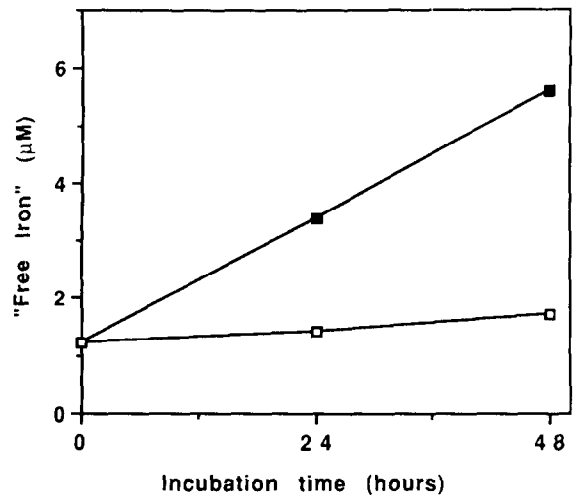

Fig. 3. Iron release (DFO-chelatable iron) in a ghost-free erythrocyte lysate during aerobic (■—■) or anaerobic (□—□) incubation at 37°C for 48 h. The values represent the mean of two experiments.

used. It seems therefore that the ATP–iron complex is in a form more active for redox cycling than the complex with NTA. Experiments (not shown here) indicated that the iron released in our experimental conditions is in the 3+ form, thus unsuitable to be redox active as NTA-chelate in the absence of any added reducing agent.

It is possible that when iron is released by phenylhydrazine during the incubation of whole erythrocytes, as seen in our former experiments [8], it is rapidly bound by ATP whose intracellular concentration is about 2 mM. This concentration remains high during the first 15 min of incubation with phenylhydrazine (data not shown) when iron release is almost maximal [8].

During the hypotonic lysis of the erythrocytes, as performed in the experiments here reported, the concentration of ATP rapidly drops to about one-half; the addition of 1.5 mM ATP to the lysate restores the ratio ATP/iron released as seen during the incubation of whole erythrocytes with phenylhydrazine. It is possible, therefore, that a high concentration of ATP needs to be present when iron is released and that the immediate chelation of iron preserves it in an active form capable of initiating lipid and protein oxidation.

The targets for the iron released in an active form could be not only lipids and proteins of cellular membranes but also other biological important molecules. Recent studies have shown that iron can interact with DNA and that low molecular weight iron chelates are effective in producing DNA damage [25–28]. DNA strand breakage as well as modifications of DNA bases can occur [29–31]. It is well known, for instance, that the risk for cancer and fibrosis is increased in conditions of iron overload, such as hemochromatosis or porphyria cutanea tarda [32–35].

Acknowledgements: This research was supported by grants from the Italian Ministry of University and Scientific Research (Progetto 'Cirrosi Epatica') and from the Italian Research Council (CNR), Strategy Project 'A.C.R.O.'.

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