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Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides

John M.C. Gutteridge

National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England

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Hydrogen peroxide and organic hydroperoxides react with haemoglobin to release iron which can be complexed to apotransferrin, bleomycin and desferrioxamine. This released iron promotes deoxyribose degradation by a Fenton reaction, DNA degradation in the presence of bleomycin and stimulates lipid peroxidation. It is likely that iron released from haemoglobin is the true generator of hydroxyl radicals in the Fenton reaction.

Hydroxyl radical *Lipid peroxidation* *Transferrin iron binding* *Heme-protein iron release* *Hemoglobin Fenton catalyst* *Desferrioxamine* *Bleomycin-detectable iron*

1. INTRODUCTION

The highly reactive and biologically damaging hydroxyl radical ($OH^{\cdot}$) can be formed in biological material when suitable transition metal ion catalysts are available (reviews [1,2]). The most abundant metal ion likely to catalyse this reaction is iron. Normally, iron is transported and stored in specific proteins which prevent, or minimise, its reaction with reduced oxygen metabolites. However, in spite of these safeguards increasing evidence suggests that reactive iron becomes available during disease states [3]. Studies have shown that the specific iron chelator desferrioxamine can modify tissue changes observed in inflammatory, degenerative and ischaemic damage as well as organ storage for transplantation [4–7].

Several reports have suggested that haemoglobin [8] and the iron-binding proteins transferrin [9] and lactoferrin [10] can act as catalysts for the Fenton reaction, although the experiments performed with haemoglobin were inadequate in that the only evidence for $OH^{\cdot}$ radical participation in the system under study was inhibition by thiourea [8] which is not a specific scavenger of $OH^{\cdot}$. I show here that iron bound to haemoglobin is unlikely to produce $OH^{\cdot}$ radicals in free solution, but that peroxides can cause a release of iron from the protein. This released iron, which can be bound by transferrin or desferrioxamine, is the true promoter of $OH^{\cdot}$ generation and contributes to peroxidation in haemoglobin-containing systems.

2. MATERIALS AND METHODS

2.1. *Materials*

Catalase (bovine liver, 17000 units/mg protein, thymol-free), albumin (human, fatty acid-free), apotransferrin (human), haemoglobin (human), caeruloplasmin (human), 2-deoxy-D-ribose, *tert*-butyl-hydroperoxide were from Sigma, Poole, Dorset. Units of enzyme activity were as defined in the Sigma catalogue. Haptoglobin (human, mixed types) was from Calbiochem, and desferrioxamine from Ciba-Geigy. All other chemicals were of the highest purity available from BDH, Poole.

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2.2. *Measurement of iron release from haemoglobin by peroxides*

Haemoglobin (1 mg/ml) dissolved in Chelex resin-treated ion-free water was further treated with Chelex resin using a batch technique to remove any loosely bound iron salts. 0.1 ml of this solution was incubated with hydrogen peroxide or organic hydroperoxides as indicated in fig.1 and table 1. Iron released from haemoglobin during the incubation was measured by the ferrozine or bleomycin methods [11,12].

2.3. *Deoxyribose degradation*

Deoxyribose in phosphate buffer (pH 7.4) was incubated for 2 h at 37°C with haemoglobin and hydrogen peroxide under the conditions shown in table 2. Inhibitors were added before H_2O_2. After incubation, thiobarbituric acid (TBA) reactivity was developed by adding 0.5 ml TBA (1%, w/v, in 0.05 M NaOH) and 0.5 ml trichloroacetic acid (2.8%, w/v) then heating at 100°C for 10 min. The resulting chromogen was extracted into butan-1-ol and relative fluorescence intensity (RFI) measured at excitation 532 nm and emission 553 nm using a reference standard of rhodamine B.

2.4. *Lipid peroxidation*

Linolenic acid micelles were prepared by vortex-mixing 10 μl fatty acid in 10 ml phosphate-saline buffer, pH 7.4 (0.025 M sodium phosphate in 0.15 M NaCl). Micelles were substituted for deoxyribose and incubated as described in section 2.3 with haemoglobin and hydrogen peroxide. Full details are given in table 3. After incubation, 0.5 ml TBA reagent and 1.0 ml acid buffer (pH 3.5) were added and the tube contents heated at 100°C for 20 min. The resulting chromogen was extracted into butan-1-ol and measured as described above for deoxyribose.

3. RESULTS

Iron was released from haemoglobin after incubation with H_2O_2 or organic hydroperoxides. This iron could be measured by the ferrozine method (fig.1). In the case of H_2O_2, the results were confirmed with the more sensitive bleomycin assay (fig.1). Unfortunately, organic hydroperoxides interfere with the bleomycin assay and so it could not be used in their presence. The slightly higher levels of iron obtained by the bleomycin method are ascribed to its greater sensitivity.

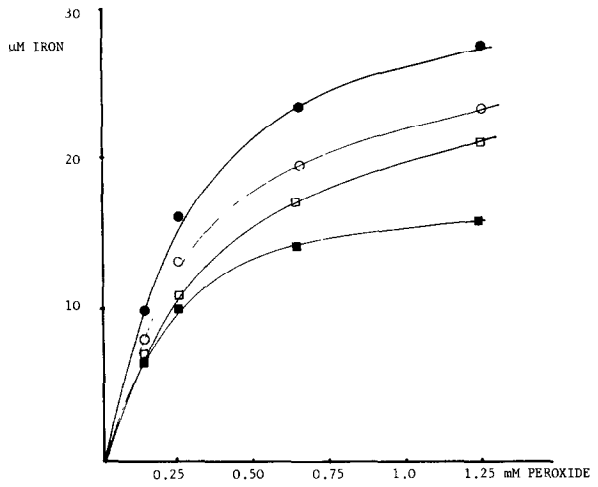

Fig.1. Haemoglobin (1 mg/ml) was incubated at 37°C for 2 h with peroxide at the different final reaction concentrations indicated. At the end of the incubation period, 50 μl catalase (0.5 mg/ml) was added to destroy any remaining H_2O_2. Standard curves were established with ferric chloride and the amount of iron released from haemoglobin determined by the ferrozine and bleomycin methods. (●) Haemoglobin incubated with H_2O_2 and released iron measured by the bleomycin method. (■) Haemoglobin incubated with H_2O_2 and released iron measured by the ferrozine method. (○) Haemoglobin incubated with *tert*-butyl-hydroperoxide and the released iron measured by the ferrozine method. (□) Haemoglobin incubated with cumene hydroperoxide and the released iron measured by the ferrozine method.

Catalase prevented the release of iron from haemoglobin but did not inhibit the detection of an iron salt in the bleomycin assay. Apotransferrin and desferrioxamine, however, completely prevented the detection of bleomycin-available iron (table 1). Albumin, added as a control for non-specific protein effects, showed no significant inhibition of iron release from haemoglobin or of the reaction of released iron with bleomycin (table 1). The haem-binding protein haptoglobin weakly inhibited the peroxide-dependent release of iron from haemoglobin (table 1) but had no effect on an iron salt.

The iron released from haemoglobin by H_2O_2 accelerated deoxyribose degradation and this could be detected and measured using the sensitive

Table 1

Release of iron from haemoglobin in the presence of H_2O_2 and its availability for binding to bleomycin: the effect of inhibitors on iron release and binding

	Iron (μM) available to bleomycin	% inhibition of iron released or detected
(1) Control haemoglobin (0.17 mg/ml)	0	
(2) Haemoglobin + H_2O_2 (0.8 mM)	23.4	
Reaction 2 + catalase (0.08 mg/ml)	0	100
Reaction 2 + albumin (0.17 mg/ml)	23.1	NS
Reaction 2 + haptoglobin (0.17 mg/ml)	19.5	17
Reaction 2 + transferrin (1.0 mg/ml)	0	100
Reaction 2 + desferrioxamine (0.17 mM)	0	100

0.1 ml haemoglobin (1 mg/ml) was incubated with 0.1 ml H_2O_2 (5 mM) in a volume of 0.6 ml for 2 h at 37°C. 10 μl catalase (0.5 mg/ml) was added to destroy any remaining peroxide. 0.1 ml of the inhibitors at the concentration shown above were added to the reaction before the addition of H_2O_2. After incubation, an aliquot of the mixture was sampled for the bleomycin assay. Iron standards and appropriate blanks were similarly treated. Results shown are the mean of 3 separate assays which differed by less than ± 5%: NS, not significant. Final reaction concentrations are shown

Table 2

Deoxyribose degradation stimulated by haemoglobin in the presence of hydrogen peroxide: the effect of inhibitors

	OH· radicals detected as the formation of TBA reactivity after deoxyribose degradation (Ex.532 nm, Em.553 nm)	
	Fluorescence (RFI units)	% inhibition of fluorescence
(1) Deoxyribose only	2	
(2) Deoxyribose + H_2O_2 (0.67 mM)	7	
(3) Deoxyribose + ferric chloride (6.7 μM)	3	
(4) Deoxyribose + ferric chloride + H_2O_2	16	
(5) Deoxyribose + haemoglobin (133 μg/ml)	7	
(6) Deoxyribose + haemoglobin + H_2O_2	41	
Reaction 6 + catalase (67 μg/ml)	9	94
Reaction 6 + albumin (133 μg/ml)	40	NS
Reaction 6 + transferrin (800 μg/ml)	17	71
Reaction 6 + desferrioxamine (133 μM)	8	97
Reaction 6 + caeruloplasmin (133 μg/ml)	15	76
Reaction 6 + mannitol (13.3 μM)	19	65

All reactions contained 6.7 μM deoxyribose and 53 mM phosphate buffer (pH 7.4). Results were calculated after the subtraction of the haemoglobin blank. Final reaction concentrations are shown. The results are a mean of 3 separate assays which differed by less than ± 5%. NS, not significant

fluorimetric TBA reaction. The deoxyribose degradation was strongly inhibited by catalase, apotransferrin, desferrioxamine or caeruloplasmin and to a lesser extent by the OH· scavenger mannitol. Hence H_2O_2 is involved not only in the release of iron but also in the formation of OH· radicals (table 2). Albumin showed no significant inhibitory activity. Iron released from haemoglobin also stimulated the peroxidation of linolenic acid micelles. The peroxidation was inhibited by catalase, apotransferrin, desferrioxamine, propyl gallate and caeruloplasmin (table 3).

4. DISCUSSION

Suggestions that the haemoglobin molecule is a catalyst for the biological Fenton reaction [8] do not appear to be compatible with the known

Table 3

Fatty acid peroxidation stimulated by haemoglobin in the presence of hydrogen peroxide: the effect of inhibitors

	Linolenic acid peroxidation as TBA reactivity (Ex.532 nm, Em.553 nm)	
	Fluorescence (RFI units)	% inhibition of fluorescence
(1) Fatty acid only	20	
(2) Fatty acid + H_2O_2 (1.1 mM)	22	
(3) Fatty acid + haemoglobin (110 μg/ml)	21	
(4) Fatty acid + haemoglobin + H_2O_2	37	
Reaction 4 + catalase (60 μg/ml)	21	100
Reaction 4 + albumin (100 μg/ml)	37	NS
Reaction 4 + caeruloplasmin (200 μg/ml)	25	75
Reaction 4 + transferrin (1.7 mg/ml)	22	94
Reaction 4 + desferrioxamine (100 μM)	18	100
Reaction 4 + propyl gallate (100 μM)	17	100

All reactions contained 0.2 ml linolenic acid micelles and 40 mM phosphate buffer (pH 7.4). Final reaction concentrations are shown. Results are the mean of 3 separate assays which differed by less than 5% and were calculated after the subtraction of appropriate blank values. NS, not significant

chemistry of the OH· radical since this implies that OH· formed at the iron centre travels through the protein core into free solution to react with a detector molecule. Studying the oxidative breakdown of haemoglobin some 50 years ago, Lemberg and colleagues [13,14] observed that 'labile iron' was released during the oxidative sequence.

Recently, loosely bound iron released from haem-protein and ferritin has been shown to account for an important part of the observed stimulatory properties towards lipid peroxidation of these proteins [15–18]. Extending these studies it is here shown that both H_2O_2 and organic hydroperoxides can oxidatively degrade haemoglobin with the release of iron. It is this iron not the haem iron or the protein which is able to promote OH· formation.

These findings support the view that low-molecular-mass iron complexes are the most likely Fenton catalysts in vivo and point to a major source of such iron. Using the bleomycin-iron assay loosely bound iron has been detected in fluids taken from patients with degenerative brain disease [19], active joint disease [20], iron overload [21,22] and exercise-stressed athletes [23]. 'Malplaced' iron likely to stimulate radical reactions can arise as a consequence of tissue damage or iron overload. In addition, the iron-promoted formation of OH· radicals and lipid peroxidation is also likely to occur as a direct result of increased oxygen radical formation in the presence of haemoglobin released from protected sites.

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