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Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation

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Haem (ferrous protoporphyrin IX) is a reactive low-molecular-mass form of iron able to participate in oxygen-radical reactions that can lead to the degradation of proteins, lipids, carbohydrates and DNA. Oxygen-radical reactions are likely to occur upon tissue damage. Extracellular fluids rely on antioxidant mechanisms different from those found inside the cell, and circulating proteins limit radical reactions by converting pro-oxidant forms of iron into less-reactive forms. Of the compounds tested, only apohaemopexin and the chain-breaking antioxidant butylated hydroxytoluene inhibited (by more than 90%) haemin-stimulated peroxidation as measured by formation of conjugated dienes, thiobarbituric acid-reactive material from linolenic acid or peroxidation-induced phospholipid flourescence. Haptoglobin, the haemoglobin-binding serum protein, was ineffective. Conversely, only haptoglobin significantly inhibited haemoglobin-stimulated lipid peroxidation. Iron-salt-induced lipid peroxidation was inhibited only by apotransferrin and the iron-chelator desferrioxamine. All lipid peroxidations were inhibited by the radical scavengers butylated hydroxytoluene and propyl gallate. These findings support the concept that transport and conservation of body iron stores are closely linked to antioxidant protection.

INTRODUCTION

Free-radical reactions have been implicated in the pathology of several human conditions, such as reperfusion injury to lung and kidney, and cerebral trauma or ischaemia (for a recent review see Halliwell, 1987). Haem (ferrous protoporphyrin IX) is a reactive low-molecular-mass form of iron able to participate in oxygen-radical reactions that can lead to the degradation of proteins, lipids, carbohydrates and DNA (Tappel, 1955; Aft & Mueller, 1983, 1984; J. M. C. Gutteridge, unpublished work). Safe removal from the circulation of haem by haemopexin (Muller-Eberhard *et al.*, 1969; Muller-Eberhard, 1970; Smith & Morgan, 1979), as part of the body's need to conserve iron stores (Davies *et al.*, 1979), has been suggested to be an important antioxidant function (Gutteridge, 1987) of this acute-phase protein (Carmel & Gross, 1977; Merriman *et al.*, 1978; Baumann *et al.*, 1984, 1987; Baumann & Muller-Eberhard, 1987).

Haemopexin, a plasma β-glycoprotein with a molecular mass of around 60000 Da, binds haem tightly (K_d < 1 pM; Hrkal & Muller-Eberhard, 1971), in stoichiometric amounts, to form a pink-coloured complex. Haem–haemopexin is a low-spin haem–protein (Morgan & Vickery, 1978), and haem is bound by co-ordination of the haem iron ion by two histidine residues (Morgan & Vickery, 1978). Haem is transported to the liver parenchymal cells by a receptor-mediated process (Smith & Morgan, 1979, 1981) involving endocytosis of haemopexin (Hunt *et al.*, 1988; A. Smith & R. Hunt, unpublished work). Like the iron-transport protein transferrin, the haem-transport protein haemopexin is not degraded when delivering haem to cells, since it returns to the circulation as an intact protein (Smith & Morgan, 1979, 1981).

Several circulating proteins have been shown to bind haem *in vitro*, including albumin (Fairley, 1938), histidine-rich glycoprotein (Morgan, 1981) and thrombin (Green *et al.*, 1983). Albumin binds haem with one high-affinity binding site, K_d 10 nM, and two lower-affinity binding sites, K_d approx. 1 μM (Beaven *et al.*, 1974). Haemopexin, although present in 50-fold lower molar concentrations than albumin, effectively competes with albumin for haem because of its higher binding affinity (Morgan *et al.*, 1976).

In the present paper we show that haemopexin, while performing its essential haem-transport role, acts as an effective antioxidant by significantly decreasing the ability of haem iron to stimulate oxidative degradation of unsaturated lipids. The integrated and protective role of haemopexin, and other acute-phase proteins, against tissue damage is discussed in detail.

MATERIALS AND METHODS

Materials

Haemin (bovine, type I), haemoglobin (bovine), linolenic acid (99% pure), albumin (human, fatty acid-free), butylated hydroxytoluene and Lubrol PX were from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of the highest purity available from BDH Chemicals, Poole, Dorset, U.K. Haemin (ferric protoporphyrin IX chloride) was dissolved in 10 mM-NaOH to give a 1.53 mM stock solution. Rabbit haemopexin was isolated from serum as previously described (Morgan & Smith, 1984). The resulting protein was greater than 95% pure as judged by polyacrylamide-gel electrophoresis in the presence of SDS and dithiothreitol (Laemmli, 1970), and contained less than 2% endo-

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genous haem–haemopexin. Phospholipid liposomes were prepared as previously described (Gutteridge, 1977) by vortex-mixing bovine brain phospholipids (5 mg/ml) in phosphate-buffered saline (0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.4). Fatty acid micelles were prepared by adding 9.2 mg of linolenic acid to 10 ml of phosphate-buffered saline (Gutteridge *et al.*, 1983) and vortex-mixing.

O_2-uptake measurements

The uptake of O_2 was measured with a Clark-type electrode (Hansatek, Kings Lynn, Norfolk, U.K.) calibrated according to the manufacturers' instructions with 100% air saturation at 37 °C, equal to 0.219 mol of O_2/ml.

Thiobarbituric acid reactivity

Fatty acid samples, after incubation to produce lipid peroxidation products, were heated for 15 min at 100 °C with the addition of 0.5 ml of thiobarbituric acid reagent (1%, w/v, 50 mM-NaOH) and 0.5 ml of 25% (v/v) HCl. The pink chromogen formed was extracted into 3.0 ml of butan-1-ol, and the organic phase was removed for quantitative determination by spectrophotometric measurement at 532 nm and spectrofluorimetric measurement (excitation wavelength 532 nm, emission 553 nm).

Phospholipid fluorescence

Incubation of phospholipid liposomes with an iron salt produces fluorescent material. After incubation for 2 h at 37 °C the fluorescent material was solubilized by adding 0.2 ml of Lubrol (1%, w/v) to 1.4 ml of reaction volume. Changes in fluorescence were measured (excitation wavelength 360 nm, emission 430 nm), and the results are expressed as relative fluorescence intensity units against a standard of 0.1 μM-tetraphenylbutadiene (Gutteridge, 1978).

Diene conjugation in micelles

The increase in absorbance at 234 nm was recorded as the formation of conjugated dienes in fatty acid micelles. A molar absorption coefficient of 25000 $M^{-1} \cdot cm^{-1}$ can be applied to quantify the yield of lipid peroxides (Fridovich & Porter, 1981). When appropriate, scan difference spectra were made.

All results shown are the means of three or more separate experiments, which differed by less than $\pm 5\%$.

RESULTS

Haem, as haemin (ferric protoporphyrin chloride), strongly binds to apo-haemopexin to give a pink-coloured complex absorbing maximally at around 410 nm. Reduction of haemin (Fe^{3+}) to ferrous (Fe^{2+}) protoporphyrin occurs in the presence of the reducing agent 2-mercaptoethanol. Haemopexin binds both ferric and ferrous protoporphyrin (Morgan & Vickery, 1978).

When haemin and 2-mercaptoethanol are added to linolenic acid micelles in phosphate buffer, pH 7.4, O_2 is rapidly consumed, causing a fall in dissoved O_2 concentration (Fig. 1) and an increase in oxidation of lipid. The uptake of O_2 is unaffected by the presence of the iron-chelator desferrioxamine, and only poorly inhibited by the iron-binding protein apo-transferrrin. Haemopexin (13 μM), however, substantially inhibits (by more than

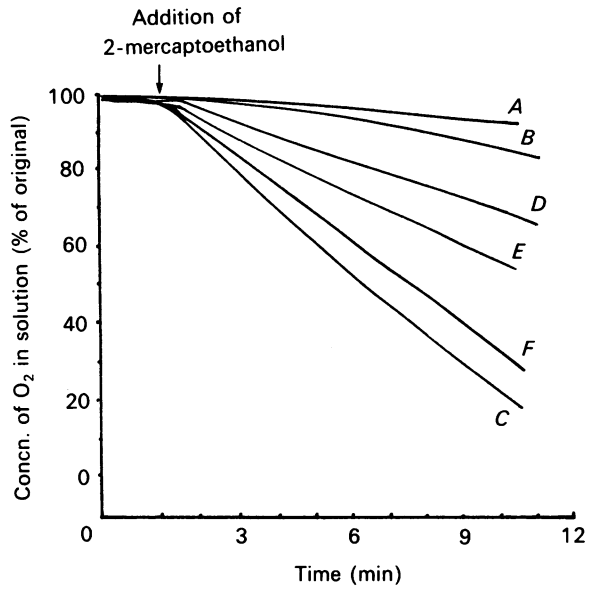

Fig. 1. Uptake of O_2 by peroxidizing linolenic acid stimulated by haemin and 2-mercaptoethanol

Curve *A*, linolenic acid micelles, pH 7.4, were incubated at 25 °C with 2-mercaptoethanol (0.25 mM) and desferrioxamine (0.08 mM) for the time indicated; curve *B*, reaction as for *A* with 2-mercaptoethanol omitted but haemin (0.012 mM) added; curve *C*, control reaction containing micelles at pH 7.4, desferrioxamine (0.08 mM), 2-mercaptoethanol (0.25 mM) and haemin (0.012 mM); curve *D*, control reaction *C* with apo-haemopexin (0.013 mM); curve *E*, control reaction *C* with albumin (0.012 mM); curve *F*, control reaction *C* with apo-transferrin (0.010 mM). Final reaction concentrations are shown.

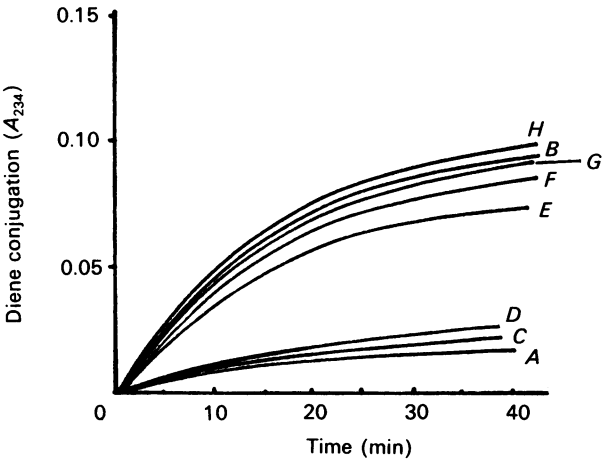

Fig. 2. Haemin-stimulated peroxidation of fatty acid micelles measured as diene conjugation

Curve *A*, fatty acid micelles at pH 7.4 incubated for the time indicated at 25 °C; curve *B*, control reaction containing fatty acid micelles at pH 7.4 and haemin (0.012 mM); curve *C*, control reaction *B* with butylated hydroxytoluene (0.07 mM); curve *D*, control reaction *B* with apo-haemopexin (0.013 mM); curve *E*, control reaction *B* with albumin (0.012 mM); curve *F*, control reaction *B* with haptoglobin (0.011 mM); curve *G*, control reaction *B* with desferrioxamine (0.153 mM); curve *H*, control reaction *B* with apo-transferrin (0.010 mM). Final reaction concentrations are shown.

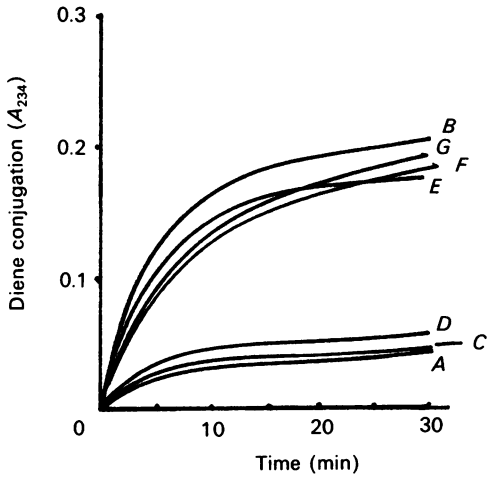

Fig. 3. Haemoglobin-stimulated lipid peroxidation measured as diene conjugation

Curve *A*, fatty acid micelles at pH 6.4 incubated for the time indicated at 25 °C; curve *B*, control reaction containing fatty acid micelles at pH 6.4, desferrioxamine (0.076 mM) and methaemoglobin (0.001 mM); curve *C*, control reaction *B* with haptoglobin (0.001 mM); curve *D*, control reaction *B* with butylated hydroxytoluene (0.03 mM); curve *E*, control reaction *B* with apo-haemopexin (0.001 mM); curve *F*, control reaction *B* with apo-transferrin (0.001 mM); curve *G*, control reaction *B* with albumin (0.001 mM). Final reaction concentrations are shown.

Table 2. Effect of haemopexin on iron-salt-stimulated phospholipid peroxidation

Final reaction concentrations are shown. The reaction was carried out in 100 mM-phosphate buffer, pH 7.4, containing 0.7 mg of phospholipid/ml. Peroxidation was started by adding 0.07 mM-ferrous salt and incubating at 37 °C for 2 h. The results shown are as relative fluorescence units (RFI) units. Abbreviation: BHT, butylated hydroxytoluene.

	Peroxidation	
	RFI units ($\lambda_{exc.}$ 360 nm, $\lambda_{em.}$ 430 nm)	Inhibition (%)
1. Blank (no Fe^{2+} added)	10 (value subtracted)	
2. Control	9	
Reaction 2+desferrioxamine (0.07 mM)	1	89
Reaction 2+propyl gallate (0.07 mM)	0	100
Reaction 2+BHT (0.07 mM)	0	100
Reaction 2+albumin (0.01 mM)	8	11
Reaction 2+haemopexin (0.012 mM)	8	11

95%) O_2 uptake, and albumin (at 12 μM) inhibits by some 50% (Fig. 1).

Haemin stimulates lipid peroxidation (measured as the formation of conjugated dienes, A_{234}), and this is not inhibited by the metal-binding agents, desferrioxamine and apo-transferrin, nor by the haemoglobin-binding protein haptoglobin (Fig. 2). However, the rate of lipid peroxidation is substantially inhibited by the haem-binding protein haemopexin and by the chain-breaking antioxidant butylated hydroxytoluene (Fig. 2). As before (in Fig. 1 above), albumin, which also binds haemin, only partly inhibits this peroxidation, consistent with its considerably lower affinity than haemopexin for haem (Beaven *et al.*, 1974). Binding was also seen with bovine albumin. Haptoglobin, but not haemopexin, was able to inhibit haemoglobin-stimulated peroxidation (Fig. 3). With the use of an entirely different measurement of haemin-stimulated lipid peroxidation, micelles were subjected to the thiobarbituric acid reaction, and the pink chromogen formed was measured by visible-region-absorption and fluorescence spectrophotometry. The results obtained in the presence of the iron-chelator desferrioxamine confirm the results from experiments based on O_2 uptake and diene conjugation, i.e. haemo-

Table 1. Inhibition of haemin-stimulated linolenic acid peroxidation by metal-binding proteins in the presence of desferrioxamine

Final reaction concentrations are shown. Reactions were carried out in 70 mM-phosphate buffer, pH 7.4, containing 0.2 ml of linolenic acid micelles and 0.14 mM-desferrioxamine. Peroxidation was stimulated by 0.011 mM-haemin. Relative fluorescence intensity (RFI) units were measured against a standard of rhodamine B. Abbreviation: BHT, butylated hydroxytoluene.

	Thiobarbituric acid-reactivity (30 min, 37 °C)			
	A_{532}	Inhibition (%)	RFI units ($\lambda_{exc.}$ 532 nm, $\lambda_{em.}$ 553 nm)	Inhibition (%)
1. Blank (no haemin)	0.002	(Value subtracted)	6	(Value subtracted)
2. Control	0.024		50.0	
Reaction 2+albumin (0.010 mM)	0.016	33	31.5	37
Reaction 2+haemopexin (0.012 mM)	0.007	71	7.5	85
Reaction 2+transferrin (0.010 mM)	0.025	0	49.0	2
Reaction 2+haptoglobin (0.011 mM)	0.030	0	56.0	0
Reaction 2+BHT (0.07 mM)	0.002	92	1.0	98

pexin and butylated hydroxytoluene strongly inhibit peroxidation, albumin shows an intermediate inhibition, and transferrin and haptoglobin are without effect (Table 1). As an additional control to address any non-specific protein effects of haemopexin or any non-specific iron binding by haemopexin, lipid peroxidation of phospholipid liposomes was stimulated by the addition of an iron salt and the increase in fluorescence was measured at 430 nm (Table 2). Only the iron-chelator desferrioxamine and the antioxidant scavengers butylated hydroxytoluene and propyl gallate substantially inhibit fluorescence formation, indicating that haemopexin inhibits lipid peroxidation *in vitro* by binding the pro-oxidant haem.

DISCUSSION

When redox-active iron becomes available in the body owing to excessive intake, tissue damage or oxidant stress, it can stimulate the formation of reactive oxygen species (for reviews see Gutteridge *et al.*, 1986; Halliwell & Gutteridge, 1986*a*). The hydroxyl radical ($^{\bullet}OH$), or species with similar reactivity, can be formed when suitable iron catalysts react with H_2O_2, and highly reactive $^{\bullet}OH$ can damage most biological molecules at the site where the metal complex is located.

When a lipid molecule is attacked in this way, it can undergo an oxidative chain reaction known as lipid peroxidation. Of equal biological importance is the possibility that malplaced iron complexes can decompose lipid peroxides (LOOH) to produce chain-propagating radicals such as alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$), which continue the process of lipid peroxidation. Lipid peroxides can be formed *in vivo* by both enzymic and non-enzymic reactions.

In the present paper we show that ferric protoporphyrin, as haemin, is one such iron complex that can stimulate lipid peroxidation by decomposing lipid peroxides. Lipid peroxidation stimulated by haemin was independent of any adventitious iron salts, since the reaction was not inhibited by desferrioxamine or apo-transferrin. Apo-transferrin and desferrioxamine do, however, inhibit lipid peroxidation stimulated by an iron salt when desferrioxamine is in molar excess and transferrin has less than 2 mol of bound iron/mol of protein (Gutteridge *et al.*, 1981). Apo-haemopexin at physiological concentrations strongly inhibited lipid peroxidation (measured by three different techniques) stimulated by the addition of haemin, whereas the haemoglobin-binding protein haptoglobin did not. The converse was found for apo-haemopexin and haptoglobin when peroxidation was stimulated by haemoglobin. Albumin was included in these studies as it is known to bind haem, forming methaemalbumin (Fairley, 1938). When present in equimolar amounts to haemin, albumin was shown here to inhibit haemin-stimulated lipid peroxidation partly, and must, therefore, be ascribed some antioxidant role in protection against radical formation by the iron complex haemin. Nevertheless, there is no evidence for a haem transport role for albumin. Rather, albumin functions in this respect as a temporary storage depot before transfer of the haem to haemopexin (Morgan *et al.*, 1976). As expected, all the peroxidation reactions were strongly inhibited by radical-scavenging antioxidants such as butylated hydroxytoluene and propyl gallate.

Inside cells antioxidant defences are primarily concerned with removing reactive oxygen species before they can react with the essential pool of low-molecular-mass iron. For this purpose the cell relies on the superoxide dismutase enzymes, glutathione peroxidase (selenium enzyme) and catalase. However, in extracellular fluids these enzymes do not exist, except perhaps in trace amounts as extracellular glycosylated forms (Marklund *et al.*, 1982; Takahashi *et al.*, 1987). Extracellular fluids appear to rely on entirely different antioxidant mechanisms from those found inside the cell (Gutteridge, 1982; Halliwell & Gutteridge, 1986*b*), limiting radical reactions by converting pro-oxidant forms of iron and copper into less or non-reactive forms. Many of these extracellular antioxidants are acute-phase reactive proteins responding to tissue damage, a time when oxygen-radical reactions are most likely to occur. Thus the ferroxidase activity of caeruloplasmin (Gutteridge & Stocks, 1981) and the iron-binding properties of transferrin and lactoferrin inhibit many iron-catalysed oxgen-radical reactions (Gutteridge *et al.*, 1981). Since haemoglobin and other haem–proteins can readily release catalytic iron in the presence of peroxides (Gutteridge, 1985, 1986), it was recently proposed that the haemoglobin- and haem-transporting proteins, haptoglobin and haemopexin respectively, would play an important role in protecting extracellular fluids against radical reactions (Gutteridge, 1987). Previous studies have confirmed an antioxidant role for the haptoglobins when lipid peroxidation is stimulated by haemoglobin (Sadrzadeh *et al.*, 1984; Gutteridge, 1987). In the present paper we show a similar role for the protein haemopexin when peroxidation is stimulated *in vitro* by haemin. These findings strengthen our suggestions that the essential transport and conservation of body iron stores are closely linked to antioxidant protection against pro-oxidant forms of iron, and that extracellular antioxidant defences depend on the removal or inactivation of reactive metal complexes.

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