Effect of ferritin-containing fractions with different iron loading on lipid peroxidation

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Ferritin-containing fractions with different degrees of iron loading were prepared. All ferritin fractions stimulated the peroxidation of bovine brain phospholipid liposomes, as measured by the formation of thiobarbituric acid-reactive material. This stimulation was increased in the presence of ascorbate. Iron salts of equivalent concentration to those of the ferritin fractions were more stimulatory to lipid peroxidation at the higher iron concentrations. None of the fractions inhibited ascorbate-dependent peroxidation in the presence of added iron salts.

Ferritin is the major iron-storage protein in animal tissues. In humans about 25% of the total body iron is contained in this protein, which is present mainly in liver, spleen and bone marrow. Ferritin, usually of low iron content, is also found in serum.

The ferritin molecule consists of a spherical protein shell that encloses an iron 'core', a complex polymer of iron(III) in the form of hydrated oxide and phosphate. Up to 4500 atoms of iron(III) per molecule have been found (Clegg *et al.*, 1980). Elevated serum ferritin levels have been observed in certain cancers (Koligo *et al.*, 1976; Hann *et al.*, 1980) and in inflammatory joint disease (Blake & Bacon, 1981) and there have been suggestions that increased ferritin synthesis and/or redistribution of body iron play a part in the pathogenesis of such diseases, perhaps by promoting such processes as the peroxidation of membrane lipids and formation of the highly-damaging hydroxyl radical ($\text{OH}\cdot$) from superoxide ($\text{O}_2^{\cdot-}$) and H_2O_2 generated by activated phagocytes (Blake *et al.*, 1981). Indeed, there have been reports of the ability of ferritin (iron loading not specified) to stimulate ascorbate-dependent peroxidation of lipids (Wills, 1966) and chromosome damage (Whiting *et al.*, 1981).

However, the degree of iron loading of ferritin varies between different tissues and changes in disease states (Aisen & Listowsky, 1980). In the

present paper we report the results of studies on the ability of several ferritin fractions of defined iron content to stimulate the peroxidation of membrane lipids.

Materials and methods

Reagents

Diethylenetriaminepenta-acetic acid (DETAPAC) was from Sigma, desferrioxamine ('Desferal') was from CIBA-Geigy and horse spleen ferritin (Cd-free) from Boehringer. All other reagents were of the highest quality available from BDH Chemicals.

Ferritin fractions were prepared by fractional centrifugation. Initially 10 ml of a solution containing approx. 10 mg of ferritin per ml in water was centrifuged for 90 min at 130 000 *g*. Five, approx. 1.5 ml, supernatant fractions were collected and the pellet and lower 2 ml of supernatant were made up to the initial volume, redissolved and re-centrifuged for 60 min at 100 000 *g* and 20 min at 80 000 *g*. The fractions were then kept as 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$ -precipitated material. Before use, $(\text{NH}_4)_2\text{SO}_4$ was removed by dialysis for 40 h against three changes of water at 4°C and the protein content of each fraction was then adjusted to 0.403 mg/ml (as measured by the Lowry method, using a ferritin standard assayed by amino acid analysis) with Chelex-treated water. Iron content was determined by the 2,2-bipyridyl method of Drysdale & Munro (1965).

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Assay of lipid peroxidation

Bovine brain phospholipid liposome suspension (5mg/ml in 0.15M-NaCl) was prepared as described by Gutteridge (1977); 0.2ml of the suspension was included in a reaction mixture of final volume 0.54 ml containing the following reagents at the final concentrations stated: 37mM-KH₂PO₄ buffer adjusted to pH7.4 with KOH, 0.1ml of the ferritin fraction and, where indicated, ascorbic acid (29.6 μ M). After incubation at 37°C for 2h, 0.5 ml of a 1% (w/v) solution of thiobarbituric acid in 0.05M-NaOH was added, together with 0.5ml of 25% (v/v) HCl. After heating at 100°C for 15 min, 1.5ml of butan-1-ol was added and the reaction mixture was vortex-mixed for 1min and then centrifuged at 2000 rev./min for 5 min. The absorbance of the upper organic layer was then measured at 532nm against a blank. Duplicate results agreed within $\pm 5\%$. The incubation time of 2h was chosen to give a representation of all phases of the peroxidation process (Gutteridge, 1977).

Results

Fig. 1 shows the effects of a series of horse spleen ferritin fractions with defined iron contents on the rate of peroxidation of bovine brain phospholipid liposomes. All fractions stimulated peroxidation, as measured by formation of thiobarbituric acid-reactive material, even in the absence of a reducing agent. Stimulatory activity increased with iron content. Stimulation of peroxidation was not due to traces of non-protein-bound iron in the fractions, since they had been extensively dialysed. Further, the stimulatory effect, although reduced, was not completely overcome by addition of the chelating agents desferrioxamine and DETAPAC, which are known to inhibit lipid peroxidation induced by free iron salts (Wills, 1969; Gutteridge *et al.*, 1979). Table 1 shows the effect of a range of chelator concentrations on a ferritin fraction containing 4150mol of Fe/mol of ferritin. Finally, after incubation of a ferritin fraction containing 4230 mol of Fe/mol of ferritin in phosphate buffer for 2h in either the presence or the absence of liposomes, no free iron could be detected in the reaction mixture by the bleomycin method (Gutteridge *et al.*, 1981a,b).

Consistent with previous results (Wills, 1966), the ferritin fractions also stimulated the rate of peroxidation in the presence of ascorbate, the effect again increasing with their iron contents (Fig. 1). Addition of desferrioxamine or DETAPAC decreased the rates of ferritin-induced peroxidation compared with those seen in the absence of ascorbate (Table 1). Application of the bleomycin assay for free iron to a reaction mixture containing ferritin (4230 mol of Fe/mol of ferritin), liposomes and ascorbate gave a free iron content of 7 μ mol/litre after a 2h incubation.

Fig. 1. Effect of ferritin fractions of different iron loading on lipid peroxidation

The thiobarbituric acid reactivity was measured as A_{532} after 2h incubation as described in the Materials and methods section. Each reaction mixture contained equal amounts of ferritin protein of the iron-loading present. ■, Ascorbate present; ▲, ascorbate absent.

Table 1. Effect of chelating agents on ferritin-induced peroxidation of phospholipid liposomes

The concentrations of chelating agents stated were the final concentrations in each reaction mixture. Ferritin iron loading was 4150 mol of Fe/mol of ferritin. Results are means of two separate assays.

Chelating agent concn. (mM)	Rate of peroxidation ($\Delta A_{532}/2h$)	
	-Ascorbate	+Ascorbate
Desferrioxamine		
None	0.091	0.192
0.037	0.070	0.078
0.048	0.050	0.050
0.161	0.045	0.050
0.484	0.042	0.040
DETAPAC		
None	0.091	0.192
0.037	0.063	0.073
0.048	0.042	0.050
0.161	0.040	0.050
0.484	0.040	0.040

Poorly iron-loaded ferritin fractions (500–900 mol of Fe/mol of ferritin) did not inhibit the peroxidation of membrane lipids induced by addition of ferric salts (6 μ M) and ascorbate (29.6 μ M) (results

Table 2. Dilution of ferritin samples to constant iron value

The experiment was carried out as described in the Materials and methods section. Ferritin samples were diluted with Chelex-treated water. Results are means of three separate assays.

Ferritin fraction code	Fe content (μg)	Protein (μg)	Lipid peroxidation ($\Delta A_{532}/2\text{h}$)	
			–Ascorbate	+ Ascorbate
C	20.7	40.3	0.080	0.177
E	20.1	40.3	0.079	0.172
G	9.8	40.3	0.059	0.157
H	4.6	40.3	0.048	0.132
Diluted samples				
C diluted to Fe value of H	4.6	9.0	0.047	0.114
E diluted to Fe value of G	9.8	19.7	0.058	0.132

Fig. 2. Effect on lipid peroxidation of iron in ferritin compared with an equal concentration of iron salt. Experiments were carried out as described in the Materials and methods section, with a final ascorbate concentration of $29.6\text{ }\mu\text{M}$ where indicated. $\blacktriangle$, Ferritin, with no ascorbate present; $\triangle$, FeCl_3 , with no ascorbate present; $\blacksquare$, ferritin plus ascorbate; $\square$, FeCl_3 plus ascorbate.

Discussion

Unlike lactoferrin and transferrin (Gutteridge *et al.*, 1981b), iron-poor ferritin fractions do not inhibit the peroxidation of membrane lipids induced by iron salts, presumably owing to their relatively low affinity for ferric iron (Treffry & Harrison, 1979). Indeed, iron-loaded fractions themselves accelerated the rate of peroxidation. Stimulation of lipid peroxidation by Fe^{3+} salts is probably due to their ability to degrade traces of lipid peroxides already present in the liposomes, to give peroxy radicals, which produce more initiation of the chain reaction of lipid peroxidation (Barber & Bernheim, 1967). It seems likely that ferritin-bound iron may act in the same way as, although less effectively than, an equivalent amount of 'free' iron salt (Fig. 2) at high iron concentrations.

The action of ferritin fractions in stimulating peroxidation in the absence of ascorbate was not due to release of inorganic iron from them, since none could be detected by the bleomycin assay (Gutteridge *et al.*, 1981a). The iron-chelating agents desferrioxamine and DETAPAC only inhibited partially, perhaps by removing iron from the protein or else affecting peroxidation in some other way (Table 1).

In the presence of ascorbate, the rate of lipid peroxidation in the presence of the ferritin fractions was greater than in the absence of ascorbate. Ascorbate did cause release of iron, as measured by the bleomycin method. Table 2 shows that even low concentrations of DETAPAC or desferrioxamine reduced the rates of peroxidation to those seen in the absence of ascorbate. Hence in the presence of ascorbate, ferritin fractions stimulate peroxidation both directly and also by a release of free iron salts that can readily be chelated.

The iron content of the ferritin fractions appears to play the major part in determining the activity of the ferritin towards lipid peroxidation. This is emphasized not only by the increasing activity at constant protein or fractions of increasing iron content, but also by the finding that at constant iron,

not shown). FeCl_3 at an iron concentration equivalent to that present in each ferritin fraction was more stimulatory to lipid peroxidation at higher concentrations than was heavily iron-loaded ferritin (Fig. 2), both in the presence and absence of ascorbate. If the amounts of different ferritin fractions added to the incubation medium were adjusted to give the same iron content, essentially the same stimulatory effect on peroxidation was observed. Table 2 shows a typical example.

different fractions showed substantially the same effect (Table 2).

It therefore seems possible that the changes in the distribution of body iron and ferritin during inflammatory joint disease (Blake *et al.*, 1981), coupled with an increased generation of oxygen radical species capable of initiating lipid peroxidation by phagocytic cells (Halliwell, 1982), may play some part in inflammation-induced tissue damage.

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