The role of iron in ferritin- and haemosiderin-mediated lipid peroxidation in liposomes

Martin J. O'CONNELL,*†‡ Roberta J. WARD,* Harold BAUM† and Timothy J. PETERS*

*Division of Clinical Cell Biology, M.R.C. Clinical Research Centre, Harrow, Middx. HA1 3UJ, and

†Department of Biochemistry, Chelsea College (University of London),
Manresa Road, London SW3 6LX, U.K.

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Ferritin and haemosiderin were shown, by the measurement of malondialdehyde production and loss of polyunsaturated fatty acids, to stimulate lipid peroxidation in liposomes. At pH 7.4 ascorbate was additionally required to achieve peroxidation; however, peroxidation occurred at pH 4.5 in the presence of iron-proteins alone. The damage was completely inhibited by the incorporation of chain-breaking antioxidants (α -tocopherol and butylated hydroxytoluene) into the liposomes. Metal chelators (desferrioxamine and EDTA) also completely inhibited lipid peroxidation. These and further results indicate that, at pH 4.5, even in the absence of a reducing agent, iron is released from haemosiderin and can mediate oxidative damage to a lipid membrane.

Iron overload is well recognized to cause damage to the liver and other organs. Ferritin has been shown to be the major iron-protein in normal liver; however, in iron overload haemosiderin is predominant (Selden *et al.*, 1980). Haemosiderin is thought to accumulate in lysosomes, and a close positive correlation has been shown between enhanced lysosomal fragility and haemosiderin content of the tissue (Seymour & Peters, 1979). It has been suggested that the toxicity of iron could be mediated by haemosiderin dependent damage to the lysosomal membrane, possibly by a lipid-peroxidation mechanism (Selden *et al.*, 1980).

Ferritin has been reported to stimulate the peroxidation of lipids, at pH 7.4, in the presence of ascorbate (Wills, 1966), and also to a limited extent in the absence of ascorbate (Gutteridge *et al.*, 1983). In the present paper we present detailed comparisons of the ability of ferritin and haemosiderin to stimulate lipid peroxidation in a model membrane both at pH 7.4 and at pH 4.5. The latter is considered to be a model for intralysosomal conditions.

Materials and methods

Ferritin from horse spleen, phosphatidylcholine (99% pure, in chloroform) from egg yolk, α -

tocopherol from vegetable oil and butylated hydroxytoluene were supplied by Sigma. All other chemicals were of AnalaR grade and supplied by BDH Chemicals.

Human haemosiderin (Weir *et al.*, 1984) and ferritin (Huebers *et al.*, 1974) were isolated from iron-overloaded thalassaemic human spleens. Haemosiderin was freeze-dried and stored desiccated at room temperature. On the day of use, solutions (approx. 10 mM-iron) were prepared in 20 mM-tetramethylammonium hydroxide solution (Weir *et al.*, 1984).

Small unilamellar liposomes were prepared essentially by the method of Gregoriadis & Ryman (1972). Chloroform solutions of phosphatidylcholine were evaporated to dryness with a rotary evaporator to form a thin film of lipid. Where indicated, chloroform solutions of α -tocopherol or butylated hydroxytoluene were added before evaporation. The lipid was resuspended in the appropriate aqueous buffer, with the aid of a few glass beads, to a concentration of 10 or 20 mg/ml. The liposomes were allowed to equilibrate and swell under an atmosphere of N₂ in the dark for 1 h. The suspension was then sonicated at 20 μ m peak-to-peak, with an MSE 150 W Mk II sonicator with an exponential probe, on ice for ten periods of 60 s with intervals of 30 s. Incubations were at 37°C, aerobic, continuously shaken, and in 50 mM-Tris/HCl buffer, pH 7.4, or 50 mM-sodium acetate/acetic acid

‡ To whom reprint requests should be addressed.

buffer, pH 4.5. Although haemosiderin alone, at pH 4.5, will precipitate within a few minutes, a stable suspension was formed in the presence of liposomes.

Malondialdehyde was determined by reaction with thiobarbituric acid (Slater & Sawyer, 1971). Amounts of malondialdehyde were calculated from the A_{535} ($\epsilon 1.5 \times 10^5 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Samples for g.l.c. analysis of fatty acid composition were extracted with 4 vol. of chloroform/methanol (2:1, v/v). Methyl esters of the fatty acids were prepared and analysed with a Pye-Unicam 204 instrument (Cairns & Peters, 1983). Peak areas representing arachidonate, linoleate and stearate were related to that of palmitate (e.g. linoleate/palmitate). The relative peak areas in incubations containing iron-proteins are reported as percentages of those in control incubations. Iron concentrations were determined with the use of a Perkin-Elmer 2380 electrothermal atomic absorption spectrophotometer.

Results

Liposomes were peroxidized (as shown by malondialdehyde increase) when incubated with human or horse ferritin and ascorbate at pH 7.4 (Fig. 1). Very limited peroxidation was detected at pH 7.4 in the absence of ascorbate.

Human haemosiderin stimulated malondialdehyde increases at pH 7.4 in the presence of ascorbate (Fig. 1), although to an extent approx. 8-fold less than that with ferritin at equivalent iron concentrations. No lipid peroxidation was detectable in the absence of ascorbate.

To model intralysosomal conditions, where haemosiderin accumulates *in vivo*, horse spleen ferritin and human haemosiderin were incubated with liposomes at pH 4.5. At this pH both iron-proteins stimulated malondialdehyde increase without the addition of ascorbate (Fig. 2). Again the extent of peroxidation was greater with ferritin than with haemosiderin at equivalent iron concentrations.

Membrane lipid peroxidation was confirmed by the determination of changes in fatty acid composition (Fig. 3). In all cases where malondialdehyde increase had been detected, the amounts of arachidonate and linoleate decreased relative to those in control experiments. Again changes were found at pH 7.4 in the presence of ferritin (Fig. 3a) or haemosiderin (Fig. 3b) and ascorbate (but not in the presence of either iron-protein or ascorbate alone) and at pH 4.5 in the presence of ferritin (Fig. 3c) or haemosiderin (Fig. 3d) without the addition of ascorbate. No changes were detected in the absence of the iron-proteins. As with the malondialdehyde increase, the changes in fatty

Fig. 1. Ferritin- and haemosiderin-stimulated lipid peroxidation at pH 7.4

The increase in malondialdehyde on incubation of liposomes (2 mg/ml) in pH 7.4 buffer, with the following additions, is shown: 500 μM -ascorbate and 500 μM -(Fe) ferritin (human, $\blacktriangle$; horse, $\bullet$), 500 μM -(Fe) ferritin (human, $\triangle$; horse, $\circ$), 500 μM -ascorbate ($\blacktriangle$), 500 μM -ascorbate and 500 μM -(Fe) human haemosiderin ($\blacksquare$), or 500 μM -human haemosiderin ($\square$). Error bars show standard deviations ($n = 3$).

acid composition were greater with ferritin than with haemosiderin.

Since haemosiderin was found, at pH 4.5, to stimulate lipid peroxidation in the absence of a reducing agent, it is quite possible that this iron-protein mediates oxidative damage in lysosomal membranes of iron-overloaded tissue. Further investigation into the mechanism of damage was

therefore centred on this system. The malondialdehyde increase caused by 500 μM -(Fe) haemosiderin at pH 4.5 could be completely inhibited by the incorporation of α -tocopherol or butylated hydroxytoluene into the liposome membrane. The α -tocopherol content of mammalian membranes is approx. 0.5 mmol/mol of lipid (Gruger & Tappel, 1971), and this value was found to give partial protection (17%) of the liposomes. Butylated hydroxytoluene at 0.5 mmol/mol of lipid gave 63% inhibition of lipid peroxidation, and at 10 mmol/mol of lipid the inhibition was 94% and 92% for the two antioxidants respectively.

The malondialdehyde formed with 500 μM -(Fe) haemosiderin at pH 4.5 was completely inhibited by 50 μM -desferrioxamine and partially inhibited by concentrations as low as 5 μM (Fig. 4). This suggests that less than 10% of the haemosiderin iron is involved in stimulating lipid peroxidation. Similar results were found with EDTA inhibition of lipid peroxidation. Inhibition by relatively low concentrations of metal-ion chelators suggests that part of the haemosiderin iron is solubilized in the acetate buffer. This was confirmed by incubating 500 μM -(Fe) haemosiderin in pH 4.5 buffer for up to 5 h and then centrifuging at 20 000 g for 10 min to pellet the residual haemosiderin. Iron was detected in the supernatant (Fig. 5), increasing in concentration with incubation time to a maximum of 8 μM . When the supernatant obtained after a 2 h

Fig. 2. Ferritin- and haemosiderin-stimulated lipid peroxidation at pH 4.5

The increase in malondialdehyde on incubation of liposomes (2 mg/ml) in pH 4.5 buffer, with the following additions, is shown: 100 μM -(Fe) ferritin (○), 500 μM -(Fe) haemosiderin (●), 100 μM -(Fe) haemosiderin (■), or no additions (□). Error bars show standard deviations ($n = 3$).

Fig. 3. Ferritin- and haemosiderin-stimulated changes in fatty acid composition

Changes in stearate (■), linoleate (●) and arachidonate (▲), relative to controls (iron-protein free), on incubation of liposomes (2 mg/ml) with the following additions are shown: (a) 500 μM -ferritin, 500 μM -ascorbate and pH 7.4 buffer; (b) 500 μM -haemosiderin, 500 μM -ascorbate and pH 7.4 buffer; (c) 500 μM -ferritin and pH 4.5 buffer; (d) 500 μM -haemosiderin and pH 4.5 buffer.

Fig. 4. Desferrioxamine inhibition of haemosiderin-stimulated lipid peroxidation

Inhibition of malondialdehyde increase on incubation of liposomes (2 mg/ml), in pH 4.5 buffer, in the presence of 500 μM -(Fe) haemosiderin and the following, is shown: (●) no further additions; (○) 5 μM -, (■) 10 μM -, (□) 20 μM - and (▲) 50 μM -desferrioxamine. The error bars show standard deviations.

Fig. 5. Iron release from haemosiderin

Release of iron into the supernatant after the incubation of 500 μM -(Fe) haemosiderin in pH 4.5 buffer, and centrifugation (20000g for 10 min), is shown. The error bars show standard deviations.

incubation of haemosiderin in pH 4.5 buffer was incubated with liposomes, it was found to stimulate malondialdehyde production comparable with that in the presence of 500 μM -(Fe) haemosiderin.

Discussion

Ferritin and haemosiderin at pH 7.4 showed very little stimulation of lipid peroxidation in egg phosphatidylcholine liposomes, in the absence of

ascorbate. Previous work (Gutteridge *et al.*, 1983) has shown that ferritin-dependent peroxidation, with bovine brain phospholipid liposomes, occurs to a relatively limited extent at pH 7.4 in the absence of ascorbate. The iron cores within ferritin and haemosiderin may not therefore be expected to cause extensive direct damage to cytoplasmic membranes. This is supported by the finding that, where extensive lipid peroxidation did occur, it could be completely inhibited by metal-ion chelators. Both iron-proteins were found to stimulate peroxidation at pH 7.4, in the presence of ascorbate. This agrees with previous work (Wills, 1966; Gutteridge *et al.*, 1983; O'Connell *et al.*, 1985). Under these conditions iron would be expected to be mobilized as an Fe^{2+} -ascorbate complex (Sirivech *et al.*, 1974), and lipid peroxidation could be induced by oxygen radicals generated in Fe^{2+} -dependent Fenton-type reactions (Halliwell, 1976). There is evidence that ascorbate is involved in the mobilization of storage iron *in vivo* (Lipschitz *et al.*, 1971; Roeser *et al.*, 1983). Furthermore the administration of ascorbic acid to patients with iron-overload has proved toxic unless given in conjunction with desferrioxamine (Propper & Nathan, 1977).

At pH 4.5, lipid peroxidation stimulated by ferritin and haemosiderin in the absence of any reducing agent must be mediated by ferric iron. The nature of the iron that is released from haemosiderin into acetate buffer is unclear, but monomeric ferric acetate and oligomeric ferric oxide seem to be two possibilities. Ferric ions in solution have been shown to stimulate lipid peroxidation by catalysing the decomposition of pre-existing lipid hydroperoxide to lipid peroxyl radicals (O'Brien, 1969; O'Connell & Garner, 1983). The inhibition of malondialdehyde production by the chain-breaking antioxidants α -tocopherol and butylated hydroxytoluene is evidence that the peroxyl radicals stimulate chains of propagation reactions within the model membrane. It can also be concluded that naturally occurring concentrations of α -tocopherol would only partially protect a lipid membrane against such damage.

The finding that ferritin is more effective than haemosiderin in stimulating lipid peroxidation suggests that the conversion of ferritin into haemosiderin that occurs in iron-overload (Selden *et al.*, 1980) may be a mechanism for cytoprotection against iron toxicity. This difference between ferritin and haemosiderin may be due to differences in the rate at which iron can be mobilized from the two iron-proteins. We have found that at pH 7.4 iron is released from ferritin to 1,10-phenanthroline (method of Hoy *et al.*, 1973) at more than double the rate of release from haemosiderin. In iron-overload syndromes the

quantity of haemosiderin deposited is such that even a relatively slow rate of iron release *in vivo* may be important in iron toxicity. Certainly iron release would be favoured by the acidic intralysosomal conditions, and this could lead to membrane lipid peroxidation. Indeed, lysosomes have been shown to be disrupted by free-radical-mediated lipid peroxidation *in vitro* (Fong *et al.*, 1973). Further studies are required to determine whether similar effects can be demonstrated in lysosomes *in vivo* in experimental iron overload.

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