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Low density lipoprotein oxidation by ferritin at lysosomal pH

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Abstract

Oxidation of low density lipoprotein (LDL) has been proposed to be involved in the pathogenesis of atherosclerosis. We have previously shown that LDL can be oxidised by iron in lysosomes. As the iron-storage protein ferritin might enter lysosomes by autophagy, we have investigated the ability of ferritin to catalyse LDL oxidation at lysosomal pH. LDL was incubated with ferritin at 37 °C and pH 4.5 and its oxidation monitored spectrophotometrically at 234 nm by the formation of conjugated dienes and by measuring oxidised lipids by HPLC or a tri-iodide assay. Iron released from ferritin was measured using the ferrous iron chelator bathophenanthroline and by ultrafiltration followed by atomic absorption spectroscopy. LDL was oxidised effectively by ferritin (0.05 - 0.2 µM). The oxidation at lysosomal pH (pH 4.5) was much faster than at pH 7.4. Ferritin increased cholesteryl linoleate hydroperoxide, total lipid hydroperoxides and 7-ketocholesterol. Iron was released from ferritin at acidic pH. The iron chelators, diethylenetriaminepentaacetate and EDTA, and antioxidant N,N'-diphenyl-p-phenylenediamine inhibited the oxidation considerably, but not entirely. The antioxidant tempol did not inhibit the initial oxidation of LDL, but inhibited its later oxidation. Cysteamine, a lysosomotropic antioxidant, inhibited the initial oxidation of LDL in a concentration-dependent manner, however, the lower concentrations exhibited a pro-oxidant effect at later times, which was diminished and then abolished as the concentration increased. These results suggest that ferritin might play a role in lysosomal LDL oxidation and that antioxidants that accumulate in lysosomes might be a novel therapy for atherosclerosis.

Keywords: Atherosclerosis, Lysosomes, Ferritin, Iron, Cysteamine, LDL oxidation

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Abbreviations: BHT, butylated hydroxytoluene; CLOOH, cholesteryl linoleate hydroperoxide; DPPD, N, N'-diphenyl-p-phenylenediamine; DTPA, diethylenetriaminepentaacetic acid; LDL, low density lipoprotein; MOPS, 3-N-morpholinopropanesulphonic acid

1. Introduction

The oxidation of low density lipoprotein (LDL) has been proposed to occur in the extracellular space of the arterial wall and lead to the formation of foam cells and atherosclerosis (Steinberg, 2009). The oxidation of LDL by cells requires the presence of micromolar concentrations of the transition metals copper or iron in the medium (Steinbrecher et al., 1984, Leake and Rankin, 1990). Free copper or iron are not readily available in the plasma or interstitial fluid because they exist in a tightly bound form. A number of mechanisms have been proposed to be involved in the oxidation of LDL *in vivo*, but at present, there is no consensus on the predominant mechanism by which LDL is modified *in vivo*. Cultured macrophages have been shown, however, to take up aggregated or acetylated LDL quickly and oxidise it in lysosomes (Wen and Leake, 2007). Cholesterol crystals derived from oxidised LDL in lysosomes have been reported to rupture these organelles in macrophages and activate the NLRP3 inflammasome (Duewell et al., 2010). This might be important as atherosclerosis is a chronic inflammatory disease and can be treated in patients with antibodies against interleukin 1- β (Ridker et al., 2017). Foam cells in human atherosclerotic lesions contain ferritin and lysosomal iron (Yuan et al., 1996, Lee et al., 1998, Yuan, 1999). Redox-active iron exists in lysosomes, due to degradation of iron-containing proteins delivered by endocytosis or autophagy, such as ferritin.

The iron hypothesis of atherosclerosis was proposed by Sullivan in 1981 to explain why men get more coronary heart disease than pre-menopausal women because of the higher body iron levels in men (Sullivan, 1981). There are small labile iron pools in cells in the cytosol, mitochondria and lysosomes (Lv and Shang, 2018), with the lysosomal one being of the highest concentration (about 16 μ M) in rat liver endothelial cells (Petrat et al., 2001). Iron is delivered to lysosomes by the autophagocytosis of ferritin or organelles, e.g. mitochondria, and might be released from them by divalent metal transporter 1 (Lv and Shang, 2018). Free iron is toxic to the body due to its redox activity and is stored in a tightly bound form that is safe, for instance, in ferritin. Ferritin is made up of 24 polypeptide chains with light (L) subunits and heavy (H) subunits with molecular weights of 19 kDa and 21 kDa respectively (Theil, 1987, Arosio et al., 2017). Elevated levels of ferritin have been associated with myocardial infarction (Salonen et al., 1992) and there is an increased level of ferritin in atherosclerotic arteries (You et al., 2003). Ferritin and mitochondrial metalloproteins delivered to lysosomes by autophagy are degraded to form low molecular weight iron (Kurz et al., 2007, Lv and Shang, 2018). Some studies have shown that iron from ferritin can be involved in oxidative damage (Reif, 1992). Iron released from transferrin at acidic pH has been shown to oxidise LDL *in vitro* (Lamb and Leake, 1994). We now report the ability of ferritin to oxidise LDL effectively at pH 4.5, the pH of lysosomes (Mindell, 2012), and the effect of antioxidants on this oxidation.

N,N'-diphenyl-p-phenylenediamine (DPPD), a hydrophobic antioxidant is a potent lipid peroxy radical scavenger (Tang et al., 2000) and has been shown to inhibit oxidation of LDL (Sparrow et al., 1992, Tangirala et al., 1995, Ahmad and Leake, 2018) and slow down the progression of atherosclerosis (Sparrow et al., 1992, Tangirala et al., 1995). Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) has a reducible nitroxide group and is a superoxide dismutase mimetic and can scavenge many reactive oxygen species (Wilcox and Pearlman, 2008).

Our hypothesis is that the major iron-storing protein ferritin can oxidise LDL at lysosomal pH and we have shown for the first time that it can do so effectively at this pH and that some antioxidants have surprising complex effects on this oxidation.

2. Materials and Method

2.1. Materials

All chemicals were purchased from Sigma Aldrich Limited. All ferritin and antioxidant solutions were freshly prepared. Ferritin was from equine spleen with a protein molecular weight of 440 kDa (Sigma Aldrich Limited).

2.2. LDL Isolation

LDL was isolated from pooled plasma of two to four healthy volunteers by sequential ultracentrifugation as previously described (Wilkins and Leake, 1994). The LDL was stored at 4 °C in the presence of 100 µM EDTA for up to one month.

2.3. Measurement of conjugated dienes

LDL (50 µg protein/ml) was oxidised with ferritin (0.05 µM - 0.2 µM) in Chelex-100 treated 150 mM NaCl/10 mM sodium acetate buffer pH 4.5 or 150 mM NaCl/10 mM MOPS (3-N-morpholinopropanesulphonic acid) buffer, pH 7.4. Chelex-100 is a transition metal chelator and was prewashed in ultrapure water (van Reyk et al., 1995). Formation of conjugated dienes was monitored by measuring attenuance (absorbance plus UV scattering) at 234 nm at 37 °C overnight in a dual beam Lambda Bio 40 8-cell position spectrophotometer (PerkinElmer) with UV software (Esterbauer et al., 1989). The test cuvettes were placed against appropriate reference cuvettes which lacked LDL and the attenuance of the reference cuvettes were automatically subtracted from that of the test cuvettes. The initial attenuance was subtracted from later time points.

2.4. HPLC analysis

The oxidised lipids in LDL (50 µg protein/ml) oxidised by ferritin (0.1 µM) were measured at different times up to 48 h. The oxidation was stopped by adding BHT (butylated hydroxytoluene, dissolved in ethanol; final concentration of BHT 80 µM) and EDTA (final concentration 4 mM). The samples were analysed by reverse-phase HPLC using a previously described method (Satchell and Leake, 2012).

2.5. Total hydroperoxide content

The total hydroperoxides in LDL (50 µg protein/ml) oxidised by ferritin (0.1 µM) were measured colourimetrically by a tri-iodide assay (El-Saadani et al., 1989). BHT (final concentration 80 µM) and EDTA (final concentration 4mM) were added to stop the oxidation at each time point.

2.6. Quantification of iron in ferritin

The amount of iron in ferritin was determined at 248.3 nm using flame atomic absorption spectroscopy (Analytik JENA AAS NovAA). Samples were prepared according to the method previously described (Herbert et al., 1997).

2.7. Measurement of iron release from ferritin

To determine the amount of iron release, ferritin (0.1 µM) was incubated at 37 °C in NaCl/acetate buffer of pH 4.5 or NaCl/MOPS buffer of pH 7.4. At varying times up to 24 hours samples of 1 ml were taken and the iron chelator bathophenanthroline disulphonic acid (30 µM) was added and the amount of ferrous complex formed was measured at 535 nm. FeSO₄ was used to construct a standard plot. Iron release was also measured by ultrafiltration using Amicon ultracentrifuge filter tubes (Mr 30 kDa) at 9500 g for 25 min followed by atomic absorption spectroscopy (Lamb and Leake, 1994).

2.8. Data Analysis

The data were presented as mean and standard error of the mean (SEM) of at least three independent experiments. Comparisons between control and treated samples were analysed by a t-test or one-way-ANOVA with Tukey's post hoc tests. Two-way ANOVA and Bonferroni post-tests were used to compare the data obtained by HPLC and tri-iodide assays. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Ferritin oxidises LDL at lysosomal pH

To test the ability of ferritin to oxidise LDL at the lysosomal pH of 4.5, LDL (50 μg protein/ml) was oxidised in NaCl/sodium acetate buffer of pH 4.5 at 37 °C with different concentrations of ferritin (0.05, 0.1 and 0.2 μM in terms of total protein content; horse spleen ferritin with all 24 subunits combined having 440,000 Da of protein per particle) and the oxidation was monitored by measuring the formation of conjugated dienes at 234 nm. LDL was oxidised effectively by all concentrations of ferritin (Fig. 1A). An aggregation phase where UV scattering occurred followed by a sedimentation phase (where the LDL aggregates fell below the beam of UV in the cuvettes) was then observed. The sedimentation phase occurred at shorter times with the highest concentration of ferritin. These phases are similar to the phases observed with added ferrous or ferric iron (Satchell and Leake, 2012). The oxidation of LDL by ferritin was very much slower at pH 7.4 than pH 4.5 (Fig. 1B).

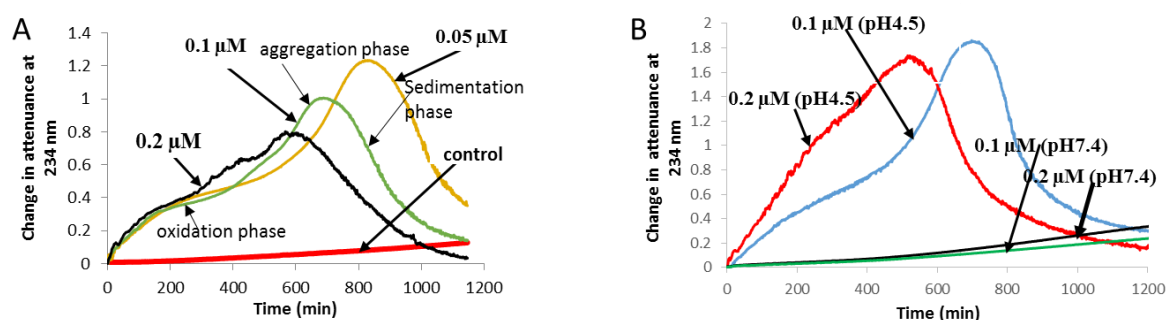


Fig. 1. Effect of ferritin on LDL oxidation. (A) LDL (50 μg protein/ml) was incubated at 37 °C in NaCl/sodium acetate buffer, pH 4.5 in the absence or presence of varying concentrations of ferritin (0.05 μM to 0.2 μM). Formation of conjugated dienes was monitored by measuring attenuation at 234 nm. (B) LDL (50 μg protein/ml) was incubated with different concentrations of ferritin at 37 °C in NaCl/sodium acetate buffer, pH 4.5 or MOPS buffer, pH 7.4. These results are representative of three independent experiments.

LDL (50 μg protein/ml) was incubated with or without ferritin (0.1 μM) at pH 4.5 at 37 °C. Cholesteryl linoleate hydroperoxide (CLOOH) and 7-ketocholesterol were generated in the presence of ferritin, but none or very little were formed in its absence (Fig. 2A). As expected (Satchell and Leake, 2012), the fatty acyl moiety of cholesteryl linoleate was oxidised before cholesterol, being more unsaturated. The levels of cholesteryl linoleate hydroperoxide and 7-ketocholesterol at 48 h were similar to those at 24 h. Total hydroperoxide levels increased with time in the presence of ferritin up to 48 h.

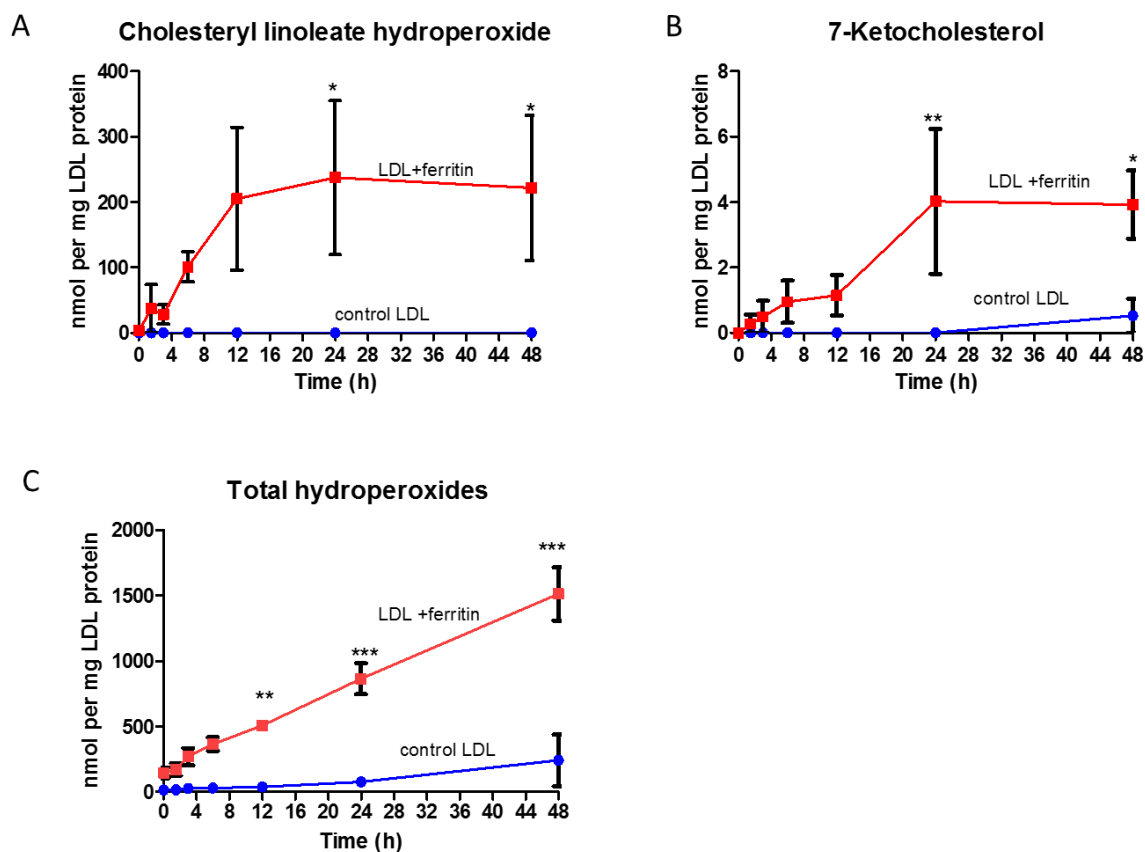


Fig. 2. Oxidised lipids in LDL incubated with ferritin at pH 4.5. LDL (50 μg protein/ml) was incubated with or without ferritin (0.1 μM) in sodium acetate buffer (pH 4.5) at 37 $^{\circ}\text{C}$. The samples were assayed for cholesteryl linoleate hydroperoxide (CLOOH) (A) and 7-ketocholesterol (B) by reverse-phase HPLC and for total hydroperoxide content by a tri-iodide assay (C). The mean \pm SEM of three independent experiments are shown. Difference between control LDL and LDL oxidised by ferritin at each time point were determined by two-way ANOVA with Bonferroni post-tests. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$

3.2. Iron release from ferritin

Atomic absorption spectroscopy showed that ferritin particles contained 1001 atoms of Fe. The concentrations of iron contained in the ferritin solutions of 0.05, 0.1, and 0.2 μM added in the experiment shown in Fig. 1A were therefore 50 μM , 100 μM and 200 μM . We compared the amount of iron released from ferritin at pH 4.5 and 7.4 to see if this could explain the faster oxidation of LDL observed at pH 4.5. Ferritin (0.1 μM) was incubated at 37 $^{\circ}\text{C}$ in NaCl/sodium acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) and samples were taken and added to the ferrous iron indicator bathophenanthroline, which does not detect ferric iron (Lynch and Frei, 1995), at different time points

(Fig. 3A). The amount of ferrous iron released was much higher at pH 4.5 than at pH 7.4. At 24 h, the concentration increased to $15.1 \pm 0.1 \mu\text{M}$ at pH 4.5 out of the total iron present in ferritin of $100 \mu\text{M}$. Ultrafiltration followed by atomic absorption spectroscopy showed the release of $12 \mu\text{M}$ iron at pH 4.5 and $3.2 \mu\text{M}$ at pH 7.4 at 24 h.

To test the effect of iron chelators, LDL ($50 \mu\text{g}$ LDL protein/ml) was oxidised with $0.1 \mu\text{M}$ ferritin in sodium acetate buffer (pH 4.5) in the presence or absence of EDTA or DTPA ($100 \mu\text{M}$). Formation of conjugated dienes was monitored (Fig. 3B). The iron chelators inhibited most of the oxidation of LDL, but did not completely inhibit it. This inhibition indicated that the iron release from ferritin was important for the oxidation of LDL.

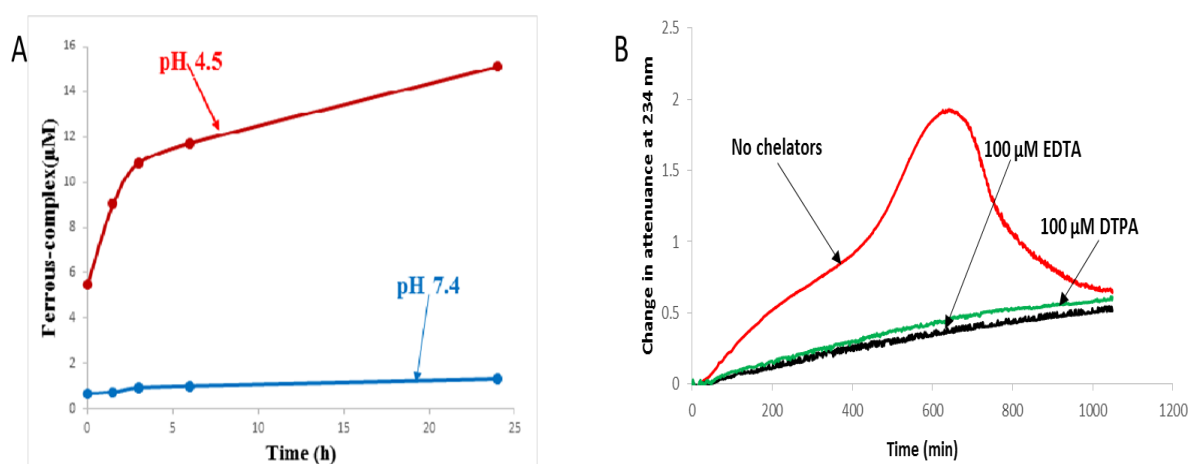


Fig. 3. Determination of iron released from ferritin and effect of iron chelators. (A) Ferritin ($0.1 \mu\text{M}$) was incubated at 37°C in NaCl/Na acetate buffer (pH 4.5) or MOPS buffer (pH 7.4). At different times, a sample (1 ml) was taken and the ferrous chelator bathophenanthroline was added left for 5 min and the absorbance was measured at 535 nm. The means of three independent experiments are shown. (B) LDL ($50 \mu\text{g}$ protein/ml) was oxidised by ferritin ($0.1 \mu\text{M}$) in sodium acetate buffer (pH 4.5) at 37°C in the absence or presence of the iron chelators, EDTA ($100 \mu\text{M}$) and DTPA ($100 \mu\text{M}$). The formation of conjugated dienes was monitored by measuring attenuation at 234 nm. These results are representative of three independent experiments.

3.3. Effects of antioxidants on LDL oxidation by ferritin at lysosomal pH

The lipid peroxyl radical scavenger DPPD (5 or $10 \mu\text{M}$) greatly inhibited the oxidation of LDL oxidised by ferritin (Fig. 4A), whereas the vehicle ethanol (1%) had no effect.

The reactive oxygen species scavenger tempol ($10 \mu\text{M}$) did not inhibit the initial oxidation of LDL by ferritin, but inhibited the oxidation later on (Fig. 4B). The inhibition observed at later times

was sometimes a partial inhibition, as shown in Fig. 4B, or sometimes a total inhibition (results not

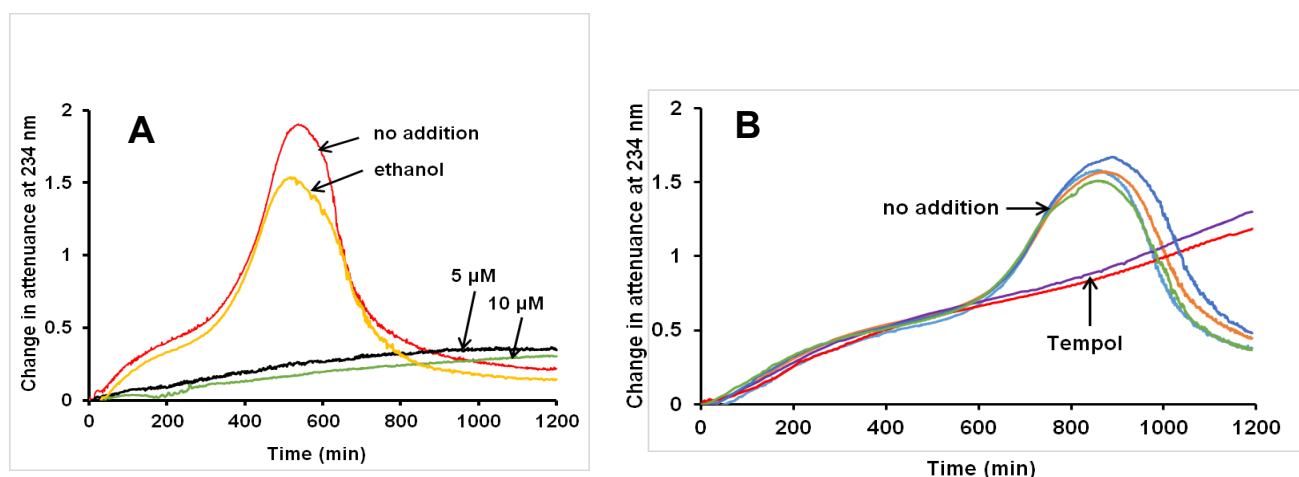


Fig. 4. The effect of DPPD and tempol on LDL oxidation by ferritin. LDL was incubated with ferritin (0.1 μ M) at pH 4.5 in the presence of (A) DPPD (5 or 10 μ M) or ethanol (1% v/v) as a control or (B) tempol (10 μ M) in duplicate or without additions in quadruplicate. These results are representative of three independent experiments for DPPD and were replicated six times for tempol. Tempol did not inhibit the early oxidation in all six experiments, but inhibited the later oxidation substantially but partially, as shown here, in three experiments or totally in three experiments.

Cysteamine (2-aminoethanethiol) is an antioxidant which concentrates in lysosomes and is used to treat the rare lysosomal storage disease cystinosis in which cysteine accumulates in lysosomes (Dohil et al., 2010). The plasma concentration of cysteamine in treated patients is about 40 μ M (Bouazza et al., 2011). It should accumulate by several orders of magnitude in lysosomes by proton trapping of its amine group due to the acidic pH in the lysosomes (Pisoni et al., 1995). Cysteamine had a complex, but consistent, effect on the kinetics of LDL oxidation. Cysteamine slowed down the initial oxidation of LDL by ferritin in a concentration-dependent manner (Fig. 5A). At later time points, cysteamine increased the rate of oxidation of LDL so that it overtook the oxidation of the control LDL, with the greatest effect seen with 25-100 μ M cysteamine. Higher concentrations of cysteamine did not increase the rate of oxidation as much. All cysteamine concentrations decreased the time taken to attain maximum attenuance, except for 1 mM and above. High concentrations of cysteamine (3 mM - 10 mM) greatly inhibited the oxidation of LDL at all studied time points (Fig. 5B). Ferritin was incubated for up to 24 h with 25 μ M (Fig. 5C) or 1 mM (Fig. 5D) cysteamine and samples were taken and the ferrous iron indicator bathophenanthroline was added and the absorbance at 535 nm measured after 5 min. Both concentrations of cysteamine increased ferrous iron release from ferritin to a similar extent compared to the control. The release was rapidly at first and more slowly later. About 30% of the iron in ferritin was released in 24 h.

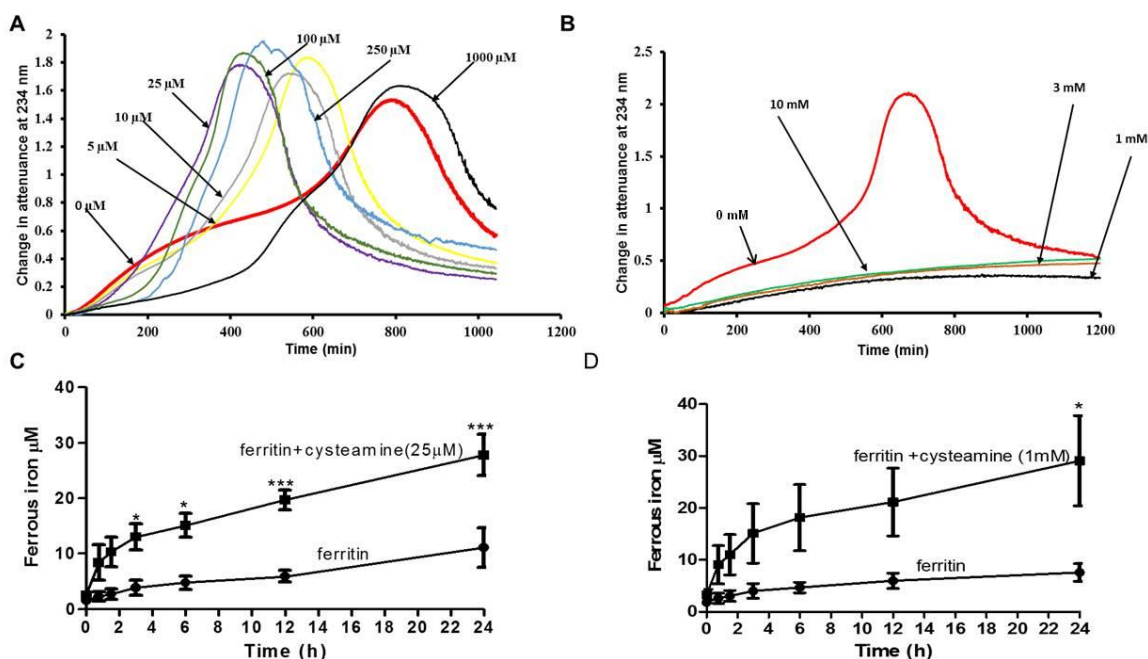


Fig. 5. The effect of cysteamine on LDL oxidation by ferritin. LDL (50 μg protein/ml) was oxidised with ferritin (0.1 μM) in sodium acetate buffer (pH 4.5) at 37 $^{\circ}\text{C}$ with 5 – 1000 μM (A) or 1 – 10 mM (B) cysteamine. The formation of conjugated dienes was monitored by measuring attenuation at 234 nm. For the colour version of (A), the colours of the lines correspond to the concentrations of cysteamine as follows: red 0, yellow 5, grey 10, purple 25, green 100, blue 250 and black 1,000 μM . For (B), the colours of the lines correspond to the concentrations of cysteamine as follows: red 0, black 1, brown 3 and green 10 mM. Ferritin (0.1 μM) was incubated at 37 $^{\circ}\text{C}$ in NaCl/Na acetate buffer (pH 4.5) in the absence or presence of cysteamine (25 μM) (C) or 1 mM (D). At different times, a sample (1 ml) was taken and the ferrous chelator bathophenanthroline was added, left for 5 min and the absorbance was measured at 535 nm (* indicates $p < 0.05$, *** indicates $p < 0.001$). These results are representative of three independent experiments

4. Discussion

The iron hypothesis of atherosclerosis has been supported indirectly by the availability of redox-active iron and high levels of both the L and H subunits of the iron storage protein ferritin in atherosclerotic lesions (Smith et al., 1992, Pang et al., 1996, You et al., 2003). Ferritin levels are increased by cytokines (Miller et al., 1991) and high levels of iron (Casey et al., 1988), which are present in atherosclerotic lesions. Ferritin might be delivered to lysosomes by autophagy of cytosolic ferritin or by other mechanisms (Asano et al., 2011). Redox-active iron in the lysosomes may play a key role in lysosomal LDL oxidation (Wen and Leake, 2007, Satchell and Leake, 2012). This present study was designed to explore the possible involvement of ferritin in lysosomal LDL oxidation and the effects of antioxidants on this oxidation.

LDL was oxidised effectively at all concentrations of ferritin at pH 4.5 and caused large scale aggregation of the oxidised LDL (Fig. 1A). The oxidation of LDL by ferritin was much faster at acidic pH (Fig 1B). Oxidation of LDL by ferrous ions was previously demonstrated to be much faster at acidic pH (Wen and Leake, 2007, Satchell and Leake, 2012) than pH 7.4, the pH of normal plasma and interstitial fluid. The measurement of cholesteryl linoleate hydroperoxide and 7-ketocholesterol by HPLC and total hydroperoxides by a tri-iodide assay confirmed the oxidation of LDL by ferritin (Fig. 2). The total hydroperoxides continued to increase after 24 h, whereas the cholesteryl linoleate hydroperoxide did not increase after 24 h possibly because the phospholipids oxidised more slowly than the cholesteryl esters or the cholesteryl linoleate hydroperoxide was further oxidised and emerged from the HPLC column at a different time as an unidentified peak of a different mass.

A previous study has shown that release of iron from ferritin requires lysosomal acidity and protease activity (Kidane et al., 2006), although there would have been no protease activity in our system. The increased oxidation of LDL by ferritin was due to the release of iron from the ferritin core as shown by the ferrous iron chelator bathophenanthroline (Fig. 3A) or ultrafiltration followed by measuring the released iron using atomic absorption spectroscopy. We detected very few degradation products in ferritin incubated alone at pH 4.5 when analysed by SDS-polyacrylamide gel electrophoresis (unpublished results). Our finding that ferritin releases iron spontaneously at lysosomal pH without proteolysis has relevance to the normal physiology of ferritin degradation and iron acquisition by the cell.

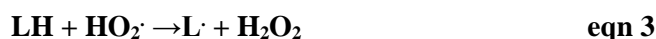
Fully saturated ferritin has the capacity to store up to 4500 atoms of iron per particle. We determined that the ferritin from equine spleen used in these experiments contained 1000 atoms of iron per particle and the concentration of ferritin typically used in our experiments, 0.1 μM , therefore contained 100 μM of iron. The rapid oxidation of LDL at lysosomal pH (Fig 1) could be attributed to the release of ferrous iron (Fe^{2+}) at this pH. The release of iron was rapid at first and much slower later (Fig. 3A). About 15% of the iron contained in ferritin was released at lysosomal pH over 24 h, but very little was released at pH 7.4, as shown by a ferrous iron assay using bathophenanthroline (Fig. 3A) and atomic absorption spectroscopy. The immediate formation of a ferrous-bathophenanthroline complex may be due to loosely bound iron in the core of ferritin that had already been released from the ferritin particles or to iron associated with ferritin which bathophenanthroline was able to bind. It is not known why iron release was faster at acidic pH, but iron is much more soluble at acidic pH and it might be speculated that an acidic pH opened the pores in the surface of ferritin through which iron is released (Jin et al., 2001).

The iron chelator DTPA completely inhibits the oxidation of LDL catalysed by ferrous iron at acidic pH (Satchell and Leake, 2012). Both DTPA and EDTA inhibited LDL oxidation by ferritin to a large extent, but not entirely (Fig. 3B). This might imply that in addition to the iron release,

reactive oxygen species are formed from inside the ferritin particles which can oxidise LDL. The ferrous iron released from ferritin can react with molecular oxygen to produce the superoxide radical ($O_2^{\cdot-}$) (eqn 1) which can protonate at acidic pH to form the hydroperoxyl radical (HO_2^{\cdot})



Hydroperoxyl radicals are much more reactive than superoxide radicals in oxidising LDL (Bedwell et al., 1989). Hydroperoxyl radicals can abstract a bisallylic hydrogen atom from a polyunsaturated fatty acyl group in a lipid in LDL to form lipid radicals (eqn 3-6).



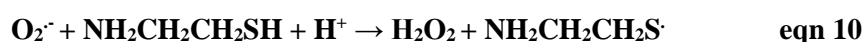
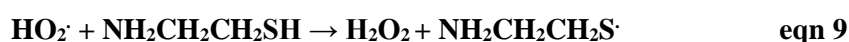
LDL oxidation by ferritin might therefore be much faster at acidic pH because (1) iron is released from ferritin much faster at acidic pH and (2) highly reactive hydroperoxyl radicals are formed at acidic pH.

DPPD effectively inhibits oxidation of LDL by copper and endothelial cells at pH 7.4 (Sparrow et al., 1992) and by ferrous iron at pH 4.5 (Ahmad and Leake, 2018). It also protects against atherosclerosis in cholesterol-fed rabbits (Sparrow et al., 1992) and decreases atherosclerosis in apoE deficient mice (Tangirala et al., 1995). In this present study DPPD, a secondary amine antioxidant, greatly reduced the formation of conjugated dienes in LDL oxidised by ferritin (Fig. 4A). Its hydrophobic nature might make it a highly effective antioxidant, as it would be expected to scavenge radicals formed within the core of LDL. Its ability to inhibit LDL oxidation by ferritin at lysosomal pH might help to explain why it decreased atherosclerosis in animal models (Sparrow et al., 1992, Tangirala et al., 1995). DPPD cannot be used in humans, however, because it is mutagenic (Sofuni et al., 1990).

Tempol is a nitroxide compound which scavengers reactive oxygen species and is a superoxide dismutase mimetic (Wilcox and Pearlman, 2008). Unexpectedly, it did not inhibit the initial oxidation of LDL by ferritin at pH 4.5, but inhibited the oxidation at later times, always substantially (Figure 4B) and sometimes totally. Tempol is an amphipathic compound and might be expected to accumulate in the phospholipid outer monolayer of LDL, rather than in its cholesteryl ester core. We suspect the oxidation of LDL by iron at lysosomal pH starts in the core of LDL and then spreads to the phospholipid monolayer (Ahmad and Leake, 2018). Tempol might not be able to

scavenge superoxide or hydroperoxyl radicals effectively enough to stop them entering the LDL particles and oxidising its core, but might be able to inhibit the later oxidation of the phospholipid monolayer by scavenging phospholipid peroxy or alkoxy radicals.

Cysteamine contains a thiol group and effectively inhibits the oxidation of LDL by ferrous iron at lysosomal pH (Ahmad and Leake, 2018). Thiols have previously been seen to act as promoters (Heinecke et al., 1993) or inhibitors of LDL oxidation (Patterson et al., 2003). Cysteamine at concentrations up to 250 μ M initially protected LDL from oxidation by ferritin in a concentration-dependent manner (Fig. 5A). The antioxidant effect might possibly be due to the lower availability of iron, as cysteamine might possibly be able to bind to and inactivate iron released from ferritin. It might also be scavenging hydroperoxyl radicals and superoxide radicals (equations 9 and 10).



A pro-oxidant effect was observed at later times (Fig. 5A) in that the rate of oxidation increased and overtook the oxidation of LDL incubated without cysteamine. The pro-oxidant effect was probably due to cysteamine removing iron from ferritin (Fig. 5C & 5D), as it might reduce ferric ion (Fe^{3+}) in ferritin to ferrous ion (Fe^{2+}) (eqn 11).



The Fe^{2+} formed might cause the generation of superoxide radicals ($\text{O}_2^{\cdot-}$) and hydroperoxyl radicals (HO_2^\cdot) by equations 1 and 2. The Fe^{2+} might also decompose lipid hydroperoxides (eqn 6) far faster than would Fe^{3+} (Esterbauer et al., 1992). The pro-oxidant and antioxidant effects of cysteamine might depend on its concentration in differing ways and this might explain the complex kinetics. As the concentration of cysteamine increased from low to moderate concentrations, the pro-oxidant effect increased, but at high concentrations, the antioxidant effects of cysteamine predominated (Fig. 5B). Plasma cysteamine concentrations of about 40 μ M are found in cystinosis patients (Bouazza et al., 2011). Cysteamine can accumulate in lysosomes due to ion trapping of its amine group (Pisoni et al., 1995) and so millimolar concentrations of cysteamine might be present in these organelles, which might inhibit the oxidation of LDL to a large extent.

These findings show that LDL can be oxidised by iron released from ferritin at lysosomal pH and raise the possibility that ferritin is a major source of iron in lysosomes that contributes to LDL oxidation in atherosclerosis. This oxidation was inhibited greatly by high concentrations of the lysosomotropic drug cysteamine, which is already used in patients for the rare lysosomal storage disease cystinosis. Lysosomal dysfunction might well play an important role in atherosclerosis (Jerome, 2010, Emanuel et al., 2014). The inhibition of lysosomal LDL oxidation by lysosomotropic antioxidants therefore raises a novel therapeutic possibility for atherosclerosis. It is interesting that

there is a strong inverse relationship between the number of years that cystinosis patients have taken cysteamine and arterial calcification (Ueda et al., 2006).

Conflict of interest

The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Authors contributions

O.O.O. performed the experiments and O.O.O. and D.S.L. designed the study and wrote the manuscript.

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