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LIPOPROTEIN OXIDATION AND INDUCTION OF FERROXIDASE ACTIVITY IN STORED HUMAN EXTRACELLULAR FLUIDS

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It was observed that during the storage of human extracellular fluids at −20°C the azide-inhibitable ferroxidase activity of caeruloplasmin declined, whilst a new azide-resistant ferroxidase activity (ARFA) developed. The literature suggested that storage-induced ARFA might be due to either a poorly defined enzymatic activity of a low density lipoprotein (LDL) or to lipid peroxides formed within the different lipoprotein fractions. To study this further, the major lipoprotein classes were separated from human serum by density gradient centrifugation. After storage of the lipoprotein fractions, it was found that the LDL fraction had the highest specific activity of ARFA and the highest content of lipid peroxidation products, as assessed by diene conjugates. The ARFA of LDL correlated with its content of diene conjugates and TBA reactive material, which initially suggested that the Fe(II) oxidising activity of peroxidised LDL arose from the reduction of peroxides by Fe(II) in the classical reaction between the metal ion and free radical reduction of lipid peroxides. However, steady state kinetic analysis indicated an enzymic role of LDL in Fe(II) oxidation, with lipid peroxides acting as a substrate for the enzyme. These results indicate that LDL may contain a peroxidase activity, catalysing the oxidation of Fe(II) by lipid peroxides, as well as a ferrous oxidase activity where O_2 is the oxidising substrate.

KEY WORDS: Human caeruloplasmin, lipoproteins, storage, azide-resistant ferroxidase activity, ferroxidase-II, lipid peroxidation.

INTRODUCTION

Lipids in normal human serum, which are mainly associated with the various lipoprotein classes, do not readily autoxidise, since serum is itself an extremely good antioxidant.[1] The inhibition of iron-dependent lipid peroxidation by human serum is largely due to the copper-containing protein caeruloplasmin (ferroxidase-I).[2] This is thought to be because caeruloplasmin is able to catalyse the oxidation of Fe(II) ions to Fe(III) ions which are bound by transferrin.[2] This ferroxidase activity of caeruloplasmin can be inhibited by at least 95% by 1 mM azide.[3]

Caeruloplasmin may undergo proteolysis and oxidative modification[4–6] and on storage of human extracellular fluids at 4°C for several weeks, the copper dissociates from caeruloplasmin, lipid peroxidation occurs and the ferroxidase activity of caeruloplasmin declines.[7,8] Concomitantly, a new azide-resistant ferroxidase activity (ARFA) develops.[7] The nature of this storage-induced ARFA is not clear. It has been

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suggested[9] that it may be due to the induction of a second ferroxidase, characterised earlier as the enzyme ferroxidase-II,[3] and/or the formation of a third "ferroxidase" activity due to lipid peroxides:

$$LOOH + Fe(II) \longrightarrow LO\cdot + OH^- + Fe(III).$$

Potentially, these two ARFA's may be distinguished: ferroxidase-II has been characterised as a subfraction of the low density lipoproteins (LDL) which catalyses the oxidation of Fe(II) ions with Michaelis-Menten kinetics.[3,10] In contrast, the ferroxidase activity of lipid peroxides is not catalytic and should be associated with all of the major lipoprotein classes since, in addition to LDL, very low density lipoproteins (VLDL) and high density lipoproteins (HDL) contain peroxidised lipids when isolated from stored plasma by density gradient centrifugation.[11]

The aim of the study reported below was to test whether storage-induced serum ARFA was accounted for by either induction of ferroxidase-II in the LDL fraction of human serum or by the formation of lipid peroxides in the serum lipoprotein fractions. Therefore, we have isolated the major lipoprotein classes and ascertained the distribution of ARFA between them and the correlation between levels of ARFA and lipid peroxidation products. We have also shown that changes in lipid peroxidation and ferroxidase activity occur, not only during refrigeration at 4°C, but also during long-term frozen storage of human serum and synovial fluid.

MATERIALS AND METHODS

Reagents and body fluids

Sera and synovial fluids were obtained from patients with definite rheumatoid arthritis (Arthritis and Rheumatism Association critiera) and from age-matched controls. Synovial fluids were aspirated for therapeutic purposes. After centrifugation at 6 000 g for 10 min to remove cells, samples from the same group were pooled, aliquoted into plastic containers, and immediately stored at 4°C, or −20°C. Samples stored at 4°C were analysed within 24 h of collection.

Caeruloplasmin (type X, human) was from Sigma, Poole, Dorset, U.K., as were $Fe(NH_4)_2(SO_4)_2$, sodium acetate, thiobarbituric acid and Na_2SO_4. Transferrin (human) was from Behring-Hoechst, Hounslow, Middx., U.K. and Chelex-100 resin was from Bio-Rad Laboratories, Watford, Herts., U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., and were AnalaR grade, except for chloroform, methanol and propan-2-ol which were Spectrosol grade.

Preparation of lipoprotein-depleted serum and lipoprotein fractions

For isolation of lipoprotein fractions, serum was collected from at least six normal healthy volunteers, after 12 hours' fasting, and pooled. The major lipoprotein classes (VLDL, LDL and HDL) and lipoprotein-depleted serum (LDS) were isolated at 4°C by KBr density gradient centrifugation (190 000 g for 48 h).[12] An aliquot of whole human serum (WHS) to which KBr had been added (in preparation for layering onto the gradient) was retained and stored at 4°C during centrifugation. After centrifugation, fractions were recovered from the gradient and dialysed overnight against three changes of 0.15 M saline (pH 7.4). The HDL_2 and HDL_3 fractions were combined.

Analysis of fractions was performed immediately after dialysis was completed. Purity of the lipoprotein fractions and LDS was tested by immunoelectrophoresis against monospecific sheep antisera to human LDL and HDL (kind gifts from Professor K. Walton, Birmingham University) and rabbit anti-WHS (Behring-Hoechst).

Acetylation and "mock" acetylation of LDL

Modification with acetic anhydride was carried out as described previously.[13] The procedure involves dilution of LDL preparations with an equal volume of saturated sodium acetate and step-wise addition of acetic anhydride during prolonged stirring on ice, followed by dialysis at 4°C against saline solution. "Mock" acetylation involves exposure of LDL to all the conditions of acetylation except the addition of acetic anhydride. Chemical modification of LDL was confirmed by its increased mobility on electrophoresis in 1% agarose.[13]

Total protein content

This was measured by the method of Lowry *et al.*,[14] with the modification that a few drops of 20% SDS were added to disperse turbidity.[13] The standard was bovine albumin.

Immunochemical determination of caeruloplasmin

Caeruloplasmin was measured immunochemically using commercial single radial immunodiffusion plates and commercial standard serum (Behring-Hoechst).

Ferroxidase assay

Ferroxidase activity was measured by the method of Johnson *et al.*,[15] in the presence of 1 mM NaN_3, to inhibit caeruloplasmin.[3] Samples (100μl) were added to a solution containing 250 ul of 2.0% (w/v) transferrin, 170 ul of 1.2 M sodium acetate buffer (pH 6.0) and 270 ul of Chelex-100-treated distilled water. The reaction was started by the addition of 300 ul of 0.4 mM $Fe(NH_4)_2(SO_4)_2$ (freshly prepared in de-aerated water) and the change in A_{460} was measured. Ferroxidase activity is expressed as the concentration of caeruloplasmin (mg/l) in fresh normal serum (determined by radial immunodiffusion) giving an A_{460}/min equivalent to the test sample (caeruloplasmin "equivalents").

Lipid peroxidation products

Diene conjugates were measured at 245 nm using chloroform/methanol (2/1, v/v)[16] or at 233 nm, using aliquots of LDL containing 200 ug of protein.[17] Thiobarbituric acid (TBA) reactivity was determined by the method of Satoh.[18] A standard curve was constructed using 1,1,3,3-tetraethoxypropane and results were expressed as nmol ml^{-1} malonaldehyde (MDA).

RESULTS

Less than 5% of the ferroxidase activity detected in freshly taken pooled samples of

TABLE I

Changes in ferroxidase activities and lipid peroxidation products in human extracellular fluids during frozen storage.

Sample[a]	Storage period and temperature	TBA reactivity (A_{532})	Diene conjugates (A_{245})	Ferroxidase activity of caeruloplasmin[b] (mg/l)	Azide-resistant ferroxidase activity (mg of caeruloplasmin "equivalents"/l)
RS	Fresh	0.022	0.54	430	20
RF	Fresh	0.006	0.23	220	30
NS	Fresh	0.018	0.47	460	10
RS	18 months −20°C	0.059	1.34	350	1630
RF	18 months −20°C	0.032	0.47	100	230
NS	18 months −20°C	0.032	1.34	230	1130
RS	18 months −70°C	0.015	0.47	480	20
RF	18 months −70°C	0.018	0.18	110	10
NS	18 months −70°C	0.018	0.41	390	10

Data represent the means of 2–4 replicate analyses which differed by less than 5%.

[a]RS = pooled rheumatoid serum, RF = matched pooled rheumatoid synovial fluid, NS = pooled normal human serum.

[b]The concentrations of caeruloplasmin in the pooled fluids, as determined by radial immunodiffusion, were: RS = 490 mg/l, RF = 220 mg/l, NS = 460 mg/l.

normal serum or rheumatoid serum was resistant to inhibition by 1 mM sodium azide (Table I). Therefore, fresh serum ferroxidase activity could be attributed to the ferroxidase activity of caeruloplasmin. In rheumatoid synovial fluid, the contribution of ARFA to total ferroxidase activity was higher.

Levels of lipid peroxidation products (measured as diene conjugates and TBA reactivity) were slightly higher in fresh rheumatoid serum than in fresh normal serum (Table I), in agreement with previous results.[16] Storage of pooled serum or synovial fluid samples at −20°C for 18 months (Table I; samples were separated into small portions before storage to avoid repeated freeze-thawing) resulted in differential increases in lipid peroxidation products and ARFA, concomitant with a decrease in the ferroxidase activity of caeruloplasmin. Storage at −70°C slowed down these changes dramatically, but not completely, as shown by the changes in ferroxidase activity. However, the freeze-thawing process itself affected the ferroxidase activity of caeruloplasmin (Table II).

When the various lipoprotein fractions were isolated from a pool of fresh fasted human serum, freshly isolated LDL and HDL were found to contain detectable amounts of lipid peroxidation products (measured by diene conjugation) and ARFA (Table III). VLDL contained relatively high amounts of diene conjugates, but no detectable ARFA. In agreement with earlier results,[19] ARFA was not detectable in any of the lipoprotein fractions when they were isolated in the presence of EDTA (results not shown). Storage of the isolated lipoproteins resulted in a marked increase in both diene conjugates and ARFA in the LDL fraction (Table III) and smaller

TABLE II
Effect of freeze-thawing on the ferroxidase activity of purified commercial caeruloplasmin.

Number of freeze-thaw cycles	Ferroxidase activity (mg/l)	Immunological activity (mg/l)
0	500 ± 14 [a] [b] [b]	500 ± 4
1	465 ± 8 [a] [b]	503 ± 3
2	459 ± 18 [b]	504 ± 9
3	455 ± 21 [b]	500 ± 7

Results represent the mean ± 1 SD of triplicate determinations. P values are from Students t test.
[a] $p < 0.02$
[b] $p < 0.05$

increases in these variables in the other lipoprotein fractions. Amongst the lipoprotein fractions isolated from stored whole serum (Table III) the largest increases in diene conjugates and ARFA occurred in the VLDL fraction, although the changes in the other lipoproteins were less marked than in the individually stored fractions.

The effects of laboratory manipulations commonly used with isolated LDL were also investigated (Figure 1). The experimental conditions of acetylation (prolonged low temperature stirring and dialysis) are known to result in an increased content of lipid peroxides in LDL.[20] ARFA, diene conjugation and TBA reactivity were determined in a freshly isolated LDL preparation (including dialysis against three changes of saline). These variables were also measured in the same preparation additionally exposed to dialysis against a further three changes of saline, or exposed to the procedures of acetylation or "mock" acetylation (see "Materials and Methods"). Figure 1 shows that both diene conjugates and TBA reactivity increased markedly during these procedures and that the changes correlated with increased ARFA.

The kinetics of Fe(II) oxidation by peroxidised LDL were studied by determining the initial rate of formation of Fe(III)-transferrin at a constant initial oxygen concentration with the initial molar ratio of [Fe(II)]/[transferrin] maintained at 2. The Fe(II) concentration was varied between 7.5 and 80 μM. In agreement with earlier results,[10] the LDL-catalysed reaction exhibited Michaelis-Menten kinetics (results not shown). Using an Eadie-Hofstee plot, a K_m of 64 μM was obtained, somewhat higher than that previously reported – 16 μM.[10] In the same system *t*-butyl hydroperoxide gave non-enzymic kinetics. At a substrate concentration of 30 μM Fe(II), the molar activity in the LDL fraction was 180 moles of Fe(III)-transferrin formed/min per mole of enzyme, using the molecular weight of ferroxidase-II (6.2×10^5).[21] This molar activity is in good agreement with those previously obtained for ferroxidase-II.[3,10] The molar activity of *t*-butyl hydroperoxide was 0.20 moles Fe(III)-transferrin/min per mole peroxide.

DISCUSSION

The marked changes in serum ferroxidase activities and lipid peroxidation which occur after several weeks of storage at 4°C[7] also occur, all-be-it at a slower rate, in samples stored at −20°C. The small changes in ferroxidase activity after storage at −70°C may be, at least in part, due to the effects of the freeze-thawing process itself. Caeruloplasmin (ferroxidase-I) is an acute phase protein and its serum concentration

TABLE III

Diene conjugation and azide resistant ferroxidase activity (ARFA) in stored lipoprotein fractions and lipoprotein fractions isolated from stored pooled human serum.

	Lipoprotein fractions isolated from fresh serum		Lipoprotein fractions stored 5 weeks at 4°C		Lipoprotein fractions isolated from serum stored 5 weeks at 4°C	
Serum Fraction	Diene conjugation (A_{245}/mg protein)	ARFA (caeruloplasmin equivalents/g protein)	Diene conjugation (A_{245}/mg protein)	ARFA (caeruloplasmin equivalents/g protein)	Diene conjugation (A_{245}/mg protein)	ARFA (caeruloplasmin equivalents/g protein)
VLDL	0.226 ± 0.039	U.D.	0.275 ± 0.39	13 ± 1	0.582 ± 0.021	75 ± 3
LDL	0.088 ± 0.004	16 ± 4	0.449 ± 0.024	205 ± 5	0.317 ± 0.011	20.2 ± 2
HDL	0.042 ± 0.002	35 ± 4	0.141 ± 0.004	62 ± 1	0.098 ± 0.013	14 ± 2
DLS	0.001 ± 0	< 1	0.003 ± 0	< 1	0.004 ± 0.001	< 1
WHS	0.003 ± 0.001	< 1	–	–	0.010 ± 0	< 1

Fractions were isolated by density gradient centrifugation in the absence of EDTA and at 4°C, as described under "Methods". After centrifugation, samples including whole human serum were dialysed as described under "Methods". Data represent mean ± S.D. for triplicate determinations. U.D. = undetectable.

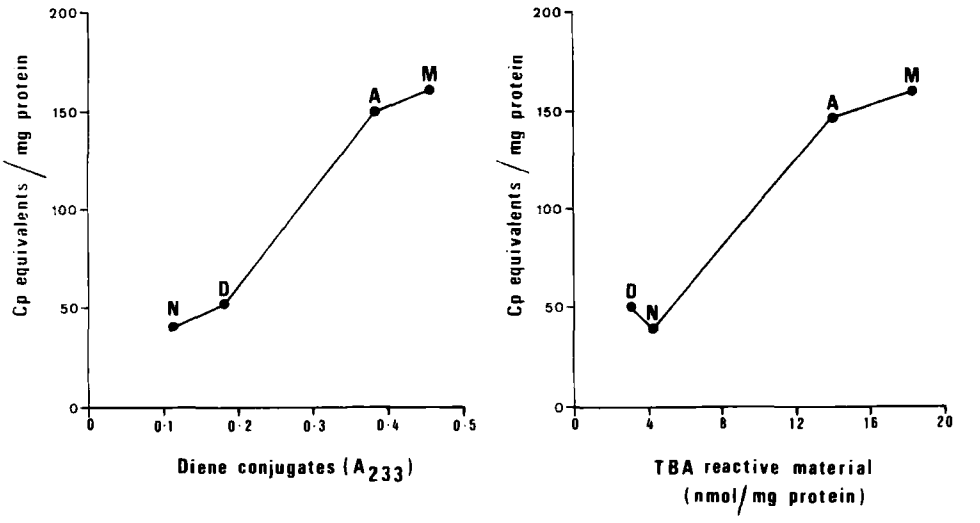

FIGURE 1 Correlation between azide resistant ferroxidase activity and lipid peroxidation products in a preparation of LDL exposed to various experimental manipulations. N, freshly isolated LDL dialysed against three changes or 0.15 M saline (pH 7.4) over a twelve hour period; D, LDL dialysed against six changes of saline over a twenty four hour period; A, LDL dialysed three times and acetylated;[13] M, LDL dialysed three times and mock acetylated. ARFA, diene conjugation and TBA reactivity were measured by duplicate determinations, which differed by less than 5%.

increases in patients with inflammatory diseases such as rheumatoid arthritis.[1] Caeruloplasmin is thought to be an antioxidant *in vivo*.[1] However, a high level of caeruloplasmin in a stored biological sample, although inhibiting peroxidation *in vitro* initially, may increase its propensity to peroxidise later, since the copper which is lost from caeruloplasmin during storage[7,8] may stimulate lipid peroxidation.[22] It has been found that the level of caeruloplasmin in sera/synovial fluids from patients with inflammatory joint disease, stored at −20°C for several months, correlates directly with the level of TBA reactive material (P.G. Winyard, unpublished data) and this may explain the differential induction of ARFA and lipid peroxidation in pooled human fluids observed in the present study. The induction of ferroxidase-II is also known to be partly regulated by serum cholesterol levels.[23]

When the individual lipoprotein components are isolated from serum they readily autoxidise. LDL contains a high proportion of unsaturated lipid and is unique among the serum lipoproteins in its susceptibility to autoxidation with storage, to give peroxides.[24] The reaction is stimulated by copper salts.[25] In the present study, not only was isolated LDL the most susceptible of the lipoproteins to peroxidation, but the extent of LDL oxidation correlated with the level of ARFA. With regard to the latter finding, similar data has been briefly alluded to by Frieden and co-workers.[19,26] The LDL in stored serum appeared to autoxidise less readily. This may be due to (a) inhibition of LDL oxidation by endogenous serum antioxidants,[27] (b) loss from LDL of water-soluble lipid peroxides,[28] or (c) precipitation of modified LDL.[11] Taken together, the results presented indicate that in stored serum both lipid peroxides and a catalytic activity in the LDL fraction may contribute to ARFA.

In LDL, the correlation between ARFA and content of lipid peroxides might be

taken to indicate a non-enzymic reaction between lipid peroxides and Fe(II) ions. However, in the present study the reaction appeared to be enzymic, since it exhibited Michaelis-Menten kinetics and a molar activity similar to that reported earlier for ferroxidase-II.[3,10] Recently, it has been shown that caeruloplasmin is able to perform a redox cycle not only between reductants and oxygen as an oxidase, but also between reducing substrates [e.g. Fe(II) ions] and organic peroxides, as a peroxidase.[29] In an analogous fashion, our results suggest that the LDL-associated ferroxidase may be able to perform a redox cycle between Fe(II) ions and lipid peroxides, as well as between Fe(II) ions and oxygen. In agreement with this conclusion, Dormandy and co-workers[30] have suggested that ferroxidase-II may be a peroxidase, rather than a ferroxidase.

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