Oxidized low-density lipoprotein is cytotoxic to human monocyte-macrophages: protection with lipophilic antioxidants

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Received 6 December 1994

Abstract Human monocyte-macrophages were incubated for 24 h with low-density lipoprotein (LDL) which had been previously oxidized for varying periods up to 24 h with copper ions, in the presence or absence of DL-α-tocopherol or probucol. The release of radioactivity from cells preloaded with tritiated adenine was used as an assay of toxicity. Toxicity of oxidized LDL increased with duration of copper oxidation and with increasing evidence of lipid oxidation, measured by assay of thiobarbituric acid-reactive substances and by gas chromatography. Oxidation and toxicity were inhibited by DL-α-tocopherol (200 μM) and probucol (50 μM).

Key words: Oxidized LDL; Monocyte-macrophage (human); Cytotoxicity; Atherosclerosis; Probucol; α-Tocopherol

1. Introduction

The foam cells of atherosclerotic lesions have been demonstrated to be macrophages, whose death has been reported to contribute to the lipid core of advanced lesions [1]. Although the cause of cell death is uncertain, it has been suggested that it may result from the production of oxidized lipids by the macrophages themselves [2]. Cell types found in the lesion, such as smooth muscle cells, endothelial cells and macrophages, have been shown to oxidize LDL in vitro [3–5]. It was also demonstrated some years ago that LDL prepared in the absence of antioxidants was toxic for smooth muscle cells and endothelial cells [6] and that neutrophil or macrophage-oxidized LDL was toxic for fibroblasts [5]. More recently, oxidized LDL has been shown to be toxic for mouse peritoneal macrophages in vitro [7] leading to apoptosis [8,9]. Native LDL was toxic to the macrophages only after a time-lag, thought to be due to oxidation of the LDL by the macrophages themselves [7].

Lipophilic antioxidants such as probucol can protect LDL from oxidation both in vivo [10] and in vitro [11]. α-Tocopherol (vitamin E), found naturally in LDL, protects LDL from oxidation when administered in vivo [12,13] or in vitro [14]. Population plasma α-tocopherol levels have been found to correlate inversely with the incidence of ischaemic heart disease [15] and α-tocopherol supplements appear to protect against coronary artery disease [16,17]. This suggests that protection of LDL by lipophilic antioxidants could be important in the prevention of atherosclerosis.

No studies on the potential toxicity of oxidized LDL to human macrophages have been reported. We have therefore investigated the toxicity to human monocyte-macrophages of LDL pre-oxidized with copper sulphate for varying periods in order to determine the extent of oxidation necessary for toxicity. We have also investigated the potential protection against LDL oxidation and toxicity by the addition of DL-α-tocopherol or probucol prior to oxidation.

2. Materials and methods

All biochemicals were from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated and were of the highest purity available. [8-^{3}H]Adenine (24 Ci/mmol) was obtained from Amersham Radiochemicals Ltd. (Aylesbury, Bucks, UK).

Human monocyte-macrophages were isolated from the blood of adult volunteers using a modification of a method described by Cathcart et al. [5]. 120 ml blood was obtained by venepuncture, EDTA (2.7 mM) added and centrifuged at 1,500 × *g*, the plasma removed and replaced with PBS. The resulting cell suspension (30 ml) was then layered on to 15 ml Lymphoprep (Nycomed AS, Oslo, Norway) and centrifuged at 600 × *g* for 30 min. Mixed mononuclear cells were removed from the interface, washed three times with PBS containing bovine serum albumin (4 mg/ml) and diluted to approximately 3×10^6 cells/ml in Iscove's Modified Dulbecco's medium (Gibco Ltd., Paisley, UK). 1 ml of this suspension was added to each of the wells of Falcon 24-well plates (Becton Dickinson, New Jersey, USA) which had been pre-coated with foetal calf serum (100 μl). After incubation for 1 h, the nonadherent cells were washed off with PBS, leaving a monolayer of monocyte-macrophages which were incubated in Macrophage-SFM (Gibco Ltd.) for up to 48 h before use. The medium was then changed to RPMI 1640 with 10% lipoprotein deficient foetal calf serum, penicillin (63 μg/ml) and streptomycin (100 μg/ml).

LDL was prepared from pooled human EDTA-plasma by the method of Havel et al. [18]. The LDL was stored at 4°C in 1 mM EDTA for up to 4 weeks before use. It was then dialysed against PBS to remove EDTA and diluted to 1 mg/ml in PBS. DL-α-tocopherol or probucol were dissolved in ethanol and added to samples of LDL at 4 μl/ml to give a final concentration of 200 μM DL-α-tocopherol or 50 μM probucol. These concentrations had previously been shown to inhibit LDL oxidation for up to 6 h. Copper sulphate solution (final concentration 5 μM) was added to the LDL and incubated at 37°C for 0, 1, 2, 4, 6 or 24 h. Chelex 100 resin was used to remove excess copper ions. Samples to be added to cells were used immediately. Butylated hydroxytoluene (200 μM) and EDTA (1 mM) were added to aliquots to be analysed. Samples for analysis by gas chromatography (GC), were stored under argon at −20°C, and samples for TBARS assay or gel electrophoresis were stored at 4°C. Viability of the cells was determined using the method of Reid et al. [19]. Briefly, 1 μCi of tritiated adenine was added to each well of cells and incubated for 1 h. Unincorporated adenine was removed by washing with PBS and the medium replaced with RPMI 1640 supplemented with 10% lipoprotein-deficient foetal calf serum. Test LDL (200 μg) was added to each well and the cells incubated for 24 h at 37°C. Medium was then removed; Triton X-100 (1% v/v) lysates of cells, and the medium, were both counted (disintegrations per minute) by liquid scintillation counting and the percentage release of radioactivity into the medium was calculated.

Lipids were extracted from the samples and processed for GC as

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SSDI 0014-5793(94)01393-4

described previously [20]. The procedure comprised addition of internal standards (*n*-heptadecanoic acid, coprostane and 5α-cholestane), Bligh and Dyer extraction, sodium borohydride reduction, saponification and derivatisation to methyl esters and trimethysilyl esters. Analysis of lipids using GC was performed as described by Carpenter et al. [21], except that a 30 m DB-1 fused silica capillary column, 0.32 mm internal diameter, 0.1 μm thickness (J&W Scientific, Folsom, CA, USA) was used.

A thiobarbituric acid reactive substances (TBARS) assay based on that described by Slater and Sawyer [22] was used to assess LDL oxidation. Each sample (50 μl) was diluted to 500 μl with de-ionised water. Trichloroacetic acid (250 μl: 40% w/v) and thiobarbituric acid (250 μl: 1.34% w/v) were added, mixed and incubated at 90–100°C for 30 min. Absorbance at 532 nm was measured and the TBARS assessed as malondialdehyde (MDA) equivalents using a standard curve of 1,1,3,3-tetramethoxypropane.

Determination of electrophoretic mobility of samples was carried out by applying 5 μl of each sample to Paragon 'LIPO' lipoprotein electrophoresis gels (Beckman, Brea, CA, USA). Gels were run at 100 V for 30 min, followed by fixing in a solution of ethanol (60%), deionised water (30%) and glacial acetic acid (10%), then drying and staining with Paragon 'LIPO' stain (Sudan Black B).

3. Results and discussion

The aims of the present study were to investigate the relationship between extent of oxidation of LDL and its cytotoxicity to human monocyte-macrophages, to assess possible protection with lipophilic antioxidants and to measure oxidized LDL components by gas chromatography, in an attempt to identify which oxidation products may be responsible for toxicity.

Using the TBARS assay as a measure, LDL oxidation was dependent on the duration of incubation with copper ions (5 μM), increasing over 24 h of incubation (Fig. 1). The progress of oxidation was rapid up to 4 h and thereafter increased more slowly. DL-α-tocopherol (200 μM) reduced the oxidation of LDL from 2–6 h, but at 24 h there was little inhibitory effect. In contrast, probucol (50 μM) conferred longer-lasting inhibition, of about 50% at 24 h. A similar time-course of LDL oxidation and inhibition with lipophilic antioxidants was found using measurements of relative electrophoretic mobility on agarose gels (data not shown).

Analysis of the various oxidized LDL extracts by GC

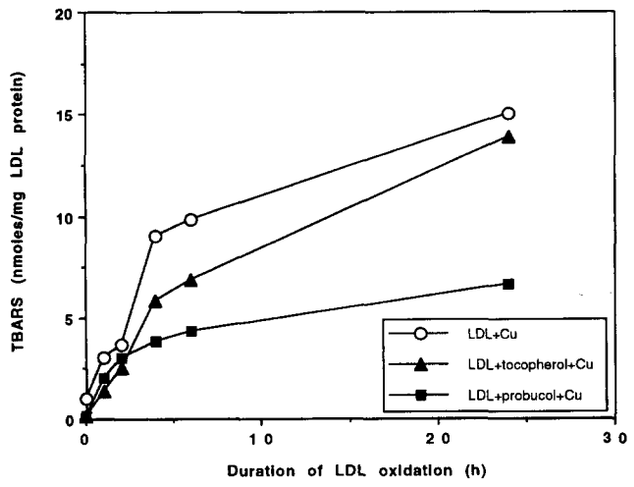

Fig. 1. LDL oxidation: LDL (1 mg/ml) was incubated with copper sulphate (5 μM) either alone or with DL-α-tocopherol (200 μM) or probucol (50 μM) for up to 24 h at 37°C. TBARS results are expressed as equivalent nmol MDA/mg LDL protein and are from one experiment typical of three.

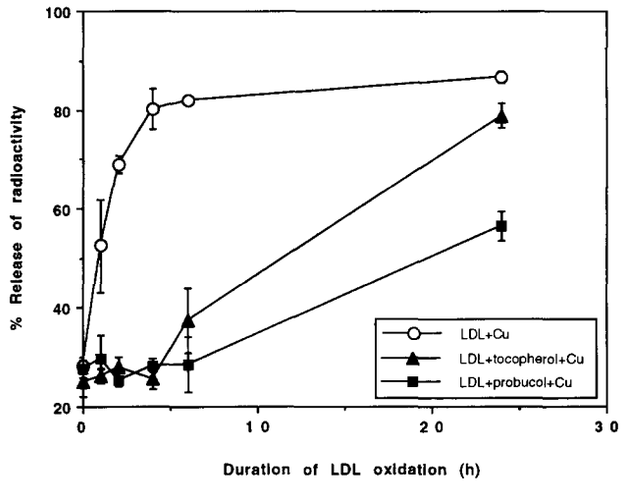

Fig. 2. Toxicity of oxidized LDL: LDL (1 mg/ml) was oxidized with 5 μM $CuSO_4$ for up to 24 h in the presence or absence of DL-α-tocopherol (200 μM) or probucol (50 μM). Cells were exposed to 200 μg/ml of this oxidized LDL for 24 h. Results are expressed as the mean percentage release of radioactivity from cells pre-loaded with tritiated adenine. Results are from one experiment typical of three and are the mean ± standard deviation of triplicate wells. Control cultures containing no LDL gave a mean percentage release of 23.2.

revealed depletion of linoleate (18:2) and arachidonate (20:4), accompanied by production of 7β-hydroxycholesterol (Table 1). Arachidonate was depleted more rapidly, and was undetectable at 24 h in the absence of exogenous antioxidants. 18:2 and 20:4 fell steeply in the first 4 h of oxidation and thereafter continued to fall but at a slower rate. 7β-hydroxycholesterol was undetectable up to 2 h oxidation, but from 4 h onwards its production increased with time. Probucol (50 μM) was effective in inhibiting both depletion of polyunsaturates and production of 7β-hydroxycholesterol, but the protective effect of DL-α-tocopherol (200 μM) appeared to be exhausted by 24 h.

Previous studies on the time-course of formation of lipid oxidation products from LDL suggested that the initial products, after depletion of antioxidants, are lipid hydroperoxides [23]. Using the TBARS assay as a general measure of LDL oxidation, we observed no significant lag phase (Fig. 1), but a 1 h lag was observed in the depletion of 18:2 and 20:4 fatty acids (Table 1).

LDL oxidized in the presence of copper sulphate (5 μM) for up to 24 h produced striking toxicity in human monocyte-macrophages (Fig. 2), measured as release of radioactivity, after 24 h exposure to oxidized LDL, from cells pre-loaded with tritiated adenine. The toxicity of LDL oxidized in the absence of lipophilic antioxidants was near-maximal after 4 h incubation. Thus it appears that even mild oxidation of LDL produces toxicity.

The protection conferred by DL-α-tocopherol and probucol diminished when LDL oxidation times were prolonged. It was virtually total at LDL oxidation times up to and including 4 h (Fig. 2). With a 24 h LDL oxidation time, no significant protection was conferred by DL-α-tocopherol, but probucol afforded better protection at this concentration, which was still total after 6 h and partially effective even after 24 h of oxidation. The initial rates of LDL oxidation (Fig. 1) up to 2 h, measured as TBARS, were similar in the absence and presence of antioxi-

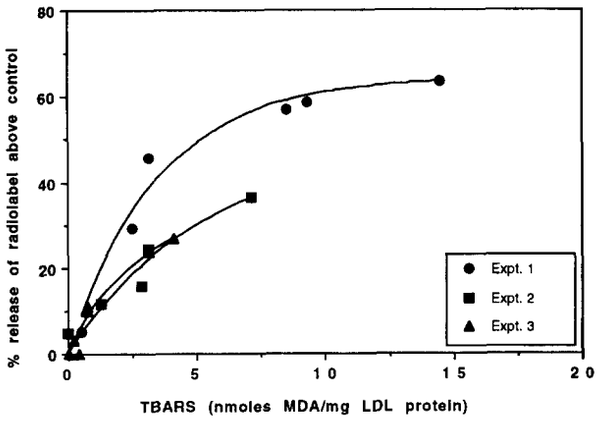

Fig. 3. Correlation of LDL oxidation and toxicity for three separate experiments: curve fits were calculated using SigmaPlot software, as were the r^2 values. The fitted curves were of the form $y = a(1-e^{-bx})$, where the constants a and b took different values for each experiment. For Expt. 1, $r^2 = 0.966$, for Expt. 2, $r^2 = 0.907$, and for Expt. 3, $r^2 = 0.938$. y axis = percentage release of radiolabel above control, i.e. percentage release for cells exposed to oxidized LDL minus percentage release for control cells exposed to native LDL. x axis = TBARS (expressed as equivalent nmoles MDA/mg LDL protein) values for oxidized LDL have had the small background TBARS values found for native LDL subtracted from them.

dants. However over the same 2 h period probucol and DL-α-tocopherol inhibited the oxidation of both linoleate (18:2) and arachidonate (20:4), measured by GC (Table 1) and inhibited the toxicity of these samples (Fig. 2).

The toxicity of oxidized LDL is presumably due to oxidation products of some of its various components, which include lipid hydroperoxides, aldehydes, oxysterols, and perhaps other, unknown toxic components. The relationship of duration of LDL oxidation to toxicity in the present study, together with analysis of oxidized LDL (by the TBA assay and by GC) give some possible clues to the nature of the toxic agents.

LDL oxidized for 4–24 h shows production of 7β-hydroxycholesterol (Table 1), and the known cytotoxicity of this compound [24], like various other oxysterols [24,25], suggests that it may be a significant contributor to the toxicity of these oxidized LDLs, particularly 24 h oxidized LDL, in which the 7β-hydroxycholesterol level was highest in these experiments. This is in agreement with the results of Hughes et al. [24], who found that the toxicity of 24 h copper-oxidized LDL to smooth muscle cells was associated with the 7-oxysterol fraction. However, in the 1 h and 2 h-oxidized LDLs, 7β-hydroxycholesterol was negligible (Table 1), and so other oxidation products may be responsible for the toxicity of the LDLs in this early stage of oxidation. However, 7β-hydroxycholesterol is found in human lesions [25,26] suggesting that the LDL in the lesion is oxidized. Therefore it may well be the more prolonged oxidation times which are more relevant to the developing lesion.

Figs. 1 and 2 show that toxicity rises more steeply than TBARS for the first 4 h of oxidation, perhaps suggesting that a non-TBA-reactive substance might be responsible. However, the high degree of correlation of toxicity with the TBARS content of the oxidized LDLs (Fig. 3) suggests a link between MDA (or other TBA-positive substances) and toxicity. However, the curvilinear shape of the relationship, rather than a straight line, perhaps hints that toxicity of MDA alone might not be the explanation. Alternatively it may be that the toxic response to MDA is curvilinear. Interestingly, Hessler et al. found that MDA toxicity to fibroblasts was insufficient to account for the toxicity of a lightly-oxidized form of LDL [27]. Lipid hydroperoxides are also possible candidates as toxic agents. Their formation during copper oxidation of LDL, though not measured here, has been found to be maximal at around 4 h, with their levels typically falling appreciably by 24 h, owing to decomposition [28], though there may be enough remaining to contribute to later toxicity. In the course of the TBA assay lipid peroxides are liable to decompose to MDA and other TBARS, the extent depending on the precise conditions of the TBA assay [29]. The steep fall in polyunsaturates (18:2 and 20:4 fatty acids) over the first four hours of LDL oxidation (Table 1) supports the role of lipid hydroperoxides in early toxicity.

Kuzuya et al. [30] attributed the toxicity of copper oxidized

Table 1
Levels of linoleate (18:2) arachidonate (20:4) and 7β hydroxycholesterol (7β-OH) in LDL oxidized with 5 μM copper sulphate in the presence or absence of DL-α-tocopherol (200 μM) or probucol (50 μM) in one experiment.

	Linoleate, arachidonate and 7ß-hydroxycholesterol content (mg/mg LDL protein)								
Time	LDL + Cu			LDL + Cu + α-tocopherol			LDL + Cu + probucol		
	18:2	20:4	7ß-OH	18:2	20:4	7ß-OH	18:2	20:4	7ß-OH
0 h	128.6	10.0	0.0	127.9	11.2	0.0	100.8	7.2	0.0
1 h	125.5	12.4	0.0	107.7	8.6	0.0	111.7	7.7	0.0
2 h	81.1	3.6	0.0	115.4	11.8	0.0	116.1	10.3	0.0
4 h	58.3	2.3	5.8	107.9	8.8	0.0	112.1	8.6	0.0
6 h	66.4	4.4	2.6	118.4	13.0	0.0	126.2	16.4	0.6
24 h	37.3	0.0	16.6	62.4	3.1	17.7	115.3	13.0	1.6

LDL to its content of lipid peroxides and the presence of transition metals. The toxic effects reported in the present study were probably not due to the persistence of low concentrations of free copper ions, because the oxidized LDL was treated with Chelex resin to remove copper ions and because we have incubated monocytes with 5 μM copper ions alone, without causing toxicity (data not shown). This would not, however, exclude a role in cytotoxicity of a copper-LDL complex in the cytotoxicity or a combined effect of lipid peroxides plus residual traces of copper ions.

The results indicate that the oxidation of LDL results in toxicity for human monocyte-macrophages and although the exact mechanism is not yet clear, lipophilic antioxidants inhibit both the oxidation and the resulting toxicity. Since to judge from histological appearances the macrophage is the only cell-type which dies in the atherosclerotic lesion [1], this is clearly of potential relevance to the mechanism of progression of atherosclerosis and therefore requires further study.

Acknowledgements: Funding for this study was provided by the British Heart Foundation and the Ministry of Agriculture, Fisheries and Food. We thank Mrs. V Mullins for secretarial assistance, Dr. P. van der Schoot (Cavendish Laboratory, Dept. of Physics, University of Cambridge) for advice and Dr. T.A. Carpenter (Herchel Smith Laboratory for Medicinal Chemistry, University of Cambridge) for advice on curve fitting.

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