RESEARCH COMMUNICATION

Non-oxidative modification of native low-density lipoprotein by oxidized low-density lipoprotein

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The oxidative modification of low-density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis, although little is known as yet about the precise mechanism of oxidation *in vivo*. The studies presented here demonstrate that, in the absence of cells or transition metals, oxidized LDL can modify native LDL through co-incubation *in vitro* such as to increase its net negative charge, in a concentration-dependent manner. The interaction is not inhibited by peroxyl radical scavengers or metal chelators, precluding the possibility that the modification of native LDL by oxidized LDL is through an

oxidative process. Studies with radioiodinated oxidized LDL showed no transfer of radioactivity to the native LDL, demonstrating that fragmentation of protein and the transfer of some of the fragments does not account for the modified charge on the native LDL particle. The adjacency of native to oxidized LDL in the arterial wall may be a potential mechanism by which the altered recognition properties of the apolipoprotein B-100 may arise rapidly without oxidation or extensive modification of the native LDL lipid itself.

INTRODUCTION

Oxidation of low-density lipoprotein (LDL) is implicated in the pathogenesis of atherosclerosis [1]. It is not yet clear how LDL becomes oxidized *in vivo*, what the nature of the initiating process is or how the oxidation is propagated. Although superoxide radical may be generated at inflammatory loci which may be involved in the complex sequence of events underlying endothelial damage, the superoxide radical *per se* is not capable of mediating oxidation of polyunsaturated fatty acyl components of lipoproteins unless protonated [2–4]. Superoxide, and hydrogen peroxide, the product of its dismutation, can be activated to more reactive radical forms by transition-metal ions and haem proteins (reviewed in [5,6]), but they are unlikely to be available *in vivo* to catalyse early oxidative events in atherosclerosis. Other possible mechanisms may be contributions to the oxidation from peroxynitrite arising from the interaction of NO with superoxide [7], enzymic oxidation mediated by lipoxygenase [8] or incorporation into LDL of enzymically oxidized fatty acids or of dietary peroxides. Once a minimal amount of peroxide is seeded into the LDL, studies *in vitro* have shown that peroxidation may be amplified by such catalysts as haem proteins or transition-metal complexes. Although these components might be available in more advanced lesions, their potential presence in the early stages of atherosclerosis has not been rationalized or substantiated.

The results of the present study show that oxidized LDL can modify native LDL such that the surface charge on the apolipoprotein B-100 is altered, the extent being related to the degree to which the LDL is indeed oxidized and the ratio of the concentrations of native to oxidized LDL. The findings also reveal that the modification of native LDL arising from its interaction with oxidized LDL is not inhibited by the peroxyl radical scavenger butylated hydroxytoluene (BHT) or by EDTA, a copper-ion chelator. The adjacency of native to oxidized LDL in the arterial

wall may be a mechanism by which the altered charge on the apolipoprotein B-100 may arise without oxidation or extensive modification of the native LDL lipid itself.

MATERIALS AND METHODS

Materials

Hydrogen peroxide, BHT, *N*-acetylcysteine, albumin and acetic anhydride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), and L-lysine monohydrochloride was from BDH.

Isolation of LDL

Blood was collected by venepuncture into sampling vials containing acid-citrate/dextrose and 100 μ M EDTA. LDL was isolated by ultracentrifugal flotation in KBr at a density of 1.063 g/ml [9]. After isolation, the LDL was dialysed in PBS (10 mM phosphate/154 mM NaCl, pH 7.4) containing 100 μ M EDTA at 4 °C. The LDL preparation was then sterilized by passing it through a 0.2- μ m-pore-size filter (Millipore), stored in the dark at 4 °C and used within 7 days. Prior to oxidation, the LDL was dialysed into PBS containing 10 μ M EDTA and assayed for protein concentration [10].

Oxidation of LDL

LDLs were analysed for modifications of the surface charge on the apolipoprotein B-100 by their altered electrophoretic mobility and for changes in their lipids by monitoring the levels of conjugated diene.

The oxidation of LDL (125 μ g of protein/ml) *in vitro* was performed by employing a modification of the procedure described by Esterbauer et al. [11]. Oxidation of LDL was initiated by adding Cu²⁺, the final total concentration of CuSO₄ being 3 μ M and of EDTA 1.5 μ M. Oxidation was terminated after 5 or

Abbreviations used: LDL, low-density lipoprotein; BHT, butylated hydroxytoluene; HPODE, hydroperoxyoctadecadienoic acid.

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18 h by addition of BHT (final concn. 50 μ M) to one portion and 100 μ M EDTA to another. For the incubations of native LDL with oxidized LDL, equal volumes of each were combined and incubated for 1 h. Analysis of oxidative modification of apolipoprotein B-100 was performed by agarose-gel electrophoresis in Beckman pre-cast gels in order to assess the net negative surface charge on the LDL particle. Electrophoresis was carried out at 100 V for 45 min (pH 8.8). Lipoprotein was revealed by Sudan Black staining. The relative electrophoretic mobility (R_m) is calculated by determining the quotient of the distance from the origin to the midpoint of the band in relation to that for the native, untreated LDL. Appropriate controls, as shown in the results, include incubation of native LDL with 1.5 μ M Cu^{2+} for 1 h. The oxidation of LDL was continuously monitored as the formation of conjugated dienes by measuring the increase in absorbance at 234 nm at 37 °C.

α -Tocopherol levels of LDL, oxidized LDL and mixtures thereof were measured by HPLC [12]. LDL (125 μ g of protein/ml), containing α -tocopherol as internal standard, was extracted with 1 ml of hexane after the addition of 1 ml of methanol. After centrifugation of the sample at 1500 *g* for 15 min, α -tocopherol was measured by normal-phase HPLC (Novapak silica column; 4 μ m particle size; 3.9 mm $\times$ 156 mm). The mobile phase consisted of 92% hexane and 8% methyl *t*-butyl ether pumped at a flow rate of 1 ml/min. Detection and quantification of α -tocopherol was performed fluorimetrically (295 nm excitation; 340 nm emission).

Radioiodination of LDL

LDL was radioiodinated using the method described by De Whalley et al. [13]. After mixing LDL gently with Na^{125}I in the presence of ICl, the LDL was dialysed at 4 °C against 4–5 litres of phosphate buffer over 48 h with four changes of buffer. The LDL was subsequently filtered through a sterile 0.22 μ m-pore-size Millipore filter. The radioiodinated LDL (125 μ g/ml) was oxidized with 3 μ M CuSO_4 for 18 h. The oxidized ^{125}I -labelled LDL was incubated with equal concentrations of non-radio-labelled native LDL. After 1 h, the incubation mixtures were subjected to agarose-gel electrophoresis and the bands of gel which included oxidized ^{125}I -labelled LDL and modified native LDL were analysed for radioactivity.

Acetylation of LDL

LDL was acetylated using acetic anhydride, as previously described [14]. LDL (1–3 mg of protein/ml in PBS) was added to 1 ml of saturated solution of sodium acetate. Acetic anhydride was diluted ten-fold and added in multiple small portions of 2 μ l over a period of 1 h up to a total mass of acetic anhydride equal to 1.5 times the mass of protein used. After stirring for 30 min the solution was dialysed for 24 h at 4 °C against phosphate buffer containing 10 μ M EDTA and saturated with nitrogen.

RESULTS

Native LDLs (125 μ g of LDL protein/ml) were incubated for 1 h at 37 °C with the same concentration of LDL that had been oxidized by 3 μ M Cu^{2+} for 18 h. The modification of native LDL by oxidized LDL was examined by investigating the change in charge on the apolipoprotein B-100 by agarose-gel electrophoresis, and the oxidation of the polyunsaturated fatty acids by measuring the levels of conjugated dienes. The native LDL became more negatively charged on the agarose gel after incubation with the oxidized LDL. The R_m (migration compared with non-incubated native LDL) of the native LDL was increased

Table 1 R_m of LDLs and co-incubations for 1 h of native LDL (125 μ g of LDL protein/ml) with pre-oxidized LDL (18 h, 125 μ g of LDL protein/ml, 3 μ M Cu^{2+})

Results are means $\pm$ S.D. (*n*).

Treatment	R_m	
	Oxidized LDL	Native LDL
Pre-oxidized LDL (18 h) + 1 h further on ice	5.5 $\pm$ 0.5 (4)	
Native LDL + pre-oxidized LDL	5.0 $\pm$ 0.5 (9)	2.0 $\pm$ 0.1 (9)
Native LDL + EDTA (100 μ M) + pre-oxidized LDL	5.0 $\pm$ 0.4 (9)	2.1 $\pm$ 0.3 (9)
Native LDL + BHT (50 μ M) + pre-oxidized LDL	5.1 $\pm$ 0.5 (4)	2.0 $\pm$ 0.1 (4)
Pre-oxidized LDL (18 h) + 100 μ M EDTA, 1 h, 37 °C	5.2 $\pm$ 0.4 (9)	
Pre-oxidized LDL (18 h) + 50 μ M BHT, 1 h, 37 °C	5.6 $\pm$ 0.3 (3)	

to 2.0 $\pm$ 0.1 (*n* = 9 independent experiments) (Table 1); the R_m of the LDL oxidized by Cu^{2+} for 18 h was 5.0 $\pm$ 0.5 (*n* = 9).

In order to examine the nature of the modification of native LDL by oxidized LDL, a chain-breaking antioxidant which scavenges lipid peroxy radicals (BHT) and a copper chelator (EDTA) were added to the oxidized LDL immediately before incubation with the native LDL. The modification of native LDL by oxidized LDL was not suppressed by EDTA (100 μ M) or by BHT (50 μ M). When low-molecular-mass components in the oxidized LDL preparation were removed by dialysis for 5 h before exposure to native LDL, the dialysed oxidized LDL retained its ability to modify the charge on the native LDL particle. If less hydrophilic reactive components within the oxidized LDL are involved in the modification of the surface charge on native LDL, these might elude the dialysis treatment. Incorporation of *N*-acetylcysteine (50, 100 or 200 μ M), albumin (at a range of concentrations from 62.5 μ g/ml to 5 mg/ml) and lysine (0.25, 0.5 or 1 mM) did not inhibit the altered migration of the target LDL.

The concentration-dependence of the modification of native LDL was examined by incubating oxidized LDL (125 μ g of LDL protein/ml) for 18 h in the presence of 100 μ M EDTA with various concentrations of native LDL (125, 250, 500 and 1000 μ g of LDL protein/ml). The extent of modification was dependent on the relative concentrations of native to oxidized LDL. As the concentration of native LDL increased, in the presence of a constant amount of oxidized LDL (i.e. as the ratio of native to oxidized LDL increased from 1:1 to 8:1), the extent of the increase in the net negative charge on the native LDL induced by the oxidized LDL was less, the R_m of the modified native LDL being 1.9, 1.7, 1.5 and 1.25 respectively. It is noteworthy that the net negative charge on the oxidized LDL was decreased slightly as the concentration of native LDL was increased. With LDL oxidized by exposure to Cu^{2+} for only 5 h (rather than 18 h), a similar, but less extensive, pattern of modification of the native LDL was obtained. The R_m of the LDL oxidized by Cu^{2+} for 5 h was 3.7 and this increased the R_m of the native LDL to 1.35 (*n* = 3). Incorporation into the co-incubations of EDTA (100 μ M) and BHT (50 μ M) did not inhibit the modification of the native LDL by the oxidized LDL.

The effect of the time of co-incubation on the altered R_m of the target LDL induced by oxidized LDL showed that the modification was essentially complete at *t* = 15 min, with a small further increase by 1 h and minimal changes thereafter up to 3 h. The effects of mixing the oxidized LDL with the native LDL on the state of oxidation of the polyunsaturated fatty acids within the native LDL were monitored by measuring the levels of

conjugated dienes by difference spectroscopy. No changes were observed in the spectral properties of the polyunsaturated fatty acids within the native LDL, indicating no further formation of conjugated dienes, but the negative charge on the native LDL protein increased during the course of the interaction (results not shown).

A further investigation was undertaken to ascertain whether oxidative processes, not inhibitable by BHT or prevented by copper chelation, were involved in the modification of the native LDL. α -Tocopherol levels in the native (0.125 mg of LDL protein/ml), oxidized and co-incubated native and oxidized LDL were measured. The results [17.7 ± 0.34 , 0 and 8.15 ± 0.11 nmol/mg of protein ($n = 3$) respectively] gave summative levels indicative of a non-oxidative process.

In order to investigate whether the change in charge on the native LDL when in close proximity to oxidatively modified LDL was due to transfer of protein or peptide units from the apolipoprotein B-100 of the oxidized LDL to the native particle, LDL was radiolabelled with ^{125}I before oxidation for 18 h and then allowed to interact with unlabelled native LDL for 1 h at 37 °C. The reaction mixture was subjected to electrophoresis and the radioactivity of the individual bands determined. The results showed that the modified native LDL contained virtually no radioactivity, indicating that peptide transfer from one particle to another does not apparently take place.

Modification of apolipoprotein B-100 of the LDL, by acetylation of the lysine residues, and its combination with native LDL had no effect on the migration of the target native LDL, in contrast with the effects of the oxidized LDL.

DISCUSSION

There are many mechanisms by which the oxidative modification of LDL has been investigated, generally via the oxidation of the LDL lipid and the binding of the secondary products of LDL peroxides to the apolipoprotein B-100. We have shown that oxidized LDL can rapidly modify native LDL to increase its net negative charge. The lack of inhibition of this effect by BHT and EDTA precludes the possibility that the lipid hydroperoxides in oxidized LDL in some way induce the rapid propagation of lipid peroxidation in the native LDL particles or that the oxidized LDL converts the Cu^{2+} into a form that can rapidly initiate the oxidation of the native LDL particles.

The role of peroxides in enhancing the oxidizability of LDL has also been exemplified in the observed pro-oxidant effects of lipoxygenase-derived peroxides on copper-initiated oxidation [15] and the amplification effect of hydroperoxyoctadecadienoic acid (HPODE) on metmyoglobin-mediated LDL oxidation [16]. The findings reported here suggest that the modification of native LDL by oxidized LDL is not through an oxidative process, nor is it dependent on the presence of transition-metal ions or the propagation of lipid hydroperoxide formation occurring during the interaction of pre-oxidized LDL with native LDL. The possibility that decomposition products of lipid hydroperoxides, formed during the oxidation of the LDL, may have transferred to the apolipoprotein B-100 of the target native LDL on co-incubation was precluded by dialysis and from incorporation of potential scavengers of reactive aldehydic products. However, it is conceivable that more lipophilic aldehydic decomposition products of the oxidized LDL transferred to closely aligned native LDL molecules without necessarily the intervention by the scavenging thiol or amino compounds in the aqueous phase.

Apolipoprotein B-100 becomes fragmented by non-enzymic oxidative cleavage into a large number of fragments of relative molecular mass 14–200 kDa [17]; the possibility was considered

that some of these may have been able to transfer from the oxidized LDL particles to the native LDL particles and, if they had a high net negative charge, to increase the net negative charge on the native LDL particles. Virtually no radioactivity was transferred, however, from oxidized ^{125}I -labelled LDL to native non-labelled LDL. It is possible that some peptides that did not contain tyrosine may have been transferred (the ^{125}I would have been incorporated mainly into tyrosine residues), but this is unlikely, since apolipoprotein B-100 contains about 150 tyrosine residues [15].

Others have shown that addition of native LDL (50 μg of protein/ml) during the oxidation of LDL (50 μg of protein/ml) by Cu^{2+} (10 μM) almost instantaneously enhances the oxidation, which they postulated to be due to the presence of high level of peroxides or the α -tocopheroxyl radicals already present in the oxidized LDL [18]. This is consistent with previous studies showing the enhancement of the rate of LDL oxidation by the addition of peroxides in the form of HPODE [15,16,19], but is in contrast with the findings reported here in which, in the presence of high concentrations of a peroxyl-radical scavenger and a copper chelator, increased lipid oxidation was not observed, but modification of the surface charge was the predominant observation.

Recent studies [20] have demonstrated an LDL fraction, isolated from the plasma of patients with established extensive carotid atherosclerosis and with confirmed evolving acute myocardial infarction, carrying a modified protein moiety; the finding that this protein moiety of the modified LDL is not fragmented suggests that it is not a consequence of extensive oxidation of that LDL particle itself. These workers also found increased breakdown products of lipid peroxidation and blocked lysine residues in the modified LDL fraction, the latter converting macrophages into foam cells. Previous work of others has also shown that direct interaction with malonaldehyde or 4-hydroxynonenal modifies LDL to an extent that it is recognized by scavenger receptors [21,22].

The significance of the findings reported here may be that, in atherosclerotic lesions, with oxidized LDL already present [23], native LDL entering from the bloodstream may be modified rapidly before it has the chance to escape from the lesion back into the blood or into the lymphatics.

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