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Apolipoprotein oxidation in the absence of lipid peroxidation enhances LDL uptake by macrophages

James V. Hunt*, James R. Bailey, Donna L. Schultz, Alan G. McKay, Malcolm J. Mitchinson

Division of Cellular Pathology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

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Abstract

A characteristic of the antioxidant, probucol, is its inability to inhibit apolipoprotein B fragmentation in low density lipoprotein (LDL), despite a pronounced ability to inhibit lipid oxidation on relatively lengthy exposure to Cu(II). Here we show that a short exposure of LDL to hydrogen peroxide and Cu(II) leads to ^{125}I-labelled apolipoprotein B fragmentation, the production of malondialdehyde and hydroperoxides and leads to increased uptake by macrophages on subsequent culture. However, pre-loading LDL with probucol protects LDL from lipid oxidation but not protein fragmentation or macrophage uptake. The use of probucol to conduct studies on apolipoprotein B oxidation without extensive lipid oxidation may prove useful when studying LDL apolipoprotein damage on exposure to an aqueous free radical insult.

Key words: Protein oxidation; Low density lipoprotein; Probucol; Macrophage uptake

1. Introduction

There is now substantial evidence in favour of low density lipoprotein (LDL) oxidation playing a role in the development of atherosclerosis [1,2]. A major source of lipid in the lesion is thought to be oxidatively modified LDL, which has been detected in lesions using immunohistochemistry [3]. Studies in animal models of the disease have shown antioxidants to be protective and this is supported by some human epidemiological studies [4–6]. However, the cause of oxidation of LDL remains uncertain. One possible factor is the availability of transition metals as catalysts for such oxidative events [7]. However, transition metal availability has, thus far, been shown only in very advanced lesions [7] and may participate only in later stages of lesion development rather than lesion initiation.

Many in vitro studies suggest that macrophages play a role in LDL oxidation, possibly involving the respiratory burst [8,9] and cystine metabolism [10,11]. Many other cell-independent alternatives have been proposed, including autoxidation of ascorbate [12] and glucose [13]. For instance, there is some evidence that in diabetes mellitus, in which the severity of atherosclerosis is elevated, ascorbic acid levels are reduced and its oxidised product, dehydroascorbate, is increased [14]. This suggests that ascorbic acid oxidation occurs, with its concomitant production of aldehydes, superoxide, hydrogen peroxide and hydroxyl radicals [15,16]. There is also some suggestion that glucose, via its ability to generate oxidants of a similar hydrophilic nature, may contribute to oxidative stress and the complications of diabetes [17].

*Corresponding author. Fax: (44) (223) 33-3346.

However, one factor common to many, if not all proposals is the formation of an initiating free radical species of a hydrophilic nature within an aqueous environment. The lipid content of LDL, as opposed to the protein content, is protected by lipophilic antioxidants such as vitamin E [18] which, physiologically, appear to be maintained in the reduced form by such aqueous reducing agents as ascorbic acid [18]. Should the initiating species causing LDL oxidation in vivo prove to be predominantly in the aqueous phase then the study of damage to apolipoprotein B by such oxidising species may prove to be of importance. The current emphasis on developing an interventional approach based upon the interruption of oxidative reactions might therefore have to take such an effect into account.

Probucol, 4,4′-(isopropylidenedithio)bis[2,6-di-*tert*-butylphenol], has a structural similarity to butylated hydroxytoluene and is known to inhibit LDL lipid peroxidation in vitro and LDL uptake and degradation in Watanabe hereditary hyperlipidaemic rabbits [19]. Independent of any of its hypocholesterolaemic effect, probucol has also been shown to inhibit the progression of atherosclerosis in Watanabe rabbits [19,20]. However, there has been some suggestion that probucol cannot lower arterial coverage of thoracic aortic lesions in rabbits [20] and that its effects are rather more complicated; the anti-atherosclerotic effect of probucol has also been shown to differ when comparing the rates of animal growth [21]. That probucol is unable to reduce lesion coverage whilst reducing cholesteryl ester content of lesions [20] might be taken to imply that probucol has little effect upon the initiating cause of atherogenesis. Thus, given the extremely lipophilic nature of probucol, these observations might be consistent with an initiating oxidative process of an aqueous origin. The preservation of

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endogenous antioxidants in LDL by ascorbic acid, but not probucol [22], during LDL oxidation suggests that the inability of probucol to decrease lesion coverage consistently in the rabbit model of atherosclerosis is related to its inability to 'link' with aqueous antioxidants and to scavenge oxidising species in the aqueous milieu.

Using probucol, it is possible to fragment apolipoprotein B in LDL oxidatively with only a limited extent of lipid oxidation [23]. We have therefore investigated the possibility that protein oxidation, monitored by fragmentation, affects LDL uptake by macrophages in a manner that is independent of lipid oxidation.

2. Experimental

Radiochemicals were obtained from Amersham (Aylesbury, UK). All biochemicals were obtained from Sigma (Poole, UK) or Aldrich (Gillingham, UK) unless stated otherwise and were of the highest purity available.

Human low density lipoprotein (LDL) was prepared from normal individuals as previously described [13,23]. Briefly, blood from healthy volunteers was centrifuged in the presence of 1 mg/ml EDTA to obtain plasma. Lipid fractions were obtained from pooled plasma by ultracentrifugation and flotation through potassium bromide gradients. Centrifugations were performed at $100{,}000 \times g$ for 18 h at 4°C in the presence of EDTA. Low density lipoprotein (LDL) was taken as the fraction that floated at a relative density of 1.063 g/ml. Prior to experimentation, LDL was dialysed overnight at 4°C against phosphate-buffered saline containing chelating resin (Sigma) to remove any EDTA and transition metals.

Pre-loading of LDL with probucol was performed as previously described [24]. Essentially, radioiodinated LDL (3.5 mg/ml) was incubated at 37°C for 2 h with no further additions, 100 μM probucol in ethanol (0.1% v/v in incubation) or with ethanol alone. The LDL was then subjected to sieve chromatography (PD10 columns) to remove LDL-unassociated ethanol or probucol.

LDL (2.5 mg/ml) was then incubated with 2.5 mM hydrogen peroxide and 50 μM Cu(II) for 3 h at 37°C in the presence of 10 mM potassium phosphate (pH 7.4). At various times throughout this exposure to an aqueous free radical generating system, samples were removed and treated with 200 IU catalase and chelating resin for 10 min. Samples were then immediately assessed for thiobarbituric acid-reactive material (malondialdehyde equivalents) [13,23,25], hydroperoxide content as measured by the xylenol orange assay [13,23,25]. Fragmentation of apolipoprotein B was determined by measuring peptides soluble in 5% trichloroacetic acid (TCA), as previously described [26]. Samples were retained for cell uptake studies using P388D1 macrophage-like cells and also for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [27,28] and analysis of electrophoretic mobility on agarose gels using a CIBA-Corning system. The uptake of such pre-incubated [^{125}I]LDL by P388D1s on culturing with 100 μg/ml LDL for 5 h was determined as previously described [23]. Uptake of LDL in cells is expressed as ng LDL/mg cell protein, the later assessed by the Lowry assay for protein [29].

Cell culture of the P388D1 macrophage cell line was conducted as previously described at 37°C with an atmosphere of 5% CO_2 and 95% air [30]. The cells were maintained in Dulbecco's Modified Eagles Medium and 10% foetal calf serum [30]. Cells were plated at 0.5×10^6 cells/ml in 24-well plates and cultured for 5 h (LDL studies) or in 3 ml plates for 24 h cultures (BSA studies).

3. Results and discussion

During these studies we have used P388D1 cells, a macrophage-like cell line derived from a lymphoid neoplasm of DBA/2 mice treated with 3-methylcholanthrene. This cell line has been shown to bind and degrade native and modified LDL in a manner analogous to that of murine peritoneal macrophages and human peripheral blood monocyte-derived macrophages [29].

The exposure of 2.5 mg/ml human LDL to 2.5 mM hydrogen peroxide and 50 μM Cu(II) over 3 h leads to the accumulation of thiobarbituric acid-reactive material (expressed as malondialdehyde equivalents) (Fig. 1A), and the formation of lipid hydroperoxides (Fig. 1B), detectable by the Xylenol orange assay. When LDL was pre-loaded with probucol (with ethanol as a carrier) there was a marked decrease in both parameters of lipid peroxidation. A control of LDL incubated with Cu(II) alone showed negligible levels of lipid oxidation beyond

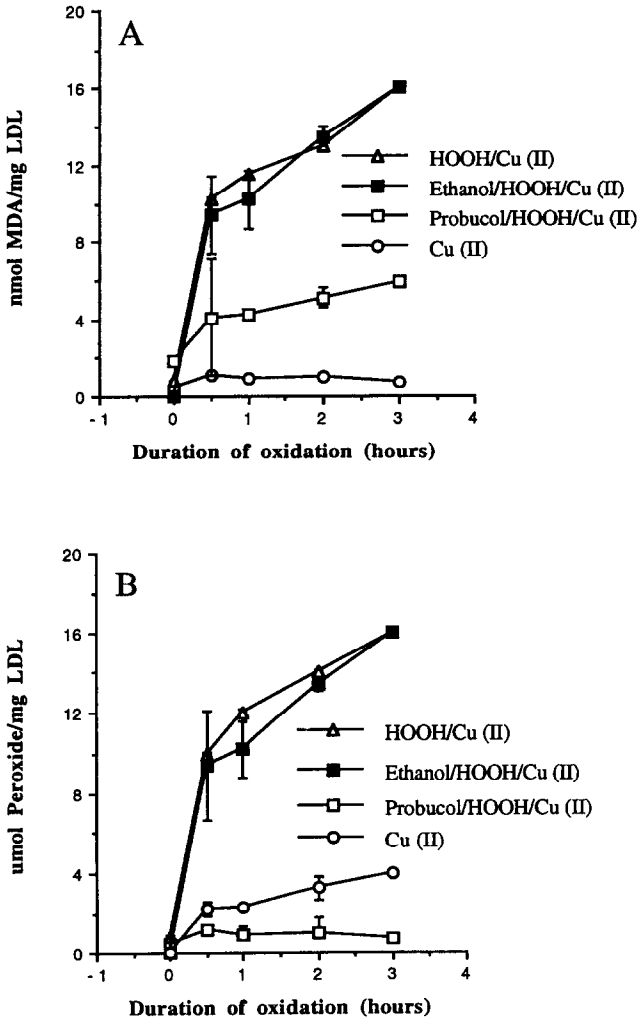

Fig. 1. LDL was oxidised as described in the text and assessed for (A) thiobarbituric acid-reactive material (expressed as malondialdehyde equivalents) and (B) lipid hydroperoxides detectable by the Xylenol orange assay. Values are expressed as the mean ± S.D. obtained from triplicate experiments. In this and all other figures: Cu(II) = exposed to Cu(II) alone; HOOH = exposed to H_2O_2/Cu(II); Ethanol/HOOH = pretreated with ethanol and exposed to H_2O_2/Cu(II); Probucol/HOOH = pre-loaded with probucol (in ethanol) and exposed to H_2O_2/Cu(II).

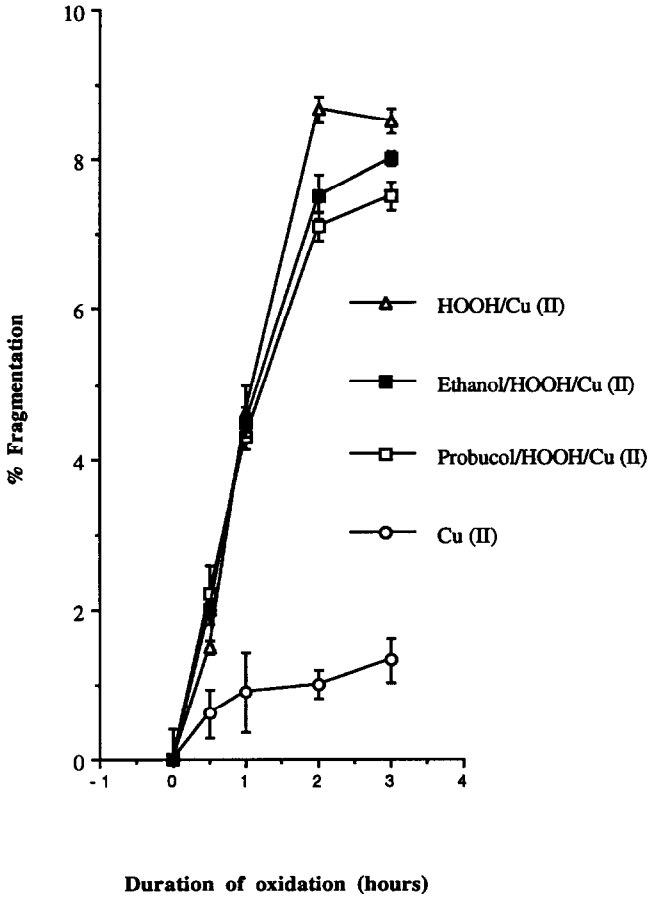

Fig. 2. Apolipoprotein B fragmentation to TCA-soluble radiolabelled fragments was determined. Values are expressed as mean ± S.D. obtained from quadruplicate assays within a representative experiment.

the baseline when monitored by the thiobarbituric acid assay. When monitored by the Xylenol orange assay, however, there was a modest increase in lipid hydroperoxides, in keeping with the known pro-oxidant activity of Cu(II) with LDL. Another control of LDL pre-loaded with ethanol alone did not exhibit any difference in susceptibility to oxidation by the H_2O_2/Cu(II) free radical generating system used in these studies. Thus, the extent of lipid peroxidation of LDL exposed to Cu(II) alone or LDL pre-loaded with probucol and exposed to H_2O_2/Cu appears to remain within a 'lag period' over this short term exposure. After 24 h exposure, lipid peroxidation was unaffected by any of the conditions used within these studies and approached the high levels of peroxide accumulation previously described [25].

Fig. 2 shows the effect of the H_2O_2/Cu(II) oxidising system upon apolipoprotein B integrity. Apolipoprotein B is the sole protein of LDL and is the site of radioiodination. During exposure to the aqueous oxidising system used in these studies the protein is fragmented to TCA-soluble radiolabelled peptides. Like the parameters of lipid peroxidation (Fig. 1), protein fragmentation occurs in a time-dependent manner. However, fragmentation differs from lipid peroxidation in that it is not limited by pre-loading LDL with probucol. There were no differences in apolipoprotein fragmentation of LDL with or without pre-loading with probucol (ethanol as a carrier) or ethanol. LDL which was incubated with Cu(II) alone showed some fragmentation, expected given the accumulation of some peroxide shown in Fig. 2 and the ability of lipid peroxide and Cu(II) to fragment protein [27].

These observations were confirmed on analysis by SDS-PAGE (Fig. 3) which again showed no inhibitory effect of probucol on fragmentation of apolipoprotein B. Interestingly, SDS-PAGE of treated LDL shows no generation of distinct peptide fragments but an increase in peptides present in LDL exposed to Cu(II) alone, as well as buffer alone (not shown), and the formation of randomly sized fragments, giving the appearance of a 'smear'. This is unlike SDS-PAGE analysis of other proteins exposed under similar conditions, which shows the formation of distinct peptides [27], becoming a 'smear' only after very extensive oxidation. The presence of small peptides in LDL exposed to Cu(II) and buffer alone over such a short period, an increase in their density and the obvious generation of peptides of a random size on exposure to H_2O_2/Cu(II) suggest that apolipopro-

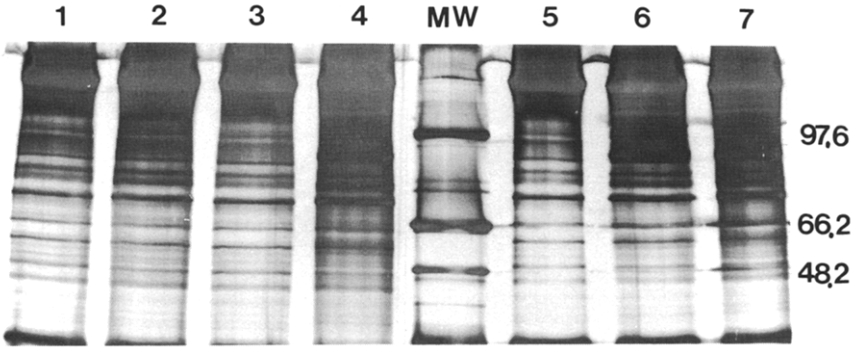

Fig. 3. Apolipoprotein B fragmentation was visualised by SDS-PAGE, using a 10% polyacrylamide [23,24] gel and staining with silver nitrate. MW = molecular weight markers of 97.4, 66.2 and 45.2 kDa, respectively. Lanes 1 and 2 = LDL exposed to Cu(II) alone for 0 and 4 h, respectively. Lanes 3 and 4 = LDL exposed to H_2O_2/Cu for 0 and 4 h, respectively. Lanes 5, 6 and 7 = probucol loaded LDL exposed to H_2O_2/Cu for 0, 2 and 4 h, respectively.

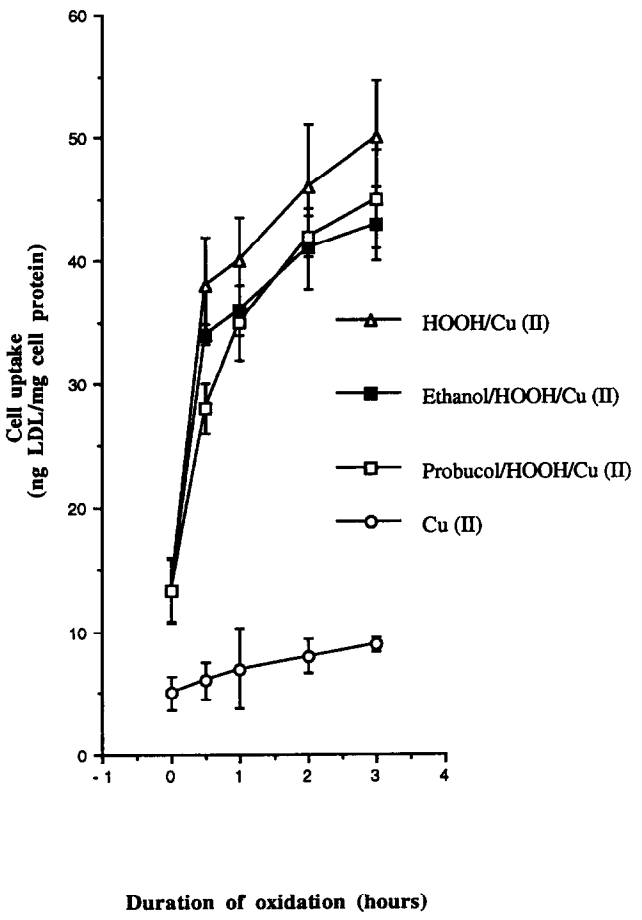

Fig. 4. The uptake of [^{125}I]LDL was monitored in P388D1s. Values are expressed as mean ± S.D. obtained from quadruplicate assays within a representative experiment.

tein B is all too readily oxidised [31]. Changes in electrophoretic mobility on agarose electrophoresis were slight and least apparent for probucol-loaded LDL exposed to H_2O_2/Cu(II) (not shown). This later observation is in keeping with the relatively low levels of lipid oxidation (Fig. 1), and also a limited subsequent apolipoprotein B aldehydic modification (a process dependent on concentration of aldehyde and time of exposure), that occurred during the short times of exposure to H_2O_2/Cu(II) used throughout these studies.

The uptake of LDL after exposure to the aqueous oxidising system used in these studies (Fig. 4) best reflected the changes observed on monitoring apolipoprotein B fragmentation, rather than the parameters of lipid oxidation shown in Fig. 1A and B. Thus, whereas probucol inhibited parameters of lipid peroxidation, it had no effect upon apolipoprotein B oxidation or subsequent uptake by P388D1s. Although lipid peroxidation and its subsequent aldehydic forms of apolipoprotein B modification have been shown to increase LDL uptake by P388D1s, via scavenger receptors [30], it would also appear that oxidative apolipoprotein B fragmentation is also associated with LDL uptake. Whether uptake of oxidised protein involves the scavenger receptor in P388D1 cells remains unclear, as does the relative importance of macrophage scavenger receptors in the lesions themselves [32–34]. It is possible that phagocytic activity plays a greater role in foam cell formation than any one specific receptor-mediated uptake [32–34]. Thus, whether the uptake of oxidised protein involves the scavenger receptor may not be highly relevant. However, P388D1 cells possess receptors for both native and modified LDL [31] and it is possible that minimally oxidised LDL (produced during these studies) is taken up via the native receptor [35]. On the other hand, there is little evidence in favour of increased uptake by this receptor on oxidation of apolipoprotein B. Generally, apolipoprotein B modification results in a decreased uptake by the native receptor [36,37].

The relative importance of whether protein oxidation aloone contributes to cell uptake is likely to depend on the initiating cause of LDL oxidation and the relative ease with which it is oxidised. For instance, recent reports suggest that hypochlorite, a powerful oxidant, can oxidatively damage apolipoprotein B and transform LDL to a high-uptake form for macrophages, without the necessity of extensive lipid oxidation or antioxidant depletion [38]. Our observations suggest that if the initiating cause of LDL oxidation in vivo takes the form of an aqueous free radical insult then there is the possibility that protein oxidation occurs first, leading to LDL uptake in the first stages of foam cell formation and atherogenesis. Perhaps lipid oxidation and accompanying aldehydic modification is pertinent to later stages of lesion development rather than earlier stages of atherogenesis. The inability of probucol to protect protein from oxidative damage, unlike DL-α-tocopherol shown in previous studies [23], makes it possible to study apolipoprotein oxidation independent of lipid peroxidation. This, in itself, makes LDL pre-loading with probucol, and perhaps other extremely lipophilic antioxidants, a useful experimental approach.

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