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Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor

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Peroxynitrite is an oxidant which could be formed in the vasculature by the reaction of superoxide with nitric oxide. It is capable of modifying amino acid residues and of initiating lipid peroxidation. In the present study we have shown that peroxynitrite converts low density lipoprotein to a form recognized by the macrophage scavenger receptor and that this process is associated with modification of the protein and lipid, and with the oxidation of α-tocopherol to α-tocopherol quinone.

Macrophage; Peroxynitrite; Low-density lipoprotein; Atherosclerosis

1. INTRODUCTION

The development of macrophage-derived foam cells within the artery wall is thought to be a key feature of the early atherosclerotic lesion [1,2]. Foam cells originate from monocyte-derived macrophages [2] which take up modified low-density lipoprotein (LDL) in an unregulated manner, via the scavenger receptor(s) [3–5]. Native LDL does not cause foam cell formation because accumulation of cholesteryl esters is prevented by a sterol dependent down-regulation of the native LDL receptor [3]. LDL can be modified so that is recognized by the macrophage scavenger receptor by any of the following means: acetylation [5], acetoacetylation [6], malondialdehyde (MDA) treatment [7], hypochlorite treatment [8] and oxidation, either by copper [9] or by cells such as endothelial cells [10,11], smooth muscle cells [12], platelets [13] and monocytes/macrophages [14,15]. LDL may also be modified by treatment with phospholipase C [16] or lipoxygenase plus phospholipase A_2 [17], and by haem proteins [18].

There is increasing evidence to indicate that oxidized LDL is the modified form of LDL which contributes to the formation of foam cells in vivo [4,19,20]. However, the physiological processes which contribute to this oxidation are not fully understood at present. A number of mechanisms have been proposed involving the generation of 'seeding lipid peroxides' within the LDL particle by the action of 15-lipoxygenase [10,14,21,22] or by superoxide-dependent oxidation [12,23,24]. However, more recent studies have provided conflicting evidence [25,26]. The 'seeding peroxide' hypothesis requires the presence of agents that are able to promote the breakdown of peroxide, once inserted into the lipoprotein particle. Possible candidates for this role include transition metal ions (e.g. copper and iron), either free or bound in the prosthetic group of proteins (e.g. haem proteins such as haemoglobin).

Alternatively, the direct abstraction of a hydrogen atom from an unsaturated fatty acid could also lead to lipid peroxidation within the LDL particle without the requirement for 'seeding peroxides' and transition metal ions. The initiation of this reaction in vivo would require the formation of a highly reactive free radical oxidant, such as the hydroxyl radical. Beckman and coworkers have recently described a possible route for hydroxyl radical formation in vivo, which involves the reaction of nitric oxide and superoxide [27]. In stimulated macrophages, superoxide is generated by NADPH oxidase, while nitric oxide is generated by the vascular endothelium. Nitric oxide modulates vascular tone and may react with superoxide to form the peroxynitrite anion [27–30]. Decomposition of peroxynitrite generates a strong oxidant with reactivity similar to the hydroxyl radical which has been shown to initiate lipid peroxidation [31–33]. We have shown that treatment of LDL with peroxynitrite [32], or with nitric oxide and superoxide in combination, can modify LDL [33,34]. In this study we demonstrate that peroxynitrite-modified

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Abbreviations: LDL, low density lipoprotein; DTPA, diethylenetriamine penta acetic acid; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; TNBS, trinitrobenzene sulphonate; PMA, phorbol myristoyl acetate; REM, relative electrophoretic mobility; BSA, bovine serum albumin.

LDL is recognized by the macrophage scavenger receptor and leads to accumulation of cholesteryl esters within these cells. We also describe the effects of peroxynitrite upon the protein, lipid and antioxidant components of the LDL particle.

2. MATERIALS AND METHODS

2.1. *Preparations*

Peroxynitrite was prepared as described in [32] and the concentration of peroxynitrite was determined spectrophotometrically at 302 nm (ε = 1670 $M^{-1} \cdot cm^{-1}$). LDL was prepared from human plasma by ultracentrifugation through a potassium bromide density gradient as described by Chung et al. [35].

2.2. *Measurement of cholesterol esterification by THP-1 macrophages*

THP-1 cells, a human monocytic cell line, were maintained essentially as described by Hassall [36]. To differentiate cells into macrophages, THP-1 cells were cultured in 6-well plates, in RPMI medium containing 10% foetal calf serum and 100 ng/ml phorbol myristoyl acetate (PMA) for 7 days [37]. The cells were then changed to RPMI medium containing 0.2% bovine serum albumin (BSA) together with 100 ng/ml PMA. After incubation for 24 h, the cells were changed to fresh medium containing 0.2% BSA and the appropriate concentration of native or peroxynitrite-modified LDL, and the incorporation of radiolabelled oleate into cholesteryl oleate was measured. This measurement was performed essentially as described by Goldstein et al. [38], except that 10 μM [9,10(n)-^{3}H]oleate/BSA complex (79 Ci/mol) was used. After incubation for 24 h, the cells were washed three times with PBS and the lipids were extracted from the cell homogenates by the method of Bligh and Dyer [39]. Cholesteryl [^{14}C]oleate was added as the internal standard (0.45 nCi/ml, 8 nM). The neutral lipids were then separated by TLC in petroleum ether (60–80°C b.p.)/diethyl ether/glacial acetic acid (90:30:1, v/v/v).

2.3. *Biochemical measurements*

The concentration of trinitrobenzene sulphonate (TNBS) reactive amino groups was measured as described in [40] and thiobarbituric acid-reactive substances (TBARS) were measured as described in [33]. α-Tocopherol and α-tocopherol quinone were determined after extraction into n-heptane and analysis by HPLC [34]. Cholesteryl ester oxidation products were measured in samples of LDL (200 μg/ml) incubated in the presence of peroxynitrite (4 mM) or $CuSO_4$ (100 μM). Diethylenetriamine penta acetic acid (DTPA) was added to all incubations at a final concentration of 100 μM. After incubation for 24 h at 37°C, samples of the LDL (0.5 ml) were extracted and dissolved in 150 μl of tetrahydrofuran/acetonitrile (80:20, v/v) and the products analyzed by HPLC as described by Carroll and Rudel [41]. The mobile phase was monitored at 235 nm and products identified by co-chromatography with oxygenated cholesteryl ester standards, prepared by treatment of cholesteryl esters of LDL with soybean 15-lipoxygenase. Quantitation of oxidation products was constrained to measurement of peak area, as extinction coefficients for these cholesteryl ester oxidation products are unknown.

3. RESULTS

Incubation of LDL with peroxynitrite results in an increase in electrophoretic mobility of the lipoprotein particle [32]. It is well known that some electronegative forms of LDL are ligands for the macrophage scavenger receptor and in the following series of experiments we determined whether peroxynitrite-modified LDL also possessed this property. Three preparations of human LDL (1 mg/ml) isolated from different donors were modified with peroxynitrite (5 mM) which resulted in a range of relative electrophoretic mobility (REM) values (1.8–4.9). This type of variability has been observed using other methods to modify oxidatively LDL when isolated from different donors [9,33]. The different samples of LDL were added to THP-1 macrophages in increasing amounts and, as shown in Fig. 1, this resulted in a marked increase in the incorporation of [^{3}H]oleate into cholesteryl oleate, indicating that this modified lipoprotein was being taken up at a much greater rate than native LDL. In addition, cholesterol esterification was saturable at high concentrations of LDL, and was dramatically reduced by polyinosinic acid, which inhibits uptake via the macrophage scavenger receptor. It is clear that, although the extent of modification (as measured by REM) and the extent of esterification are dependent upon the preparation of LDL, each experiment exhibited saturable uptake that was inhibited by polyinosinic acid.

The dependency of enhanced cholesterol esterification upon increase in REM was investigated further by increasing THP-1 macrophages with a single preparation of LDL treated with different amounts of peroxynitrite to generate samples with a range of REM values (1–3.25). The extent of cholesterol esterification depended upon the electrophoretic mobility of the lipoprotein (Fig. 2). This dependence appeared to be complex; at REM values below 2.0, a small decrease in cholesterol esterification was observed in both of the

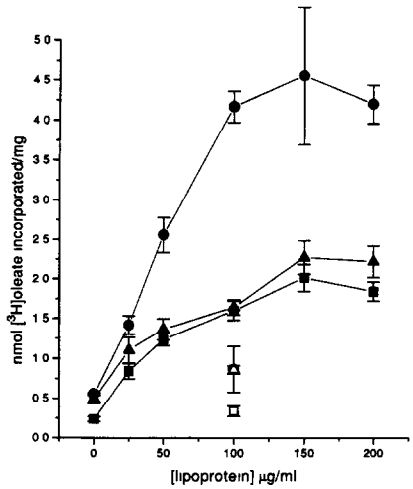

Fig. 1. Uptake of peroxynitrite-modified LDL by the macrophage scavenger receptor. THP-1 macrophages were preincubated in serum-free medium for 24 h, before the effect of peroxynitrite-modified LDL upon cholesterol esterification was assessed. Results from three independent experiments (filled symbols), using different preparations of peroxynitrite-modified LDL, are shown separately and are the mean ± S.D. of triplicate wells. The effect of polyinosinic acid (30 μg/ml) upon uptake of 100 μg/ml peroxynitrite-modified LDL (open symbols) is shown for each preparation. The REM values for each preparation were 4.9 (■), 2.54 (●) and 1.80 (▲). Native LDL (100 μg/ml) resulted in 0.82 ± 0.25 nmol [^{3}H]oleate incorporation/mg (mean ± S.D., n = 3).

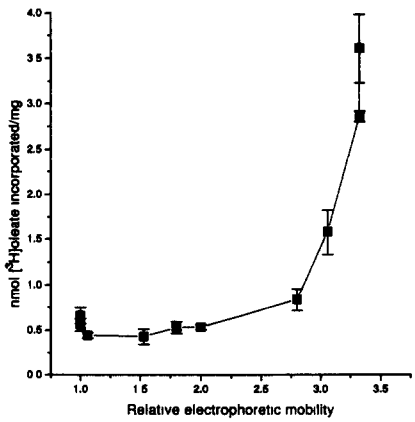

Fig. 2. Relationship between electrophoretic mobility and uptake of peroxynitrite-modified LDL by macrophages. THP-1 macrophages were preincubated in serum-free medium for 24 h, before the addition of LDL which had been treated with increasing amounts of peroxynitrite to generate samples of LDL with a range of REM values. The effect of incubation with each form of peroxynitrite-modified LDL (100 μg/ml) upon the incorporation of [^{3}H]oleate into cholesteryl esters by THP-1 macrophages is shown. Results are expressed as nmoles of [^{3}H]oleate incorporated into cholesteryl ester/mg cell protein, and are the means ± S.D. of triplicate wells from a single experiment.

independent experiments performed. At REM values above 2.0, dramatic increases in the extent of cholesterol esterification were observed. The decrease in uptake may be due to the production of a 'partially modified' form of LDL that is recognized by neither the native LDL receptor nor the scavenger receptor of the macrophage. In addition, although increasing REM is associated with increased uptake within a single preparation, this relationship does not appear to hold when different preparations of LDL are compared.

The change in mobility which occurs during the copper-dependent oxidation of LDL appears to involve the modification of positively charged amino acid residues on the protein by lipid decomposition products [9,40]. Consistent with this hypothesis, the extent and rate of the copper-dependent modification of LDL is accelerated by the addition of lipid hydroperoxides [42]. In the experiments described here peroxynitrite modification of LDL was performed in the presence of DTPA which inhibits the transition metal-dependent decomposition of lipid hydroperoxides. This suggests that modification of the LDL particle can occur in the absence of transition metals and, furthermore, increasing the lipid peroxide content of the LDL particle did not enhance the extent of oxidative modification by peroxynitrite (results not shown).

Treatment of LDL (1 mg/ml) with peroxynitrite (5 mM) decreased the concentration of TNBS-reactive amino groups by 181 ± 24 (mean ± S.E.M., n = 6) modified lysine residues per molecule of apoB. This represents a 59% of lysine residues in apoB, and an efficiency of between 11.8 and 15.2 peroxynitrite molecules per modified amino group. When lysine (1 mM) was treated with peroxynitrite (5 mM), the TNBS-reactive amino groups were reduced by only 2.3 ± 0.5% (mean ± S.E.M., n = 6), representing an efficiency of between 89 and 139 peroxynitrite molecules per modified amino group. This suggests that lysine residues within apoB are more susceptible to peroxynitrite-dependent modification, consistent with the idea that lipid peroxyl radical-dependent decomposition of lipids may play an intermediary role in this process.

We have previously shown that either treatment with peroxynitrite or exposure to superoxide and nitric oxide depletes the vitamin E content of LDL [32,34]. This effect is also observed during copper-dependent oxidation of LDL [9]. However, unlike copper, peroxynitrite

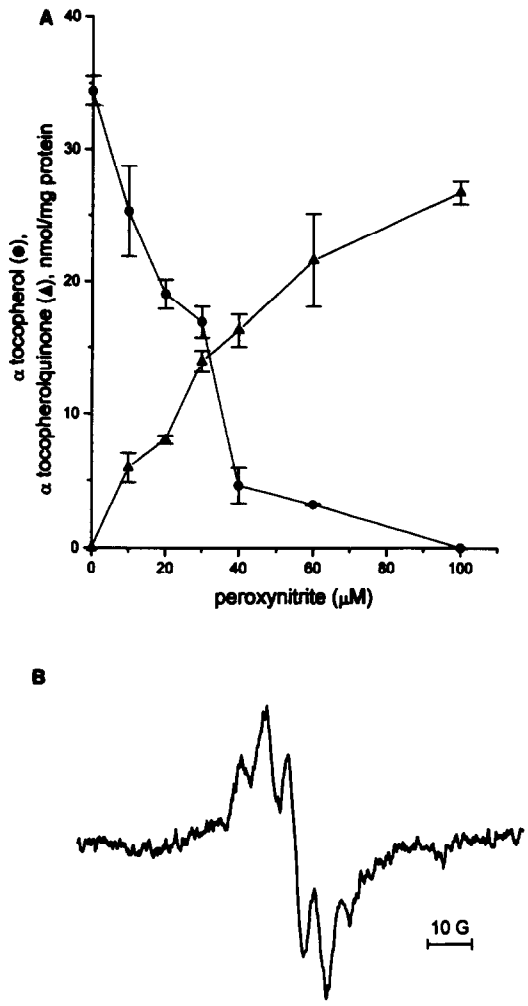

Fig. 3. Oxidation of α-tocopherol during the oxidation of LDL by peroxynitrite. Panel A shows the depletion of α-tocopherol and its conversion to α-tocopheroquinone when LDL (250 μg/ml) was incubated with the range of peroxynitrite concentrations shown. Panel B shows the ESR spectrum of a sample of LDL (6 mg/ml) treated with peroxynitrite (3 mM). The ESR conditions were as follows: modulation amplitude 2 × 105 G, microwave power 100 mW, scan range 100 G, scan time 4 min.

treatment resulted in the oxidation of α-tocopherol to α-tocopherol quinone during LDL modification (Fig. 3a). The reaction of pure α-tocopherol with peroxynitrite also generated α-tocopherol quinone (result not shown). Measurement of the esr spectrum during the initial exposure of LDL to peroxynitrite revealed the characteristic seven line spectrum of the α-tocopheroxy radical (Fig. 3b). This result suggests that free radicals are generated during the reaction of peroxynitrite and LDL.

Peroxynitrite-modified LDL was assayed for TBARS and when treated with peroxynitrite (5 mM), LDL (1 mg/ml) contained 5.9 ± 0.2 TBARS nmol/mg LDL protein (mean ± S.E.M., $n = 3$). Untreated LDL did not contain any observable TBARS and no species with maximum absorbance at 532 nm was observed during incubation of peroxynitrite and TBA.

In order to compare the effect of treatment of LDL with copper (100 μM) and peroxynitrite (1–4 mM) on production of oxidation products within the core of the LDL particle (within the cholesteryl ester fraction), we analyzed the lipid fractions from two preparations of LDL after an overnight incubation at 37°C with the above agents (Table I). Treatment with peroxynitrite generated oxidation products within the cholesteryl ester core of the lipoprotein which seemed to parallel changes in REM. Copper oxidized LDL, as expected, also contained cholesteryl ester oxidation products.

4. DISCUSSION

We have clearly demonstrated that peroxynitrite treatment modifies LDL to a form which has increased electrophoretic mobility [32] and which is recognized by the scavenger receptors which are present on THP-1 macrophages (Fig. 1). If generated in vivo, peroxynitrite-modified LDL would therefore represent a potentially atherogenic lipoprotein which could give rise to the formation of macrophage-derived foam cells.

Table I

LDL (200 μg/ml) from separate preparations were incubated with $CuSO_4$ (100 μM) or peroxynitrite (1–4 mM) for 24 h at 37°C

Condition	Preparation A		Preparation B	
	REM	Peak area	REM	Peak area
No addition	1.0	6.97	1.0	0
$CuSO_4$ (100 μM)	3.66	24.79	4.25	23.99
Peroxynitrite:				
1 mM	n.d.	n.d.	1.27	11.68
2 mM	n.d.	n.d.	1.425	12.88
4 mM	4.33	52.29	3.6	37.95

The cholesteryl ester oxidation products were identified as described in section 2. The relative electrophoretic mobility (REM) of each preparation following treatment was also measured. n.d. indicates not determined.

The mechanism by which peroxynitrite modifies LDL is as yet uncertain. The increase in electrophoretic mobility and decrease in TNBS-reactive amino groups suggest that chemical modification of lysine residues has occurred. The number of lysine residues which require modification before maximal recognition by the macrophage scavenger receptor appears to be dependent upon the modifying reagent [40,43]. Modification by succinylation or acetylation required > 60% of lysine residues to be modified whereas modification by malondialdehyde requires only 16% of lysines to give maximal scavenger receptor recognition in human monocyte macrophages. The modification of critical lysine residues of the LDL protein rather than the net negative charge of the lipoprotein was thought to determine receptor recognition [43]. Peroxinitrite treatment of LDL resulted in modification of 59% of lysine residues within the LDL particle, but of only 2.3% of free lysine in solution. This suggests that modification of apoB by peroxynitrite may be occurring indirectly. The detection of both TBARS and cholesteryl ester oxidation products indicates that peroxynitrite is able to generate other reactive species which could react with lysine amino groups and lead to LDL modification. The source of the TBARS remains uncertain, as it is possible that peroxynitrite may generate such compounds from the lipid, protein or carbohydrate components of LDL. However, the oxidation within the lipid core of the LDL particle suggests that at least some of the TBARS are lipid-derived.

Vitamin E is the major lipid-soluble radical-trapping antioxidant in plasma [44] and in LDL [9], and its depletion within the LDL particle is usually assumed to occur as a result of lipid peroxidation [9]. The virtually stoichiometric conversion of α-tocopherol to α-tocopherol quinone in LDL may indicate that peroxynitrite is reacting directly with α-tocopherol. It is possible that α-tocopherol may be acting as a two-electron reductant, generating the quinone from α-tocopherol and nitrate from peroxynitrite. However, the detection of the α-tocopheroxy radical by ESR also indicates the involvement of free radicals in the oxidation of this antioxidant although they need not necessarily be lipid-derived. For example, nitrogen dioxide has recently been shown to oxidize α-tocopherol to α-tocopherol quinone [45].

The in vivo relevance of peroxynitrite modification of LDL remains speculative. A protective role for nitric oxide in the oxidative modification of LDL by mouse macrophages has been proposed [46,47]. Jessup et al. [47] examined the effects of modulators of cellular nitric oxide synthesis, in the presence or absence of PMA (to stimulate superoxide generation). In this study, superoxide generation did not appear to play a role in the cell-mediated oxidation of LDL even in the presence of enhanced nitric oxide formation. Exposure of LDL to SIN-1, a compound which generates peroxynitrite, did result in production of substantial amounts of lipid hydroperoxides, in agreement with our findings, but did

not generate a high-uptake form of LDL. However, the largest increase in REM observed was 1.39. From our study of peroxynitrite treatment of LDL it appears likely that an REM of approxiately 2 is required for enhanced LDL uptake by THP-1 macrophages. A similar observation has been made for copper-oxidized LDL incubated with mouse peritoneal macrophages [48]. It is possible that within the arterial wall the generation of a low steady-state concentration of peroxynitrite may result from a low, but continuous, flux of both nitric oxide and superoxide. This low level of oxidant, acting over a prolonged period of time, may constitute a significant oxidative stress. Peroxynitrite may also act to increase the susceptibility of LDL to oxidation by other agents such as transition metal ions and haem proteins, possibly by depletion of vitamin E [32].

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