

Interaction of 4-hydroxynonenal-modified low-density lipoproteins with the fibroblast apolipoprotein B/E receptor

Wendy JESSUP,* Gunther JURGENS,† Johanna LANG,‡ Hermann ESTERBAUER‡ and Roger T. DEAN*

*Cell Biology Research Group, Department of Applied Biology, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K., and †Institute of Medical Biochemistry and ‡Institute of Biochemistry, University of Graz, Harrachgasse 21 and Schuberstrasse 1, A-8010 Graz, Austria

The incorporation of the lipid peroxidation product 4-hydroxynonenal into low-density lipoprotein (LDL) increases the negative charge of the particle, and decreases its affinity for the fibroblast LDL receptor. It is suggested that this modification may occur *in vivo*, and might promote atherogenesis.

INTRODUCTION

Low-density lipoproteins are implicated in the pathogenesis of atherosclerosis. Much interest has been given to the mechanisms by which LDL uptake by various cell types might be altered, to produce the pattern of cholesterol deposition characteristic of atherosclerosis. Recently, evidence has been accumulating that lipid peroxidation may be a contributory factor in atherogenesis. Enhanced levels of serum lipid peroxides have been measured in individuals at risk (Yagi, 1984), and there are some data which suggest that lipid peroxidation is involved in cell-mediated modifications to LDL which enhance its endocytosis by macrophages (Steinbrecher *et al.*, 1984). One way in which this process might affect LDL uptake is by reaction of lipid peroxidation products with the apolipoprotein B moiety. Malondialdehyde has been shown to react with lysine residues in apolipoprotein B, thereby abolishing its affinity for the fibroblast LDL receptor and promoting uptake by the macrophage scavenger receptor (Haberland *et al.*, 1982). We have recently shown that another product of lipid peroxidation, HNE, also reacts with LDL, and at much lower concentrations than those required for malondialdehyde (Jurgens *et al.*, 1984, 1985). We now report on the effects of HNE modification on LDL uptake by the fibroblast LDL receptor.

MATERIALS AND METHODS

Culture of cells

Human skin fibroblasts from normal and homozygous familial hypercholesterolaemic (receptor-deficient) donors were maintained in monolayer culture in a humidified incubator (5% CO₂ in air) at 37 °C in Eagle's minimal essential medium containing 10% (v/v) heat-inactivated foetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml), and were used between passages 10 and 20. For experiments, the cells were harvested by trypsinization and seeded at near-confluent density (10⁵ cells/dish) in 24 mm dishes. After 24 h incubation, the cultures were washed with phosphate-buffered saline and incubated in Eagle's minimal essential medium containing 10% (v/v) lipoprotein-deficient

serum (final concentration 24 mg/ml) plus antibiotics for 48 h before use in experiments. Each dish contained approx. 60–70 µg of cell protein.

Isolation and modification of lipoproteins

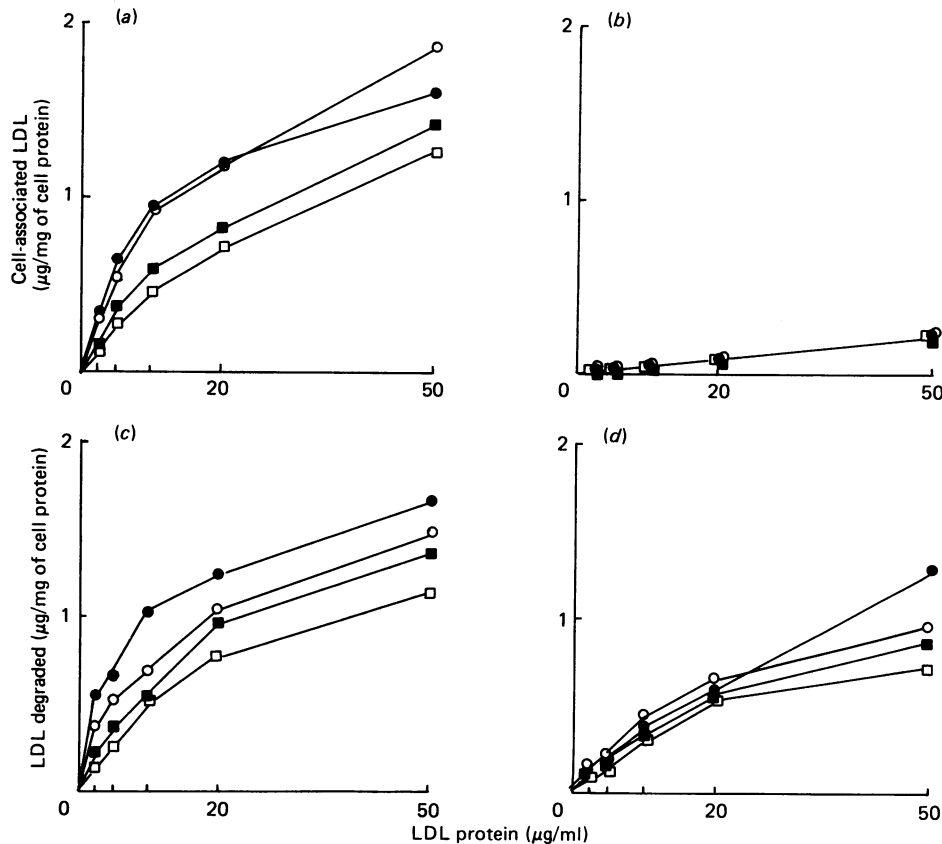
Freshly-drawn blood from normal donors was collected in 2 mM-EDTA and LDL was isolated from the plasma by differential density ultracentrifugation (Havel *et al.*, 1955) between densities of 1.02 and 1.05. HNE was prepared by chemical synthesis as previously described (Esterbauer & Weger, 1967), and was dissolved in 0.1 M-Tris/HCl (pH 7.5) containing 0.16 M-NaCl, EDTA (1 mg/ml) and chloramphenicol (0.1 mg/ml) (incubation buffer). The exact HNE concentration was determined by A_{222} , with ϵ 13600 M⁻¹·cm⁻¹. LDL was prepared in incubation buffer at various concentrations (22–57 mg/ml); HNE was added in the same buffer to give a final concentration of 1–7 mM, and incubations were performed at 37 °C for 4 h under N₂, together with control incubations, in which either HNE was omitted (reference LDL) or LDL was omitted (to correct for possible self-decomposition of the aldehyde). Unchanged HNE was removed from LDL by chromatography on Bio-Gel A-15m, or by dialysis against several changes of the incubation buffer. Native and modified lipoproteins were routinely labelled with ¹²⁵I by the Iodogen method (Salacsinski *et al.*, 1981). The labelled LDLs were separated from low- M_r ¹²⁵I by chromatography on Sephadex G-25 equilibrated with phosphate-buffered saline containing 0.01% EDTA, then dialysed against several changes of the same solution at 4 °C for 48 h. Immediately before experiments, the labelled and unlabelled lipoproteins were combined to the appropriate specific activity, dialysed for 1 h against phosphate-buffered saline, then sterilized by passage through 0.45 µm membrane filters and samples were taken to determine the exact specific radioactivity. Of the radioactivity, 95–99% was precipitable in 10% (w/v) trichloroacetic acid. In some experiments, LDL labelled by the ICl method (Bilheimer *et al.*, 1975) was also used; no differences between the uptake properties of LDLs labelled by these alternative methods were found. Lipoprotein-deficient human serum was prepared as described (Knight & Soutar, 1982).

Table 1. Dependence on HNE and LDL incubation concentrations of the relative electrophoretic mobilities of HNE-modified LDL preparations

Abbreviation used: n.d.; not determined.

| Batch | Concentration of HNE in the incubation (mM) | Concentration of LDL in the incubation (mg/ml)* | Relative electrophoretic mobility (R_m) of HNE-LDL† | HNE consumed after 4 h incubation (mM) |
|----------|---|---|---|--|
| <i>a</i> | 1.0 | 31 | 1.1 | n.d. |
| <i>b</i> | 1.0 | 57 | 1.1 | 0.81 |
| <i>c</i> | 6.0 | 57 | 1.35 | 4.92 |
| <i>d</i> | 6.5 | 31 | 1.45 | n.d. |
| <i>e</i> | 7.0 | 22 | 1.5 | 4.32 |
| <i>f</i> | 3.0+6.0‡ | 8 | 1.52 | n.d. |

* Total LDL concentration was determined by dry weight estimation.
† Anodic mobility in 1.0% agarose is expressed relative to reference unmodified LDL incubated under the same conditions except that HNE was omitted.
‡ After removal of excess HNE by chromatography on Bio-Gel A-15m, material from batch *c* was reincubated with 3.0 mM-HNE.

**Fig. 1. Uptake of HNE-modified low-density lipoproteins by fibroblasts from normal and familial hypercholesterolaemic subjects**

Confluent monolayers of normal (*a, c*) and familial hypercholesterolaemic (*b, d*) fibroblasts were incubated in lipoprotein-deficient medium supplemented with the indicated concentrations of ^{125}I -labelled lipoproteins (specific activities 290–490 d.p.m./ng of LDL protein) for 4 h at 37 °C. LDL associated with (*a, b*) and degraded by (*c, d*) the cells was determined as described in the Materials and methods section. ●, Normal LDL; ○, HNE-LDL (batch *b*, R_m 1.1); ■, HNE-LDL (batch *c*, R_m 1.35); □, HNE-LDL (batch *f*, R_m 1.52). Further details of the modified LDLs are given in Table 1. Data are means of quadruplicate determinations.

Measurement of lipoprotein uptake

For experiments, the medium was removed and replaced with fresh Eagle's minimal essential medium, supplemented with 10% (v/v) lipoprotein-deficient serum, 20 mM-Hepes, penicillin (100 units/ml), strepto-

mycin (100 µg/ml) plus lipoproteins as appropriate and the cultures were incubated in a humidified incubator at 37 °C. The final volume in each well was 0.5 ml. At the end of the incubation, the medium was removed, and trichloroacetic acid-soluble iodide-free radioactivity was determined as described by Knight & Soutar (1982).

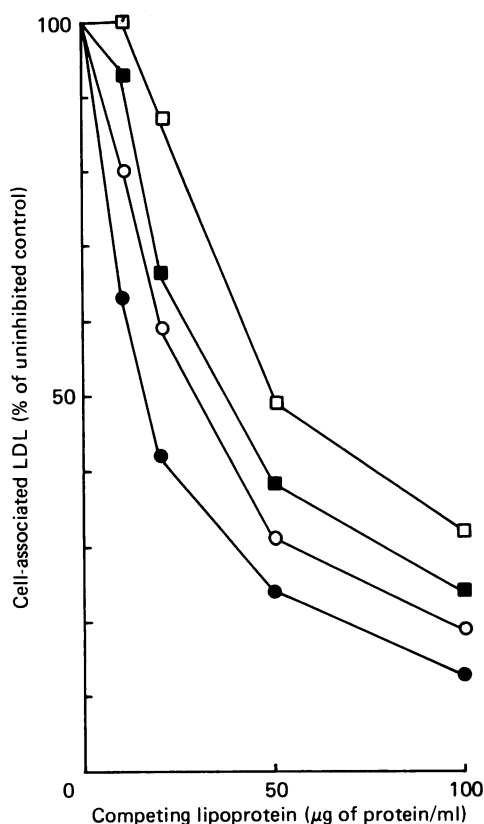


Fig. 2. Uptake of ^{125}I -labelled LDL by normal fibroblasts in the presence of HNE-modified LDLs

Confluent monolayers of fibroblasts were incubated with lipoprotein-deficient medium containing ^{125}I -LDL ($5 \mu\text{g/ml}$; 360 d.p.m./ng) plus the indicated concentrations of unlabelled HNE-modified lipoproteins at 37°C for 4 h, after which time cell-associated radioactivity was determined as described in the Materials and methods section. At the end of the incubation, the amount associated with control cells (incubated without competing unlabelled lipoproteins) was 654 ng/mg of cell protein. Data are means of duplicate determinations. For explanation of symbols, see Fig. 1.

Fibroblasts were washed as described by Goldstein *et al.* (1976) and dissolved in 0.1 M-NaOH for assay of protein and radioactivity.

Other methods

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Agarose electrophoresis of LDL was performed at pH 8.6 using the Lipidophor system (Immuno AG, Austria).

RESULTS

Table 1 shows the properties of several HNE-modified LDL preparations. HNE reacts with lysine residues of apolipoprotein B, but we also have evidence that other amino acids are involved in the reaction (tyrosine and, to a lesser extent, serine, histidine and cysteine), and also the lipid moiety of the lipoprotein particle (Jurgens *et al.*, 1985).

The uptake of ^{125}I -labelled normal and HNE-modified

LDLs by human fibroblasts was measured. The amounts of lipoprotein (a) cell-associated and (b) degraded were determined separately. It was found that in normal fibroblasts the HNE-modified LDLs were endocytosed less rapidly than normal LDL, and that the extent of this effect was proportional to the amount of HNE incorporated into the LDL particle. This response was seen both in the incorporation of label in the cells and in the release of degraded materials to the medium (Fig. 1). The effect of HNE modification on uptake was most pronounced at concentrations of $20 \mu\text{g/ml}$ and below; between 20 and $50 \mu\text{g/ml}$, the increment in LDL uptake was hardly affected by HNE incorporation. This suggests that HNE modification of LDL alters its affinity for the LDL receptor, without affecting the rate of endocytosis by the low-affinity non-saturable route which predominates at higher lipoprotein concentrations (Goldstein & Brown, 1974; Knight & Soutar, 1982). This is supported by the lack of any effect of HNE modification on LDL endocytosis by fibroblasts of a receptor-negative familial hypercholesterolaemic homozygote (Fig. 1).

The competitive inhibition of the internalization and degradation of ^{125}I -labelled normal LDL by various unlabelled preparations of LDL modified with HNE is shown in Fig. 2 and Table 2. These data also indicate that HNE-modified LDL has a lower affinity for the LDL receptor than has normal LDL, but a similar low-affinity non-saturable rate of uptake.

There was no evidence that HNE-modified LDL is toxic to the cells used in these experiments, as judged by release of the cytosolic enzyme, lactate dehydrogenase, into the medium, or by recovery of cell protein at the end of the incubations.

DISCUSSION

There is increasing evidence that lipid peroxidation is involved in the atherogenic process, and may even be an initiating factor (Yagi, 1984). Of particular interest are recent reports that the modification *in vitro* of lipoproteins by cultured endothelial and smooth muscle cells involves lipid peroxidation initiated by free radicals produced by the cells (Steinbrecher *et al.*, 1984; Morel *et al.*, 1984). In this report we have studied the interaction of one product of lipid peroxidation, HNE, with human LDL, and its effect on the uptake and degradation of LDL. Our results indicate that HNE-modified LDL has a reduced affinity for the fibroblast LDL receptor. This is consistent with several other reports (Goldstein *et al.*, 1979; Gonen *et al.*, 1983; Knight & Soutar, 1982) in which blocking of lysine residues by a range of functional groups lowers the affinity of apolipoprotein B in the LDL particle for its receptor.

Our interest in HNE interaction with LDL lies in the demonstration that this molecule is produced during lipid peroxidation, and is therefore likely to be available *in vivo* for reaction with LDL in some circumstances, such as during platelet aggregation at sites of arterial injury, or within the LDL particle itself as a result of free radical-initiated peroxidation. Although both MDA and HNE will be generated under such circumstances, HNE was shown to be much more reactive with LDL (Jurgens *et al.*, 1984). In addition, HNE has a higher lipid solubility than MDA, and therefore may have greater accessibility to regions of the lipoprotein under some circumstances.

Table 2. Uptake of ¹²⁵I-labelled LDL by normal human fibroblasts in the presence of competing unlabelled HNE-modified LDLs

¹²⁵I-LDL was supplied at 10 µg of protein/ml and 100 d.p.m./ng; unlabelled lipoproteins were added simultaneously at 100 µg/ml. The HNE-LDL used was batch *e* (see Table 1 for details). Cultures were incubated for 4 h at 37 °C; other details are as in the Materials and methods section.

| Competing LDL | ¹²⁵ I-LDL uptake (ng/mg of cell protein) | | Uptake as % of uninhibited control |
|---------------|---|-----------|------------------------------------|
| | Cell-associated | Degraded | |
| No addition | 939 ± 75 | 743 ± 103 | 100 |
| Reference LDL | 283 ± 3 | 161 ± 60 | 26 |
| HNE-LDL | 496 ± 70 | 254 ± 34 | 45 |

In preliminary experiments, we have also compared the uptake of normal, HNE-modified, and acetylated LDLs by mouse resident peritoneal macrophages. At the degree of HNE substitution used in the LDL preparations reported in this paper, no enhancement of LDL uptake was observed. However, the most highly substituted LDL contained only 45 mol of HNE-substituted lysine/mol of LDL. This is below the threshold value for recognition of MDA-modified LDL by the macrophage 'scavenger' receptor (Haberland *et al.*, 1982), and therefore would not be expected to enhance macrophage endocytosis. We are currently studying the endocytosis of more highly substituted LDL preparations to determine whether HNE modification may directly promote macrophage uptake by the 'scavenger' receptor.

The initial effect of modification of LDL by HNE *in vivo* is likely to be to increase the circulation time of the LDL particle in the plasma and residence time in the subendothelial space. In other instances where circulation is prolonged, such as familial hypercholesterolaemia and diabetes mellitus, the outcome is an increased incidence of atherosclerosis. This may result from increased availability and/or susceptibility of the particle to further modification (by products of lipid peroxidation such as HNE or by other mechanisms) or to the formation of antibodies to the altered LDL. In either case, the net effect is likely to be accelerated endocytosis of the LDL by mononuclear phagocytes, and subsequent foam cell generation. We propose that a similar outcome might result from modification *in vivo* of low density lipoproteins by HNE.

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