Modification of Low Density Lipoprotein with 4-Hydroxynonenal Induces Uptake by Macrophages

Henry F. Hoff, June O'Neil, Guy M. Chisolm III, Thomas B. Cole, Oswald Quehenberger, Hermann Esterbauer, and Günther Jürgens

There is indirect evidence that the oxidation of low density lipoprotein (LDL) may be involved in the development of atherosclerosis. Modification of LDL by oxidation may lead to its unregulated uptake by intimal macrophages to form foam cells. Because of the complexity of events occurring during LDL oxidation, we have tested whether LDL modified directly with 4-hydroxynonenal (HNE), a major propagation product formed during lipoperoxidation and known to be present in oxidized LDL, could bring about lipid loading of macrophages. Modification was accomplished by incubating LDL with various concentrations of HNE up to 7.5 mM. When LDL was derivatized with lower concentrations of HNE, concentration-dependent increases were observed in the covalent binding of HNE to apolipoprotein B (apo B), the blockage of the ε-amino groups on lysine residues of apo B, and the relative electrophoretic mobility of LDL. Decreases were observed in degradation of the modified LDL by the J774 cell line, mouse peritoneal macrophages, and smooth muscle cells. Modification of LDL by incubation with the higher concentrations of HNE resulted in LDL aggregation. This modification was associated with marked increases in the macrophage degradation of LDL. Degradation of aggregated HNE-modified LDL increased linearly with incubation time, leading to lipid loading of these cells as observed by oil red O staining and cholesterol accumulation. Uptake appeared to occur by phagocytosis, since cytochalasin D, an inhibitor of phagocytosis, quantitatively inhibited uptake and degradation of labeled HNE LDL. Uptake did not appear to be mediated by either the LDL receptor or the scavenger receptor, since competition with excess amounts of LDL or acetyl LDL failed to inhibit degradation of labeled, aggregated HNE LDL. Saturation of degradation of HNE LDL by macrophages could be attributed, in part, to steric hindrance, since both excess HNE LDL and other particulate ligands could inhibit this degradation. These studies suggest that interaction of LDL with HNE formed during lipid peroxidation could be responsible for structural modifications leading to unregulated uptake of the lipoprotein by tissue macrophages. This could partially explain lipid loading or foam cell formation in atherosclerosis. (Arteriosclerosis 9:538-549, July/August 1989)
directed at B-100 in LDL and malondialdehyde (MDA)-modified proteins co-localize to sites in lesions from WHHL rabbits, suggesting that the LDL in the atherosclerotic lesion has been modified by MDA, an aldehyde produced during lipid peroxidation. 

Another major propagation product of lipid peroxidation formed in oxidized LDL is 4-hydroxy-2,3-trans-nonenal (HNE). Reports on the structural and functional properties of LDL modified directly with HNE indicate that certain properties of HNE LDL are similar to those of oxidized LDL. These properties include increased electron negativity, blockage of lysyl residues on apolipoprotein (apo) B, increased fluorescence at 360 nm excitation and 430 nm emission, and reduced recognition by the LDL receptor on fibroblasts. In the present study, we wished to determine whether modification of LDL directly with HNE would lead to enhanced recognition by macrophages and, if so, to determine the mode of cellular recognition and the structural changes in the modified LDL responsible for the enhanced uptake.

**Methods**

**Materials**

4-hydroxy-2,3-trans-nonenal was synthesized as described and was stored as a 10 to 20 mg/ml solution in dichloromethane at −70°C. Aqueous solutions were prepared by procedures described previously. HNE concentrations were determined by spectroscopy by using ultraviolet absorbance at 223 nm and a molar absorptivity coefficient of 13.75 x 10^4. 4-hydroxy-2,3-trans-nonenal was produced from U-14C-oleic acid by lipoygenase-catalyzed formation of its 15-monohydroperoxide. The 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) was subsequently decomposed aerobically in the presence of ascorbate/Fe++. Dulbecco’s Modified Eagle’s Medium (DMEM), fetal calf serum, and penicillin-streptomycin solution were obtained from GIBCO Laboratories (Grand Island, NY). Bovine serum albumin (BSA, fatty acid-free), oleic acid, cholesteryl oleate, cytochalasin D, and zymosan were purchased from Sigma Chemical Corporation (St. Louis, MO). Carrier-free Na^125I was purchased from ICN Pharmaceuticals, Incorporated (Irvine, CA), and 1-14C-oleic acid (48 mCi/ml) and [1,2,6,7-3H(N)]cholesteryl oleate (82.7 mCi/nmol) were purchased from New England Nuclear (Wellington, DE). Pathogen-free C57Bl/6 mice (9 to 12 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, NY).

**Preparation of Low Density Lipoproteins and Modified Forms**

Human LDL (density 1.019 to 1.063 g/ml) was prepared by the procedure of Hatch and Lees from plasma obtained from the American Red Cross Blood Services of Cleveland. It was stored in 0.15 M NaCl containing 0.3 mM ethylenediaminetetraacetic acid (EDTA). Acetyl LDL was formed by repeated additions of acetic anhydride to LDL until over 80% of the lysyl residues on apo B were blocked. HNE-modified LDL was formed by incubating LDL in the dark under N₂ with an aqueous solution of HNE at final concentrations ranging from 1 to 8 mM in 0.1 M Na phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.3 mM EDTA for 5 hours at 37°C. The HNE-modified LDL was then dialyzed for 12 hours against 0.01 M Na phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA to remove unreacted HNE. Ethanol-treated LDL was prepared by incubating LDL (2 mg protein/ml) with absolute ethanol 1:1 (vol/vol) for 15 minutes and subsequently dialyzing the lipoprotein overnight against 0.15 M NaCl-EDTA, pH 7.4. This procedure produced a turbid solution.

LDL was labeled with ^125I to a specific activity of 100 cpm/pg by using the iodine monochloride procedure described by Bilheimer et al. Acetylation was performed before iodination, whereas modification with HNE was performed after iodination. The protein content of lipoproteins was determined by the bicinchoninic acid (BCA) assay as described except that a 60-minute, 60°C heating step was used, and BSA was employed as a standard. The cholesterol content was determined by a modification of the procedure of Roelsch et al. using the Reagent Set kit from Boehringer-Mannheim (Indianapolis, IN). Lipoprotein concentrations were routinely expressed as µg protein/ml.

Agarose gel electrophoresis of LDL was performed on pre-made agarose gels (Corning, Palo Alto, CA) according to the manufacturer’s instructions at 90 V for 50 minutes. LDL migration was assigned a value of one, and all other preparations were compared to LDL to obtain a relative electrophoretic mobility (REM). Lipoproteins were visualized by staining with fat red 7B in methanol as described by the supplier (Corning, Palo Alto, CA).

Vertical sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with 3% to 12% gradient gel was performed on ^125I-labeled lipoproteins according to the procedure of Laemmli with the following modifications. The samples were reduced and denatured in the presence of SDS and mercaptoethanol as described, except that samples were heated for 2 minutes at 100°C and then incubated for 1 hour at 55°C. After cooling, the lipoproteins were delipidated by adding an equivalent volume of diethyl ether. Phase separation was achieved by centrifugation for 10 minutes at 10 000 g. The top of the aqueous phase was washed with ether to remove residual lipids. Samples were then heated to 50°C for 5 minutes in open vessels to evaporate the residual ether. Intact ^125I-labeled lipoprotein samples were assessed in 3% to 8% nondenaturing polyacrylamide gels. After electrophoresis, the gel was placed in 50% methanol overnight. Wet gels were then sealed in plastic bags and subjected to autoradiography with X-omat K film in an X-omat cassette containing an intensifying screen (Kodak, Rochester, NY). Apo B in derivatized LDL was isolated by using the procedure of Bhag and Dyer and solubilizing in 3% SDS. In one series of experiments, ^14C-HNE was added to unlabeled HNE and was made to interact with...
suspended cells were disrupted with a probe sonicator at 20 W for 10 seconds. The lipids in the sonicated mixture were identified by co-migration with a cholesteryl oleate standard.

Results

Chemical and Structural Changes in Low Density Lipoprotein Modified with HNE

When LDL was reacted with increasing concentrations of HNE, the LDL exhibited the following changes:

1) a reduction in unblocked lysine residues on apo B.

2) an increase in blocked lysine residues on apo B.

3) a decrease in unblocked lysine residues on apo B.

4) a decrease in unblocked lysine residues on apo B.

5) a decrease in unblocked lysine residues on apo B.

6) a decrease in unblocked lysine residues on apo B.

7) a decrease in unblocked lysine residues on apo B.

8) a decrease in unblocked lysine residues on apo B.

9) a decrease in unblocked lysine residues on apo B.

10) a decrease in unblocked lysine residues on apo B.

11) a decrease in unblocked lysine residues on apo B.

12) a decrease in unblocked lysine residues on apo B.

13) a decrease in unblocked lysine residues on apo B.

14) a decrease in unblocked lysine residues on apo B.

15) a decrease in unblocked lysine residues on apo B.

16) a decrease in unblocked lysine residues on apo B.

17) a decrease in unblocked lysine residues on apo B.

18) a decrease in unblocked lysine residues on apo B.

19) a decrease in unblocked lysine residues on apo B.

20) a decrease in unblocked lysine residues on apo B.

21) a decrease in unblocked lysine residues on apo B.

22) a decrease in unblocked lysine residues on apo B.

23) a decrease in unblocked lysine residues on apo B.

24) a decrease in unblocked lysine residues on apo B.

25) a decrease in unblocked lysine residues on apo B.

26) a decrease in unblocked lysine residues on apo B.

27) a decrease in unblocked lysine residues on apo B.

28) a decrease in unblocked lysine residues on apo B.

29) a decrease in unblocked lysine residues on apo B.

30) a decrease in unblocked lysine residues on apo B.

31) a decrease in unblocked lysine residues on apo B.

32) a decrease in unblocked lysine residues on apo B.

33) a decrease in unblocked lysine residues on apo B.

34) a decrease in unblocked lysine residues on apo B.

35) a decrease in unblocked lysine residues on apo B.

36) a decrease in unblocked lysine residues on apo B.

37) a decrease in unblocked lysine residues on apo B.

38) a decrease in unblocked lysine residues on apo B.

39) a decrease in unblocked lysine residues on apo B.

40) a decrease in unblocked lysine residues on apo B.

41) a decrease in unblocked lysine residues on apo B.

42) a decrease in unblocked lysine residues on apo B.

43) a decrease in unblocked lysine residues on apo B.

44) a decrease in unblocked lysine residues on apo B.

45) a decrease in unblocked lysine residues on apo B.

46) a decrease in unblocked lysine residues on apo B.

47) a decrease in unblocked lysine residues on apo B.

48) a decrease in unblocked lysine residues on apo B.

49) a decrease in unblocked lysine residues on apo B.

50) a decrease in unblocked lysine residues on apo B.
Table 1. Chemical and Structural Changes in Low Density Lipoprotein after Modification with 4-Hydroxynonenal

<table>
<thead>
<tr>
<th>Concentration of HNE used to modify LDL (mM)</th>
<th>% lysine residues on apo B that remain unblocked (mean±SD)</th>
<th>Relative electrophoretic mobility</th>
<th>% LDL precipitable at 10,000 g</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>100±5</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td>0.75</td>
<td>81±1</td>
<td>1.13</td>
<td>7</td>
</tr>
<tr>
<td>1.5</td>
<td>75±2</td>
<td>1.25</td>
<td>6</td>
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<tr>
<td>3.0</td>
<td>61±1</td>
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<td>4.5</td>
<td>53±10</td>
<td>1.75agg*</td>
<td>32</td>
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<td>53±2</td>
<td>agg*</td>
<td>92</td>
</tr>
<tr>
<td>7.5</td>
<td>52±1</td>
<td>agg*</td>
<td>98</td>
</tr>
</tbody>
</table>

*agg=LDL aggregated and remaining at the origin during electrophoresis.

Low density lipoprotein (LDL) was modified to varying degrees by incubation with different concentrations of 4-hydroxynonenal (HNE) as described in Methods. Blockage of lysine residues on apolipoprotein (apo) B was determined in triplicate by the TNBS procedure, and was expressed as the percent lysine residues on apo B that remained unblocked. The relative electrophoretic mobility of each LDL was determined as described in Methods, as was the percent of LDL precipitable by centrifugation at 10,000 g as an estimate of particle aggregation. Aggregation resulted in a fraction of LDL remaining at the origin during electrophoresis.

- An increase in REM, and 3) an increase in the proportion of LDL that was pelleted by centrifugation at 10,000 g (Table 1). This last property reflected the increasing turbidity and aggregation observed when LDL was modified with HNE concentrations exceeding 3 mM. The reduction in blocked lysines increased with increasing modification until LDL aggregation was extensive (4.5 mM HNE). With increasing aggregation, more LDL remained at the origin during electrophoresis; at modifications that used 6 mM and 7.5 mM HNE, LDL migration from the origin was undetectable. The intrinsic fluorescence at 360 nm excitation/430 nm emission of both LDL and delipidated apo B was determined in triplicate by the TNBS procedure, and was expressed as the percent lysine residues on apo B of modified LDL that was pelleted by centrifugation at 10,000 g.

In separate experiments, the amount of HNE associating with LDL was quantitated for the various concentrations of HNE reacted with LDL. Using [14C]-HNE, we determined that 157 mol of HNE were associated with each mole of LDL, when modification altered the REM of LDL to 1.7 without causing aggregation. Higher levels of modification led to significant aggregation. For example, when HNE LDL was modified so that 90% was precipitable by centrifugation, the pellet and supernatant fractions contained 312 mol and 292 mol of HNE per mole of LDL, respectively.

Because of minor variations among experiments in the degree to which a particular batch of LDL was modified by incubation with a particular concentration of HNE, we also report the alteration in the degree of precipitation by centrifugation for turbid fractions. For convenience, LDL modified by reaction with 3 mM or 6 mM HNE are referred to as 3 mM HNE LDL or 6 mM HNE LDL.

When samples of 0 mM, 3 mM (unaggregated), and 6 mM HNE LDL (95% precipitable) were viewed by transmission electron microscopy after negative staining, the 0 mM HNE LDL showed only monomeric LDL particles (Figure 1A). The 3 mM HNE LDL samples showed slight aggregation, appearing as particles binding to one another (Figure 1B). The 6 mM HNE LDL preparation contained some monomeric LDL particles but consisted predominantly of clusters of varying sizes (Figure 1C), which occasionally reached diameters over 1 μm (data not shown). In addition to monomeric and aggregated LDL particles, larger particles that may represent LDL particles that had coalesced were observed (Figure 1C). These larger structures were more prevalent with increasing modification (data not shown).

To further examine these modified particles, [125I]-LDL was modified with 3 mM HNE (resulting in a sample that was 25% precipitable) and was subjected to nondenaturing PAGE with 3% to 8% gradient polyacrylamide gels followed by autoradiography. A band was present in the stacking gel, probably representing the precipitable aggregates. The most intense band comigrated with apo B of unmodified LDL (Figure 2A). Two weaker bands with slower migration rates were also present. These weaker bands probably represented smaller aggregates of LDL, since such aggregates were seen by electron microscopy in the supernatant after centrifugation (data not shown).

To determine whether particle aggregation was accompanied by changes in the structure of apo B, we subjected delipidated samples of HNE LDL to SDS-PAGE. Samples of [125I]-LDL were modified with 0 (sham-treated), 2.5, 3.5, 4.5, or 5.5 mM HNE, and equal amounts of radioactivity in each delipidated sample were applied to 3% to 12% gradient polyacrylamide gels. Autoradiograms showed that only the characteristic B-100 band was seen in sham-treated LDL (Figure 2B). By contrast, the apoprotein from LDL modified with 2.5 mM HNE appeared as a band of molecular weight higher than B-100, analogous to the intermediate bands seen in nondenaturing gels of intact LDL (Figure 2A), which showed low-level aggregation. The integrity of these higher molecular weight bands in SDS-PAGE gels were accompanied by a corresponding decrease in intensity of the B-100 band. The apoprotein from LDL treated with 3.5, 4.5, and 5.5 mM HNE showed increasing retention in the stacking gel. The proportion of total applied label that was recovered in the stacking gel was 0.4%, 2%, 19%, 53%, and 60%, respectively, for apo B from LDL modified with 0, 2.5, 3.5, 4.5, and 5.5 mM HNE. A decrease in intensity of the high molecular weight band entering the 3% to 12% gel was seen with increasing modification of LDL from 3.5 to 5.5 mM HNE (Figure 2B). Concurrently, the original B-100 band of the apoprotein diminished and ultimately disappeared at the highest level of modification. Bands of molecular weight lower than B-100 representing fragments of apo B were not detectable in any of the samples.

**Cellular Uptake and Degradation of HNE Low Density Lipoprotein**

We have studied the uptake of HNE LDL by cells in culture, including the J774 macrophage cell line, mouse peritoneal macrophages (MPM), and rabbit aortic smooth muscle cells (SMC). Most experiments described in this study were performed in both MPM and J774 cells, and
the results do not differ qualitatively, except for enhanced recognition by the LDL receptor of weakly modified LDL in the latter, since J774 cells express much higher levels of LDL receptor activity.\(^3\) Uptake and degradation of highly aggregated LDL were found to be more highly reproducible when MPM were used than when J774 cells were used. When degradation of radiolabeled LDL was measured as an index of uptake of HNE LDL, we found that uptake in J774 cells decreased with increasing modification with HNE. In several experiments, minimum degradation was found when LDL was reacted with 2 to 3 mM HNE (Figure 3A). Reduction in degradation of \(^{125}\)I-LDL modified with low levels of HNE (0 to 3 mM) was also observed in cultured smooth muscle cells (SMC) (Figure 3B). That the decreased degradation by J774 macrophages and SMC was due to a diminished recognition of HNE LDL by the LDL receptor was suggested by the reduced ability of a 30-fold excess of unlabeled LDL to inhibit degradation of \(^{125}\)I HNE LDL. Degradation was reduced by 87% for 0 and 65% for 3 mM \(^{125}\)I HNE LDL in J774 macrophages.

Degradation of labeled HNE LDL by macrophages increased abruptly at the levels of modification in which aggregation occurred (e.g., Figure 3A, 62% precipitable). Such increases were also observed in MPM (data not shown). The abrupt increase in degradation of \(^{125}\)I-LDL with increasing modification was mimicked by stimulation of cholesterol esterification with the incorporation of labeled oleate into cholesteryl esters (data not shown). At levels of aggregation exceeding 90% precipitable, the degradation of HNE LDL decreased. Light microscopy revealed further increases in the size of the aggregates as the precipitability increased from 90% to 98% (data not shown), which may have impeded cellular uptake. SMC were also incubated with aggregated HNE LDL to determine whether these aggregates would be internalized and degraded by SMC, as had been observed with macrophages. However, with increasing levels of aggregation, a further reduction in degradation, rather than an increase, was found (Figure 3B).

The concentration-dependent degradation of 6 mM HNE LDL (95% precipitable) in J774 macrophages was saturable (Figure 4). Half-maximal degradation occurred around 40 \(\mu\)g LDL protein/ml and saturation around 200 \(\mu\)g LDL protein/ml, characteristic of a low affinity interaction. Degradation by MPM of aggregated HNE LDL modified with 8 mM HNE was linear over the time interval of 8 to 48 hours (Figure 5) as was acetyl LDL degradation by MPM at equivalent concentrations. By contrast, degra-
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dation of LDL had virtually stopped by 20 hours. The increase in degradation of HNE LDL was accompanied by large increases in MPM total cholesterol content. Exposures to HNE LDL, acetyl LDL, and LDL resulted in total cellular cholesterol levels of 78, 58, and 12 μg cholesterol/mg cell protein/24 hours, respectively. A 24-hour incubation with HNE LDL also resulted in the accumulation of oil red O-positive inclusions in MPM (Figure 6A). By contrast, MPM incubated for this time interval with unmodified LDL showed no inclusions (Figure 6B).

We tested whether cytochalasin D, an inhibitor of phagocytosis, would inhibit degradation in MPM of 125I-LDL modified with different concentrations of HNE. We used cytochalasin D, rather than cytochalasin B, since the former has been reported to be more specific than the latter. As seen in Figure 7, cytochalasin D induced a 78%, 90%, and 99% inhibition in the degradation of 3 mM HNE LDL (25% precipitable), 4 mM HNE LDL (75% precipitable), and 6 mM HNE LDL (98% precipitable), respectively. By contrast, cytochalasin D showed no inhibition of labeled LDL or acetyl LDL degradation, suggesting that at the concentration used it did not affect receptor-mediated endocytosis.

Possible Receptor Recognition of Aggregated HNE Low Density Lipoprotein

To determine whether uptake of aggregated HNE LDL was mediated by either the LDL or the scavenger receptor on macrophages, we incubated J774 macrophages with 125I-labeled 6 mM HNE LDL (95% precipitable) and assessed whether a 10- or 40-fold excess of unlabeled LDL or acetyl LDL could inhibit degradation of the labeled HNE LDL. Neither LDL nor acetyl LDL was able to inhibit degradation of labeled HNE LDL at either 10- or 40-fold excess (Figure 8). A 10-fold excess of unlabeled HNE LDL inhibited degradation of labeled acetyl LDL by only 10%, while excess unlabeled acetyl LDL reduced degradation of labeled acetyl LDL by over 95%. A 10-fold and 40-fold excess of unlabeled HNE LDL inhibited degradation of labeled HNE LDL by 70% and 95%, respectively, whereas a 40-fold excess of unlabeled HNE LDL inhibited degradation of labeled acetyl LDL by 40% and labeled LDL by 60% (data not shown).

To assess the possibility that the inhibition of degradation of acetyl LDL, of LDL, and of HNE LDL by excess aggregated HNE LDL could be due to possible steric hindrance of such large ligands for cellular binding sites, we tested whether other particulate ligands could likewise induce such inhibition. When a 30-fold excess of ethanol-denatured LDL, which forms micellar structures in excess of 1 μm in diameter (data not shown), zymosan granules, and aggregated HNE LDL (95% pelleted) were tested for their abilities to inhibit degradation of labeled HNE LDL and labeled acetyl LDL, all of these large ligands effectively inhibited degradation of either labeled ligand (about 80% for HNE LDL and about 50% for labeled acetyl LDL) (Figure 9).

Some of this inhibition could have been due to an association between the insoluble competitors and the labeled soluble ligand. To test this possibility, we incubated labeled acetyl LDL or LDL (20 μg protein/ml) with a 20-fold excess of HNE LDL (95% precipitable), ethanol-
HNE Concentration (mM) Used to Modify LDL

Figure 3. A. Dependence of degradation of 4-hydroxynonenal (HNE)-modified $^{125}_{1}$-labeled low density lipoprotein (LDL) on the degree of modification of LDL with HNE. Levels of aggregation were assessed by the percent of label pelleted by centrifugation at 10,000 g shown in parentheses. $^{125}_{1}$-LDL was modified with HNE at concentrations ranging from 0 (sham-treated) to 6 mM HNE as described in Methods. J774 macrophages were incubated in triplicate for 4.5 hours with LDL modified to different degrees with HNE at a final protein concentration of 20 μg/ml in Dulbecco’s Modified Eagle’s Medium containing 6 mg/ml bovine serum albumin. Degradation was measured as described in Methods. B. Degradation of $^{125}_{1}$-HNE LDL by rabbit aortic smooth muscle cells (SMC). $^{125}_{1}$-LDL was modified with HNE at concentrations ranging from 0 (sham-treated) to 8 mM HNE and was incubated for 4.5 hours with SMC in triplicate at a final protein concentration of 20 μg/ml in Dulbecco’s Modified Eagle’s Medium containing 10% lipoprotein-deficient serum. Degradation was determined as described in Methods.

denatured LDL, or zymosan for 4.5 hours at 37°C. Association was evaluated by centrifugation (10,000 g for 30 minutes) to precipitate the insoluble competitors followed by counting the amount of label in the infranatant. Twenty-two percent and 28% of the labeled acetyl LDL and LDL, respectively, were associated with the HNE LDL precipitate; 17% and 2% with the ethanol-denatured LDL precipitate; and 2 and 4% with the zymosan precipitate. Thus, some of the apparent inhibition of degradation of labeled acetyl LDL and LDL was attributable to association with HNE LDL.

Since concentrations of particulate ligands used in the competition studies were very high, we also considered the possibility that they were toxic to cells, which would result in reduced cell uptake of these ligands at high concentrations and be misinterpreted as competing with...
these ligands. Cell viability was assessed by measuring the release of lactic dehydrogenase and by quantifying cellular protein synthesis during exposure of macrophages to these particulate ligands. No significant release of lactic dehydrogenase was seen for any of the soluble or insoluble samples relative to control cells to which no lipoprotein was added (Table 2). Likewise, cellular protein synthesis, as measured by the incorporation of $^3$H-leucine into cell protein, did not significantly differ from control incubations without lipoproteins. Thus, high concentrations of particulate ligands were not measurably cytotoxic.

Discussion

HNE is a bifunctional and amphipathic aldehyde$^{19,20}$ that is a major propagation product of lipid peroxidation. It is known to be formed during the oxidation of LDL$^{10}$ and has been shown to cause structural and chemical modi-
was not extractable into aqueous buffers, but rather had to be removed using non-ionic detergents or proteolytic enzymes. It is possible that some of these lipoprotein complexes represent aggregated LDL.

As LDL was modified with increasing concentrations of HNE, this aggregation increased as assessed both by the percent of total LDL particles precipitable by centrifugation at 10,000 g and by the average size of the aggregates. Aggregation increased with increases in HNE concentration used to modify LDL even beyond the HNE concentration at which blockage of lysine residues had plateaued. Electron microscopic observations suggested that aggregation represented both association of individual particles as well as coalescence of particles to form larger spherical particles. Aggregation could be the result of HNE-induced exposure of hydrophobic regions of apo B, or of intermolecular cross-linking by the bifunctional aldehyde, as had been shown previously with glutaraldehyde.

The fact that 60% of apo B from highly aggregated HNE LDL remained aggregated in 3% SDS suggests that some covalent cross-linking had occurred. However, we cannot rule out noncovalent interactions, since 90% of the intact LDL particles were precipitable in the same sample in which only 60% of the delipidated apo B was aggregated. Furthermore, such precipitation is probably an underestimate of aggregation and encompasses only larger aggregates, since at low-level modification of LDL with HNE, we were able to demonstrate some particle self-association by nondenaturing PAGE in samples that showed very little precipitation.

Apo B from such samples showed, in addition to the B-100 band, the presence of a higher molecular weight species, which was presumed to represent apo B crosslinked by the aldehyde. Such a protein pattern was previously seen in LDL modified with malondialdehyde as well as in LDL from patients with cardiovascular disease. However, since the high molecular weight band appeared when minimal particle aggregation was present, intramolecular covalent linkage may also have occurred, resulting in aberrant electrophoretic properties.

A comparison of data from the present study and others reveal similarities among these three modified forms of LDL. Both mildly modified HNE LDL and mildly oxidized LDL demonstrate a blockage of lysine residues on apo B, an increase in relative electrophoretic mobility, and an increase in intrinsic fluorescence at 360 nm excitation/430 nm emission. LDL possesses similar fluorescence properties (unpublished observation). These modifications are consistent with the liberation of Schiff-bases between HNE or other aldehydes formed during lipid peroxidation and ε-amino groups of lysine in apo B in LDL. Such adducts are responsible for the observed intrinsic fluorescence and the reduced negative charge of the particle. Demonstrated that when radiolabeled arachidonic acid was inserted into LDL phospholipids and the LDL particle subjected to oxidation, a significant amount of label became covalently associated with apo B, presumably in the form of propagation products such as malondialdehyde.

Aggregation of LDL particles observed at higher levels of modification with HNE is a characteristic shared by both extensively oxidized LDL and A-LDL. We had previously shown that about one-half of immunoreactive apo B extracted from human atherosclerotic lesions by homogenization in aqueous buffers eluted in the void volume during gel exclusion chromatography indicating particle sizes much greater than LDL. Moreover, we found that about one-half of total immunoreactive apo B in plaques...
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Figure 8. Inhibition by unlabeled lipoproteins of the degradation in macrophages of $^{125}$-low density lipoproteins (LDL) modified with 6 mM 4-hydroxynonenal (HNE) in which over 95% could be pelleted by centrifugation. J774 macrophages were incubated for 4.5 hours at 37°C in triplicate with Dulbecco’s Modified Eagle’s Medium containing 6 mg/ml bovine serum albumin and labeled and unlabeled lipoproteins at concentrations of 30 μg and 300 μg protein/ml, respectively. Degradation was determined as described in Methods. Degradation of $^{125}$-HNE LDL alone was 2.5±0.4 (mean±SD) μg/mg cell protein, whereas that of $^{125}$-acetyl LDL was 3.8±0 μg/mg cell protein. Inset. Procedures were the same except that the concentration of added unlabeled lipoproteins was 1.2 mg protein/ml. Degradation of $^{125}$-HNE LDL was 2.7±0.3 μg/mg cell protein.

Table 2. Toxicity to J774 Macrophages of Ligands Used in Competition Studies

<table>
<thead>
<tr>
<th>Competitor added</th>
<th>Lactic dehydrogenase release</th>
<th>Incorporation of $^3$H-leucine into cell protein</th>
</tr>
</thead>
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<tr>
<td>LDL</td>
<td>96±2</td>
<td>116±6</td>
</tr>
<tr>
<td>Acetyl LDL</td>
<td>95±2</td>
<td>113±3</td>
</tr>
<tr>
<td>Insoluble HNE LDL</td>
<td>98±2</td>
<td>106±8</td>
</tr>
<tr>
<td>Denatured LDL</td>
<td>97±2</td>
<td>110±6</td>
</tr>
<tr>
<td>Zymosan</td>
<td>100±1</td>
<td>90±8</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SD of the percent recorded for cells incubated only with medium. These data are representative of three similar experiments.

Triplicate wells of J774 macrophages were incubated for 4.5 hours at 37°C with each of the listed soluble and insoluble substrates in medium as used in degradation studies (see Methods) at a protein concentration of 600 μg/ml (or mass concentrations for zymosan) together with $^3$H-leucine (final concentration, 2 μCi/ml). The incubation medium was then removed, and the lactic dehydrogenase release and incorporation of $^3$H-leucine into cellular protein were measured as described in Methods.

LDL = low density lipoprotein, HNE = 4-hydroxynonenal.

of modification. A similar finding was obtained when we used aortic smooth muscle cells. We could further show using competition studies with excess unmodified LDL that this reduction in uptake was due to a reduced number of LDL particles that were still recognized by the LDL receptor on these cells. These data are consistent with those of Jessup et al. who showed that low-level modification of LDL with HNE reduced the affinity of LDL for its receptor on fibroblasts. However, we found that once appreciable aggregation of HNE LDL occurred, neither excess LDL nor excess acetyl LDL could successfully compete for degradation of HNE LDL. This suggests that
neither the LDL receptor nor the scavenger receptor were responsible for internalization of the aggregated LDL. One possibility that could not be ruled out in this study is that aggregated HNE LDL binds to either the LDL receptor or the scavenger receptor with very high affinity, due to multivalent binding of such large aggregates, thus precluding competition by excess soluble LDL or acetyl LDL. Further studies using ligand blotting procedures to purified receptors will be needed to answer this question. It is also conceivable that aggregated HNE LDL may be recognized by an additional macrophage receptor, as has been recently suggested to be present for oxidized LDL. The documented competition for degradation of HNE LDL by itself could be due, in part, to steric hindrance, since other large competitors were able to inhibit degradation of aggregated HNE LDL.

In conclusion, we have demonstrated in this study that chemical modification of LDL with one propagation product of lipid peroxidation results in unregulated uptake by macrophages and lipid loading. This result suggests that interactions of alkenals with apo B, in particular, those resulting in particle aggregation, may explain in part the changes observed in oxidized LDL and LDL accumulating in atherosclerotic plaques. Although several reports have documented macrophage uptake of various forms of aggregated LDL, the present results are the first to describe the aggregation of LDL with physiologic products of lipid peroxidation leading to foam cell formation. The similarities in the properties observed for HNE LDL, oxidized LDL, and LDL in human lesions suggest that the results may have relevance to the mechanisms occurring in the artery wall during atherogenesis.

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