Interaction of Oxidized HDLs With J774-A1 Macrophages Causes Intracellular Accumulation of Unesterified Cholesterol

Roberto Musanti, Giancarlo Ghiselli

Uptake of modified lipoproteins by resident arterial monocytes/macrophages is believed to be a key event in the formation of foam cells and thus in the early phases of atherosclerosis. Low-density lipoproteins (LDLs) that undergo oxidative changes become suitable for uptake by macrophages through a specific scavenger receptor that leads to cholesteryl ester accumulation. Because the interaction of other oxidized lipoproteins with macrophages has been poorly investigated, we studied the effect of oxidatively modified high-density lipoproteins (HDLs) on the sterol metabolism of J774-A1 macrophages. Unlike native HDLs, oxidized HDLs caused a concentration-dependent accumulation of unesterified cholesterol and decreased [14C]oleate incorporation into steryl esters. Oxidized HDLs also decreased [14C]acetate incorporation into newly synthesized sterols. Cell surface binding of [125I]-oxidized HDLs to the macrophages was saturable, with an apparent dissociation constant (Kd) of 0.96 nmol/mL. Both oxidized and acetylated LDLs but not native lipoproteins could compete for binding of [125I]-oxidized HDL. The data support the conclusion that the effects elicited by oxidized HDLs on the sterol metabolism of macrophages are significantly different from those of native HDLs. The binding of oxidized HDLs to macrophages occurs at sites that are likely the same as those for modified LDLs. We speculate that, if occurring in vivo, HDL oxidation would generate modified lipoproteins capable of modulating the cholesterol homeostasis of macrophages.

KEY WORDS • HDL • oxidized lipoproteins • cholesterol metabolism • macrophages • lipoprotein scavenger receptor • atherosclerosis

Early atherosclerotic lesions in humans and animal models are characterized by the presence of lipid-laden cells in the arterial intima that are largely derived from resident monocytes/macrophages. At this stage of the formation of atherosclerotic plaque, most of the lipids appear to be located intracellularly. Macrophages, however, do not easily accumulate lipids when incubated with native lipoproteins. On the contrary, they avidly bind and degrade modified low-density lipoproteins (LDLs). Thus, incubation with acetylated LDLs results in massiveengorge ment of cholesteryl esters that leads to the formation of foam cells. Uptake of these chemically modified LDLs occurs through a distinct scavenger receptor. However, it is unlikely that acetylation occurs in vivo. Two possible mechanisms by which the more negatively charged LDLs could be produced in the bloodstream have been reported: first, the interaction of LDLs with malondialdehyde (MDA) released by aggregating platelets or produced by peroxidation of fatty acids, and second, the interaction of LDLs with endothelial cells, which alters LDLs by oxidation. Besides promoting cholesterol accumulation in macrophages, oxidized LDLs also have important biological effects on endothelial and smooth muscle cells. The presence of oxidized LDLs has been detected in the atherosclerotic lesions of rabbits and humans. Furthermore, lipoproteins resembling oxidatively modified LDLs have been found circulating in humans.

Whether other oxidatively modified lipoproteins are internalized by macrophages and cause lipid accumulation has been marginally investigated. Recently, it has been reported that oxidized rabbit β-very-low-density lipoproteins (VLDLs) produced by incubation of native lipoproteins with endothelial or smooth muscle cells or with copper ions are degraded by macrophages, smooth muscle cells, and liver Kupffer cells at higher rates than are unmodified β-VLDLs. Modified rabbit β-VLDLs are also more effective than native lipoproteins in stimulating cholesterol esterification. The corollary from these studies is that the phenomenon of oxidative modification that changes the behavior of macrophages toward native lipoproteins may not be restricted to LDLs.

Since native high-density lipoproteins (HDLs) are involved in the removal of excess cholesterol from extrahepatic cells to the liver, the oxidative modification of HDLs may have important consequences for cholesterol homeostasis in peripheral tissues. In a previous study we documented that HDLs that had been oxidatively modified by incubation with copper ions or smooth muscle cells became potent inhibitors of cholesterol biosynthesis in human skin fibroblasts through a...
pathway that is not LDL receptor mediated. In this study we investigated the interaction of oxidized HDLs with an established line of macrophages. In particular, we examined whether oxidized HDLs have different effects than native HDLs on the cholesterol metabolism of macrophages and whether the binding of oxidized HDLs to macrophages occurs at the same scavenger receptor that binds modified LDLs.

**Methods**

**Lipoproteins**

The blood from healthy volunteers was collected in tubes using Na2-EDTA (3 mmol/L) as an anticoagulant. As a preservative, gentamicin sulfate (0.005%) and phenylmethylsulfonyl fluoride (1 mmol/L) were added to the plasma, which was stored at 4°C and used for lipoprotein isolation within 36 hours.24 LDLs (d=1.019 to 1.063 g/mL) and HDLs (d=1.063 to 1.210 g/mL) were isolated by ultracentrifugation.25 All the density solutions used to adjust the flotation density of the lipoproteins contained 1 mmol/L Na2-EDTA. After isolation, the lipoproteins were dialyzed overnight against 0.15 mol/L potassium-sodium phosphate buffer (PBS), pH 7.4.

For the oxidation, LDLs and HDLs were diluted to a concentration of 3 mg cholesterol/mL with PBS and incubated for up to 24 hours at 37°C in the presence of increasing concentrations of CuSO4. The oxidative challenge was terminated by chromatography of the lipoproteins through a PD-10 Sephadex column eluted with PBS. For the acetylation, LDLs were dialyzed against 0.15 mol/L NaCl, their concentration was adjusted to 10 mg/mL, and they were dialyzed with an identical volume of NaOH overnight at room temperature. An aliquot of the material was taken for protein determination according to the method of Lowry et al.26

**Agarose Electrophoresis**

Agarose electrophoresis of the native and modified lipoproteins was performed on Paragon Lipo-Gel strips from Beckman using the Beckman electrophoresis apparatus and following the manufacturer’s directions for the electrophoretic conditions and the gel staining and destaining. Freshly drawn human serum from a normolipemic individual was used as a standard for the migration of the lipoproteins in the α, pre-β, and β positions.

**Column Chromatography**

Molecular-sieve chromatography of the lipoproteins was carried out on a BioGel A 1.5-m (1.5x120-cm) column at 4°C. The flow of the eluting buffer (PBS) was maintained with a peristaltic pump at 2 mL/h. The column had been previously calibrated with human VLDLs (d<1.006 g/mL), LDLs (d=1.006 to 1.063 g/mL), and HDLs (d=1.063 to 1.210 g/mL) isolated by ultracentrifugation and with bovine serum albumin (BSA). The optical density of the eluate was monitored continuously at 280 nm, and each fraction collected was analyzed for its cholesterol content.

**Fast Protein Liquid Chromatography Analysis**

Fast protein liquid chromatography was performed on a Pharmacia apparatus fitted with a Mono Q 5/5 anion-exchange column at a flow rate of 2 mL/min and a back pressure of 2 atm. The optical density of the eluate was recorded continuously at 280 nm. The starting elution buffer was 0.02 mol/L tris(hydroxymethyl) aminomethane (Tris)-HCl, pH 7.4. The salt concentration was increased at 10 minutes to 0.2 mol/L with NaCl and reached 0.6 mol/L within 20 minutes by establishing a linear gradient. Lipoproteins, previously dialyzed against 0.02 mol/L Tris-HCl, pH 7.4, were injected at a concentration of 1 mg protein/mL.

**Lipoprotein Chemical Analysis**

The protein content was determined according to the method of Lowry et al by using a calibrated BSA standard from Bio-Rad. Cholesterol (total and unesterified), triglycerides, and choline-containing phospholipids were assayed by using automated enzymatic assays on a Coulter CPA Autoanalyzer.27,28 The thiobarbituric acid–reactive substance (TBARS) of the lipoproteins was quantified as described by Ohkawa et al and expressed as the MDA equivalent content (nanomoles per milligram protein) by using an extinction coefficient determined from an MDA tetramethylacetal–generated standard curve.

**Cells**

J774-A1 murine macrophages were obtained from the American Type Culture Collection cell repository. The cells were grown in Dulbecco’s minimal essential medium (DMEM) with the addition of 10% fetal calf serum in a humidified 5% CO2 incubator at 37°C. For the experiments the cells were seeded into 35-mm tissue-culture dishes. When the cells reached 90% to 95% confluence, growth medium was changed to DMEM containing 0.2% BSA (DMEM-BSA). All the experiments with lipoproteins were performed 24 hours later.

**Sterol Accumulation**

The cells were washed twice with DMEM and supplemented with 2 mL DMEM-BSA. The lipoproteins were then added at a concentration calculated according to their cholesterol content. After 24 hours, the cells were washed three times with ice-cold DMEM-BSA, twice with ice-cold DMEM, and extracted in situ at room temperature by adding 1.5 mL hexane/isopropanol (3:2, vol/vol) to the plates.31 Aliquots of the extract in isopropanol were taken for the determination of both the total and the unesterified fraction of the 3-β-hydroxy sterols by the fluorometric procedure of Gamble et al. Desmosterol, the major sterol synthesized by J774-A1 macrophages, had an extinction coefficient corresponding to 73.0% that of cholesterol. Delipidated cells were dissolved by incubation with 0.1 mol/L NaOH overnight at room temperature. An aliquot of the material was taken for protein determination according to the method of Lowry et al.

**Steryl Ester Formation Assay**

The cells were washed twice with DMEM and received 2 mL DMEM-BSA together with the lipopro-
teins. Two hours later, [14C]oleate (1 μCi/mL) as a complex with BSA was added, and incubation continued for an additional 6 hours. The lipids were extracted in situ with hexane/isopropanol. Phospholipids, triglycerides, sterols, and steryl esters were separated by thin-layer chromatography (TLC) using precoated silica gel 60 F254 plastic sheets from Merck. The developing system was heptane/ethyl ether/acetic acid (90:30:1, vol/vol/vol). The lipids were identified by the use of reference lipid standards including egg phosphatidylcholine, cholesterol, desmosterol, tripalmitin, and cholesteryl oleate. The chromatographic bands were developed with 8% phosphomolybdic acid, cut out, and counted in a Packard Tricarb 1900CA beta counter with an automatic correction for quenching. The radioactivity recovered in the steryl ester band was regarded as a direct measure of the cellular steryl ester synthetic activity. The cell proteins were measured as described.

**Microsomal Acyl-Coenzyme A: Cholesterol Acyltransferase Activity Assay**

Microsomes from J774-A1 macrophages that had been incubated 24 hours in DMEM-BSA were prepared as detailed and stored at −80°C until the performance of the assay. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) activity was determined by the rate of incorporation of [14C]oleoyl-coenzyme A ([14C]oleoyl-CoA) into the steryl ester fraction. To test the effect on the enzymatic activity of exogenously added lipids, the latter were added to the microsomes in a suspension in Triton WR-1339 as described by Billeheimer et al. When cholesterol suspensions were used, they were prepared as follows: 200 μL Triton WR-1339 as a 10% solution in acetone was added to cholesterol (10 to 100 μg), and the tubes were incubated at 37°C with occasional shaking until a clear solution was obtained. One milliliter of preheated 0.1 mol/L phosphate buffer (pH 7.4) was then added, and the acetone was evaporated under a stream of nitrogen at 37°C. The suspensions were used within 15 minutes. The suspensions of native and oxidized HDL lipids were prepared similarly. Lipoprotein lipids were obtained by extracting the lipoproteins as described by Kates. The cholesterol content of the lipoprotein extracts was assayed enzymatically, and Triton WR-1339 lipoprotein lipid suspensions containing 100 μg unesterified cholesterol/mL were prepared. The ACAT assay mixture contained 100 μg microsomal proteins in 200 μL 0.1 mol/L phosphate buffer with 0.01% dithiothreitol (pH 7.4) and 50 μL of the Triton WR-1339 suspended lipids adjusted to the desired concentration by dilution with phosphate buffer. After 30 minutes of preincubation at 37°C, the reaction was initiated by the addition of 400 000 dpm [14C]oleoyl-CoA and stopped 15 minutes later by the addition of 2 mL CHCl₃. The microsomal lipids were extracted after the addition of [3H]cholesteryl oleate as an internal standard. The lipids were separated by TLC, and the bands corresponding to the cholesteryl ester were cut out and counted.

**Lipid Biosynthesis Assay**

The cells were prepared by washing two times with DMEM and received 2 mL DMEM-BSA together with the lipoproteins. Two hours later [14C]acetate (2 μCi/mL) was added, and the incubation was terminated after 6 hours. The cell lipids were extracted and separated by TLC as detailed for the sterol esterification experiments. The radioactivity associated with the cholesterol band was taken as a direct index of the overall activity of the sterol biosynthetic pathway without further correction for the much lower radioactivity (always <12% of that in the cholesterol band) recovered with the steryl esters.

**Radioiodinated Lipoprotein Uptake and Degradation**

The lipoproteins were radioiodinated following the procedure of McFarlane adapted for lipoproteins as described elsewhere. After radioiodination, lipoproteins were oxidized as described above. Specific activity of the labeled lipoproteins ranged from 37 to 72 cpm/ng protein. More than 94% of the radioactivity could be precipitated with 10% trichloroacetic acid, and the lipid-bound radioactivity was <5.7%. The cell surface binding of the lipoprotein was measured at 4°C as follows. The cells were washed three times with ice-cold DMEM and received 2 mL ice-cold DMEM-BSA (without bicarbonate) containing 10 mmol/L NaOH-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4. After a preincubation period of 30 minutes at 4°C, 125I-oxidized HDLs were added. At the end of the incubation the medium was removed, and the cell monolayers were washed three times with ice-cold DMEM-BSA and then twice with ice-cold DMEM. The cells were finally dissolved in 0.1 mol/L NaOH. An aliquot of the cell lysate was taken for the protein determination, and the remaining cells were counted. Specific binding was calculated by subtracting binding in the presence of 20-fold excess unlabeled ligand (non-specific binding) from that measured in the absence of this excess (total binding). Lipoprotein cellular uptake and degradation were measured at 37°C. The cells were washed three times with DMEM and preincubated in 2 mL DMEM-BSA for 30 minutes, after which time 125I-oxidized HDLs were added. At the end of this incubation period, the medium was removed and saved for the assay of the degraded material according to a previously described technique. The cells were washed, solubilized by incubation with 0.1 mol/L NaOH, and finally counted. Specific uptake and degradation in these experiments were determined by running parallel incubations in the presence of a 25-fold excess of unlabeled ligand.

**Cross-Competition Binding and Binding Displacement Assay**

These experiments were carried out at 4°C. For the cross-competition binding experiments, the cells were washed three times with DMEM and received 2 mL ice-cold DMEM-BSA without bicarbonate containing 10 mmol/L NaOH-HEPES, pH 7.4. After 30 minutes' preincubation, the radioiodinated lipoproteins were added at a fixed concentration of 12.5 μg protein/mL medium together with increasing concentrations, ranging from 12.5 to 250 μg protein/mL of the unlabeled competing lipoproteins. The incubation was terminated 6 hours later. The macrophages were washed and then solubilized with 0.1 mol/L NaOH for determination of the cell protein and associated radioactivity. The displacement binding assays were performed following the same protocol, except that the unlabeled lipoproteins
Production and Characterization of Oxidized HDLs

The products of the incubation of HDLs with CuSO_4 were analyzed by agarose gel electrophoresis (Fig 1A). When exposed to 50 μmol/L CuSO_4 for 24 hours at 37°C, HDLs acquired a more negative charge than the native lipoproteins and moved to a pre-α position. On the contrary, LDLs incubated under the same conditions began changing electrophoretic mobility when exposed to 50 μmol/L CuSO_4 for 24 hours at 37°C. The oxidative modification begun changing electrophoretic mobility when exposed to 50 μmol/L CuSO_4 for 24 hours at 37°C. The oxidative modification of the lipoproteins was much different from that of the acetylated LDLs, which enhanced by 20-fold the steryl ester concentration of the macrophages.

Effect on the Cellular Sterol Content

HDLs and LDLs that had been oxidized with 50 μmol/L and 10 μmol/L CuSO_4, respectively, at 37°C for 24 hours and that had the same electrophoretic mobility were used in all the cell culture experiments. The TBARS content of these lipoprotein preparations was 50.4±2.1 (n=4) and 62.2±5.1 (n=3) nmol/mg protein, respectively. In comparison, native HDLs had a TBARS content of 4.2±1.1 (n=4) nmol/mg protein, whereas acetylated LDLs had a TBARS content of 14.3 (n=2) nmol/mg protein. Preliminary experiments showed that the modified lipoproteins, added up to a concentration of 250 μg cholesterol/mL medium (DMEM-BSA), were not toxic to the macrophages. Specifically, the cell adherence to the plates was not affected, and the morphology demonstrated by light microscopy of the cultures was normal. In addition, cell protein recovery from the plates was constant, independent of the lipoproteins added.

To investigate the effect of the oxidized HDLs on the cell sterol balance, the macrophages were incubated with the lipoproteins for 24 hours. Results are presented in Table 2. Native HDLs slightly increased the cellular total sterol content, which changed from 30.3 μg/mg cell protein (in the absence of lipoprotein) to 38.0 μg/mg cell protein by adding 250 μg HDL cholesterol/mL medium. On the contrary, oxidized HDLs produced a significant cholesterol accumulation. The increase of the cell sterols was dose dependent and reached 61.8 μg/mg at the highest concentration of oxidized HDLs added (250 μg cholesterol/mL). This significant sterol accumulation was primarily due to a severe increase of the unesterified steryl fraction. The effect of oxidized LDLs was similar to that of oxidized HDLs, as the macrophages increased their unesterified sterol content. However, oxidized LDLs had a greater effect in increasing the cell steryl ester content. The behavior of the oxidized lipoproteins was much different from that of the acetylated LDLs, which enhanced by more than 20-fold the steryl ester concentration of the macrophages.
**FIG 2.** Chromatograms showing fast protein liquid chromatography (FPLC) of native and oxidized lipoproteins. Lipoproteins were oxidatively modified with different amounts of CuSO₄ by incubating them at 37°C for 24 hours and then analyzing them by FPLC on a Mono Q 5/5 column. The column operative conditions are described in "Methods." A, Native low-density lipoproteins (LDLs) stored at 4°C; B, 10 μmol/L CuSO₄-oxidized LDLs; C, native high-density lipoproteins (HDLs) stored at 4°C; D, native HDLs stored at 37°C for 24 hours; E, 5 μmol/L CuSO₄-oxidized HDLs; F, 10 μmol/L CuSO₄-oxidized HDLs; G, 50 μmol/L oxidized HDLs; and H, 100 μmol/L oxidized HDLs. OD, optical density.
Effect on Steryl Ester Formation

Experiments were conducted to assess the effect of oxidized HDLs on sterol ester formation in macrophages by using [14C]oleate as the precursor. The lipoprotein concentration in the medium ranged from 62.5 to 250 µg cholesterol/mL. Results are presented in Table 3. Whereas both native and oxidized HDLs decreased sterol ester formation, oxidized LDLs increased sterol ester synthesis in macrophages by two-fold, and acetylated LDLs (62.5 µg cholesterol/mL) enhanced the process by fivefold. [14C]Oleate incorporation into phospholipids and triglycerides was not significantly affected by any of the lipoproteins added.

Effect of the Lipoprotein Lipids on the Microsomal ACAT Activity

Because of the inhibitory effect on cholesteryl ester formation elicited by both native and oxidized HDLs, we investigated whether the lipids of these lipoproteins directly affected the activity of the cholesterol esterifying enzyme. In these experiments the lipoprotein lipids were added to the microsomes prepared from the J774-A1 macrophages as a suspension in a Triton WR-1339 solution, which allows access of the exogenously added cholesterol to the microsomal ACAT.36 Results are illustrated in Table 4. The microsomal cholesterol content in three preparations was 30 ±3 µg cholesterol/mg protein, and the basal ACAT activity measured in the presence of 50 µL Triton WR-1339/phosphate buffer was 49.3 ±2.6 pmol/min per milligram microsomal protein. Addition to the macrophages of increasing amounts of suspended cholesterol, up to 64 µg cholesterol/mg microsomal protein, enhanced the incorporation of the [14C]choleoyl-CoA into the sterol ester fraction. The HDLs and the oxidized HDL lipids, added in quantities calculated according to their unesterified cholesterol content, had the same effect as equivalent amounts of the cholesterol suspension.

Effect on Lipid Biosynthesis

The effect of the oxidized HDLs on the lipid biosynthesis of the J774-A1 macrophages was subsequently investigated (Fig 4). Native HDLs elicited a slight increase of the incorporation of [14C]acetate into unesterified sterols. Incubation with oxidized HDLs, on the other hand, decreased acetate incorporation by as much as 53%. The effect was readily evident even at the lowest concentration of oxidized HDLs tested (62.5 µg cholesterol/mL) and was enhanced by increasing lipoprotein concentrations. Native, oxidized, and acetylated LDLs, each added at a single concentration of 62.5 µg cholesterol/mL, decreased [14C]acetate incorporation into the unesterified sterols of J774-A1 cells by 71%, 80%, and 82%, respectively. None of the lipoproteins added significantly changed [14C] acetate incorporation into phospholipids or triglycerides (data not shown).

Binding, Uptake, and Degradation of 125I-Oxidized HDLs

Binding of oxidized HDLs at the surface of macrophages was investigated at 4°C. The time course of the event is illustrated in Fig 5A. Specific binding of 125I-oxidized HDLs to macrophages reached equilibration after 120 minutes of incubation. Similar results were obtained at 37°C, when uptake, ie, cell surface–bound plus internalized radioactivity, was measured (data not shown). When macrophages were incubated with increasing concentrations of 125I-oxidized HDLs at 4°C for...
TABLE 2. Effect of Native and Oxidized HDLs and Oxidized and Acetylated LDLs on Sterol Content of J774-A1 Macrophages

<table>
<thead>
<tr>
<th>Control cells</th>
<th>Native HDLs</th>
<th>Oxidized HDLs</th>
<th>Oxidized LDLs</th>
<th>Acetylated LDLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>125</td>
<td>250</td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td>Sterols</td>
<td>26.2±2.3</td>
<td>24.9±1.8</td>
<td>27.9±2.0</td>
<td>28.0±2.2</td>
</tr>
<tr>
<td>Steryl esters</td>
<td>4.1±0.2</td>
<td>4.2±0.3</td>
<td>10.3±1.1</td>
<td>10.0±1.0</td>
</tr>
<tr>
<td>Total sterols</td>
<td>30.3±3.4</td>
<td>29.1±1.9</td>
<td>38.0±3.0</td>
<td>38.0±2.7</td>
</tr>
</tbody>
</table>

HDLS indicate high-density lipoproteins; LDLs, low-density lipoproteins. Lipoproteins were added as micrograms of total cholesterol per milliliter medium; sterol contents are expressed as micrograms per milligram cell protein. Cells were incubated for 24 hours. Values are the mean±SD of four determinations from two experiments.

6 hours, surface binding of the lipoproteins increased in a saturable fashion (Fig 5B). The result of the Scatchard plot of the data (Fig 5C) was consistent with the expression of a single binding site with a maximal binding capacity of 275 ng/mg cell protein, or 5.5 pmol/mg, by assuming a molecular weight for apolipoprotein (apo) HDL of 50 000 and an apparent dissociation constant (Kd) of 48.2 μg/mL or 0.96 nmol/mL. During a 6-hour incubation at 37°C, the specific uptake of 125I-oxidized HDLs reached a plateau at 212 ng/mg cell protein, corresponding to a lipoprotein protein concentration in the medium of 50 μg/mL (Fig 6A). The degraded material at this lipoprotein concentration after 6 hours of incubation was 1.56 μg/mg cell protein, and reached 2.12 μg/mL in cells exposed to 150 μg/mL oxidized HDLs (Fig 6B).

Cross-Competition Binding and Binding Displacement Experiments

The binding specificity of 125I-oxidized HDLs to the macrophages was investigated by cross-competition binding experiments performed at 4°C (Fig 7). Oxidized LDL and acetylated LDL efficiently competed with 125I-oxidized HDLs for binding to the macrophages. When 125I-oxidized LDLs were used as a ligand, acetylated LDLs were a potent competitor, whereas oxidized HDLs were less effective. Finally, oxidized HDLs and oxidized LDLs were both weak competitors for the binding of 125I-acuteylated LDLs. Native HDLs and native LDLs were inactive in displacing any of the radioiodinated modified lipoproteins.

Discussion

The interaction of copper-oxidized HDLs with an established line of murine macrophages was investigated. We examined whether these lipoproteins affected cell cholesterol metabolism differently from native HDLs and whether the binding of oxidized HDLs occurs at the lipoprotein scavenger receptor.

The interaction of HDLs with macrophages is complex. Schmitz et al.42 and Takahashi et al.43 independently concluded that after binding to the cell surface, native HDLs are internalized through a nonlysosomal pathway via the trans-Golgi system and are later resecreted as vesicles. This process, which would result in a net efflux of unesterified cholesterol from the macrophage, is counteracted by the selective uptake by the cell of HDL cholesteryl esters.44,45 In our experiments, the incubation of J774-A1 macrophages with native HDLs had a minor effect on the cell sterol content. On the contrary, an accumulation of unesterified cholesterol occurred in cells incubated with oxidized HDLs, suggesting that the intracellular fate of oxidized HDLs may be different from that of native HDLs. It has been shown that cholesteryl ester lipid droplets that are internalized by J774-A1 macrophages through a phagocytic process are delivered to the lysosomes, where cholesteryl ester is hydrolyzed at a linear rate.46 At this site the fractional hydrolysis is constant over a wide range of cellular esterified cholesterol levels; however, only a small fraction of the free cholesterol generated in the lysosomes is esterified by ACAT. This is the opposite of what occurs to an endogenous pool of free cholesterol, the esterification of which proceeds briskly.

TABLE 3. Effect of Native and Oxidized HDLs and Oxidized and Acetylated LDLs on [14C]Oleate Incorporation Into Esterified Lipids of J774-A1 Macrophages

<table>
<thead>
<tr>
<th>Control cells</th>
<th>Native HDLs</th>
<th>Oxidized HDLs</th>
<th>Oxidized LDLs</th>
<th>Acetylated LDLs</th>
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<tbody>
<tr>
<td>62.5</td>
<td>125</td>
<td>250</td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td>Steryl esters</td>
<td>20.5±3.2</td>
<td>8.4±0.5</td>
<td>7.2±0.3</td>
<td>7.2±1.2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>142.1±10.0</td>
<td>120.7±8.1</td>
<td>120.1±7.0</td>
<td>117.3±8.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>90.3±8.5</td>
<td>80.7±7.1</td>
<td>92.7±5.2</td>
<td>82.7±6.2</td>
</tr>
</tbody>
</table>

HDLS indicate high-density lipoproteins; LDLs, low-density lipoproteins. Lipoproteins were added as micrograms of total cholesterol per milliliter medium; [14C]Oleate incorporation is expressed as disintegrations per minute per microgram cell protein. After a 2-hour incubation, [14C]Oleate was added and the incubation was continued for another 6 hours. Values are the mean±SD of four determinations from two experiments.
We investigated whether the lipids carried by these materials are active. These data are in agreement with those previously reported using human liver endothelial cells and murine peritoneal macrophages, and support this conclusion.

The net result of the phagocytic uptake of the cholesterol ester lipid droplets is an increase of the cellular unesterified cholesterol content. However, analogous to oxidized LDLs that enter the macrophage through a scavenger receptor, oxidized HDLs are delivered to the lysosomal compartment rather than to the trans-Golgi apparatus, thereby leading to unesterified cholesterol accumulation. A defective lysosomal degradation of the internalized oxidized HDLs may contribute to this accumulation. A discrepancy between the cell association and the degradation of oxidized LDLs has been previously reported using human liver endothelial cells and murine peritoneal macrophages and is believed to be secondary to a faulty lysosomal degradation of the internalized oxidized lipoproteins. In addition, the cholesterol of the internalized but nondegraded oxidized LDLs may not be easily accessible to ACAT for esterification.

Roma et al. concluded that the oxidized LDL cholesterol is processed intracellularly by macrophages in a different manner than the acetylated LDL cholesterol. Our results, which show a much higher capability of the acetylated LDLs compared with the oxidized lipoproteins to increase both sterol esterification and the steryl ester content of J774-A1 macrophages, support this conclusion.

Since both the native and the oxidized HDLs decreased cholesterol ester formation in the macrophages, we investigated whether the lipids carried by these lipoproteins had a direct inhibitory effect on the macrophage ACAT activity. The results obtained are consistent with the concept that when the lipoprotein cholesterol is made available to ACAT, the former is efficiently esterified. The oxidized lipids present in oxidized HDLs appear unable to modulate the enzymatic activity. These data are in agreement with those of Zhang et al., who did not detect a significant effect of the oxysterols contained in oxidized LDLs on the cholesterol esterification activity of murine macrophages. Most likely, as has been pointed out, the different oxysterols present in oxidized LDLs have opposing effects on cholesterol esterification, and the net result of the presence of oxysterols in lipoproteins depends on the relative concentrations of the different oxysterol species. The effect of the native and oxidized HDLs in decreasing the sterol esterification activity of the macrophages might be secondary to the ability of these lipoproteins to promote the efflux of cholesterol from the cell plasma membrane that serves as a substrate for ACAT. It is noteworthy that unlike apoB in LDL, apoA-1 in HDL does not fragment on oxidation, and its lipid-organizing properties may not be critically blunted by oxidation.

Evidence for binding and internalization of oxidized HDLs to macrophages was also gathered. Binding of 125I-oxidized HDLs occurred in a saturable fashion, with an affinity constant (Kₐ) of 0.96 nmol/mL. This value is considerably higher than that reported for oxidized LDL "high-" and "low-" affinity binding sites of mouse peritoneal macrophages (8.34 pmol/mL and 44.4 pmol/mL, respectively, from the values reported in Reference 56, and by assuming a molecular weight for apoLDL of 500,000). On the other hand, the maximal binding capacity of J774-A1 macrophages for 125I-oxidized...
HDLs (5.5 pmol/mg cell protein) is not much different from that of the mouse peritoneal macrophages for $^{125}$I-oxidized high-density lipoproteins (ox HDL) to J774-A1 macrophages. A, Cells that had reached 90% to 95% confluence were used. On day 0, cell monolayers received Dulbecco’s minimal essential medium-bovine serum albumin (DMEM-BSA). After 24 hours, the medium was replaced with DMEM-BSA and the cells were placed at 4°C. After 30 minutes they received 12.5 μg protein of $^{125}$I-ox HDL/mL. Incubations were conducted at 4°C and terminated at the indicated times. Cells were washed and counted for associated radioactivity. Specific binding was measured in the presence of 20-fold excess ox HDLs. B, Cell monolayers were prepared as described for the time course experiment with the difference that cell plates received increasing amounts of $^{125}$I-ox HDLs and were incubated for 6 hours. For A and B, ○ indicates total binding; △, nonspecific binding; and ●, specific binding. C, Scatchard plot of the specific binding from points of B. Each point is the mean of four determinations from two independent experiments. B, bound; F, free.
between HDLs and LDLs, oxidation of the same amount of surface lipids is expected to have a greater effect on the surface charge density of LDLs than on HDLs. This may account for the relatively greater oxidizability of LDLs compared with HDLs if investigated by methods based on detection of a charge shift.

Whether oxidized HDLs are in fact formed in vivo remains to be demonstrated, and thus the physiological implication of our findings remains hypothetical. Ion-exchange chromatography on a Mono Q column, which allowed excellent separation of native and modified lipoproteins, did not detect the presence of oxidized HDLs in freshly isolated HDL preparations. The same method also failed to detect the presence of negatively charged LDLs in freshly isolated LDLs. Yet there is convincing evidence that MDA-modified LDLs as well as oxidized LDLs are formed in vivo, and we have demonstrated that both smooth muscle...
cells and endothelial cells oxidatively modify HDLs. Modification of lipoproteins may occur locally, eg, in the intima of the arterial wall or in other sites where there are cells capable of mediating this reaction. Efflux of modified lipoproteins to the bloodstream from the site of modification may be restricted because of anatomic barriers, or alternatively, may be rapidly taken up by the resident cell population. Oxidized HDLs may exert their biological effects at these restricted sites.

In conclusion, our results demonstrated that oxidative modification of human plasma HDLs dramatically alters their interaction with J774-A1 macrophages. The effects observed on cholesterol homeostasis were similar, although not totally identical, to those produced by oxidized LDLs. In particular, oxidatively modified HDLs led to a significant increase in macrophage cholesterol content, mainly by increasing the unesterified sterol fraction. Both cholesterol esterification and cholesterol biosynthesis were depressed by oxidized HDLs. The binding affinity of J774-A1 macrophages for oxidized HDLs was less than that displayed for oxidized LDLs. The competition binding experiments were consistent with the idea that both oxidatively modified lipoproteins bind to the same scavenger macrophage receptor responsible for the binding of acetylated LDLs. Further studies, however, will have to be performed to ascertain whether oxidized HDLs are in fact formed in vivo and to fully establish their pathophysiological significance.

References


Interaction of oxidized HDLs with J774-A1 macrophages causes intracellular accumulation of unesterified cholesterol.

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