High Density Lipoprotein Apolipoproteins Mediate Removal of Sterol From Intracellular Pools but Not From Plasma Membranes of Cholesterol-Loaded Fibroblasts

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Cultured cells possess high-affinity binding sites (receptors) for high density lipoprotein (HDL) that appear to mediate removal of excess intracellular cholesterol from cells. To examine the role of intact HDL apoproteins in receptor-mediated cholesterol removal, HDL₃ apoproteins were digested with the proteolytic enzymes trypsin and pronase, and the residual particles were used in sterol efflux experiments. Protease treatment abolished the interaction of HDL₃ with the 110-kd cell membrane protein postulated to represent the HDL receptor molecule, indicating that this interaction is mediated by HDL apoproteins rather than lipids. Compared with native HDL₃, protease-modified HDL₃ had a markedly reduced ability to selectively remove sterol from intracellular pools, even though modified particles promoted greater cholesterol efflux from the plasma membrane than did native particles. These results indicate that whereas sterol efflux from plasma membranes is mediated by HDL lipids, removal of excess intracellular sterol from cells is mediated by HDL apoproteins. These findings are consistent with the hypothesis that receptor binding of HDL apoproteins stimulates translocation of excess intracellular sterol to the cell surface where it becomes accessible for removal by HDL or other lipid-rich acceptor particles. (Arteriosclerosis and Thrombosis 1991;11:403–414)

Studies from many different laboratories have demonstrated the existence of high-affinity binding sites on cultured cells and cellular membranes that specifically bind high density lipoprotein (HDL). Recent studies from our laboratory have characterized a 110-kd membrane-binding protein that has many features in common with the high-affinity binding sites on intact cells. The cellular binding sites and the isolated protein interact with HDL₃ and phospholipid vesicles containing either apoprotein (apo) A-I or apo A-III but do not appear to interact with low density lipoprotein (LDL). Acetylated LDL or vesicles containing apo E. Moreover, the binding of HDL to intact cells and the isolated 110-kd protein is enhanced when cells are loaded with cholesterol or when the rate of cell proliferation is inhibited. These similar specificity and regulatory properties suggest that the 110-kd binding protein is a component of the cell-surface HDL binding sites on intact cells.

Recent studies from our laboratory have provided evidence that the cellular HDL binding sites represent receptors that mediate transport of excess intracellular cholesterol from cells. Incubation of cholesterol-loaded cells with HDL₃ stimulates translocation of radiolabeled sterol from intracellular pools to the plasma membrane and into the culture medium. This stimulation appears to require the interaction of HDL₃ with cell-surface binding sites, since modification of HDL₃ with tetranitromethane (TNM) reduces its ability both to bind to cells and to stimulate translocation and efflux of intracellular sterol. Because TNM treatment causes extensive covalent cross-linking of apos to other apos and phospholipids, it could not be determined from these studies whether modification of apos, lipids, or particle conformations accounted for the reduction in either cell-surface binding or sterol transport. Evidence that lipids rather than apos mediate cell-surface binding of HDL particles was provided by Tabas and Tall in a study showing that trypsin treatment of HDL₃ failed to impair its ability to interact with cells.
The purpose of the current study was to directly examine the role of intact apolipoproteins (apo) in HDL receptor-mediated transport of excess cholesterol from cells.

To remove intact apo from HDL$_3$ under conditions that preserve the native lipid composition of the particles, apolipoproteins were digested by mild treatment with proteolytic enzymes. We then tested these protease-modified particles for their ability to bind to the 110-kd HDL binding protein and to promote efflux of cellular cholesterol from the plasma membrane and intracellular pools. Results indicate that whereas removal of cholesterol from plasma membranes is mediated by HDL lipids, HDL binding to the candidate receptor protein and selective removal of intracellular sterol require the presence of intact apo in HDL particles.

**Methods**

**Cells and Cell Membranes**

Cultured human skin fibroblasts and bovine aortic endothelial cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously. For cholesterol efflux experiments, fibroblasts were plated into 35-mm dishes at a density of 5 to 7 x 10$^5$ cells/dish and grown to confluency (7–9 days). For preparation of cell membranes used in ligand-blotting studies, endothelial cells were plated and grown to confluency in 150-mm dishes.

Cell membranes were prepared according to Basu et al. Briefly, washed endothelial cell monolayers were dislodged from dishes with a nylon policeman into 0.15 M NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in dimethyl sulfoxide, and 10 mM Tris HCl, pH 7.4. Cells from four to five dishes were combined, pelleted by centrifugation at 200g for 10 minutes, resuspended in the same buffer, homogenized with two 5-second pulses of a polytron homogenizer (Tekmar model No. SDT 1810 with 10EN shaft, Cincinnati, Ohio), and then centrifuged at 800g for 10 minutes at 4°C. The supernatant was centrifuged at 100,000g for 60 minutes at 4°C, and the pellet was stored frozen for future use in ligand-blotting studies.

**Lipoproteins**

Lipoproteins were isolated from human plasma by standard sequential ultracentrifugation techniques (LDL, $d=1.019-1.063$ g/ml; HDL$_3$, $d=1.125-1.210$ g/ml). Lipoproteins were iodinated by the modified Hahm et al. The proteolytic enzymes used for the digestion were trypsin (from porcine pancreas) (GIBCO, Grand Island, N.Y.) dissolved in 0.1 M Tris/0.01 M CaCl$_2$, pH 8.0, and pronase (from Streptomyces griseus) (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 M Tris HCl, pH 7.3. To start the reaction, 200 µl enzyme solution was added to 1.0 ml 0.15 M NaCl and 1 mM EDTA (pH 7.2) containing 10 mg HDL$_3$ at an enzyme-to-lipoprotein ratio of 1:40 (wt/wt). The reaction was continued for either 30 minutes (pronase) or 60 minutes (trypsin) at 37°C and then stopped by addition of 20 µl 0.1 M PMSF in ethanol. The reaction mixture was cooled to 4°C, and protease-modified core particles were isolated from cleaved and dissociated peptides by chromatography on a Sephadex G-75 column (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). The modified particles were dialyzed at 4°C against 0.15 M NaCl plus 1 mM EDTA, filtered (0.22-µm pore size), and stored at 4°C. Control HDL$_3$ ("native" HDL$_3$) was subjected to the same protocol as trypsin-treated HDL$_3$ except that the enzyme was omitted from the reaction mixture. An aliquot was assayed for protein and phospholipid composition.

**Electrophoresis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli using a 12.5–17% gradient gel. Analysis of HDL$_3$ particle size was performed by non-denaturing gradient gel electrophoresis as described previously. Proteins were identified by Coomassie Blue staining.

**Ligand Blotting**

Membrane pellets were solubilized by needle aspiration into SDS-PAGE electrophoresis buffer (with β-mercaptoethanol) and boiled for 3 minutes. Samples containing equal amounts of protein (500 µg) per lane were electrophoresed on 7% polyacrylamide slab gels, and separated proteins were transferred to nitrocellulose membranes (0.45 μm) by electrophoresis. To assay binding activity, nitrocellulose membranes were first incubated for 2 hours at room temperature with blocking buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 10% [wt/vol] nonfat dried milk, 0.01% [vol/vol] antifoam A, 50 µg/ml LDL) and then incubated for 30 minutes to 60 minutes at room temperature in the same buffer containing 5 µg/ml $^{32}$P-HDL$_3$ (4 µM phospholipid) plus the indicated concentration of competitor. Nitrocellulose membranes were washed at room temperature once rapidly and then five times for 10 minutes with LDL-free blocking buffer. Protein bands were visualized by autoradiography.

**Differential Radiolabeling of Cellular Cholesterol Pools**

Fibroblasts were loaded with cholesterol by incubation for 48 hours with serum-free DMEM containing 2 mg/ml bovine serum albumin (BSA) plus 50 µg/ml cholesterol added in ethanol (from a 10 mg/ml cholesterol stock). To enrich intracellular membranes with $^3$H]sterol under conditions that minimize sterol translocation to the plasma membrane, cells were pulsed with [H]mevalonolactone. Initially, pulse incubations were performed at 15°C since previous studies showed that newly synthesized sterol was transferred to the plasma membrane at a slow rate at this tempera-
were washed five times with cold phosphate-buffered glutaraldehyde for 10 minutes at 0°C. The fixed cells were collected, and cells were washed three times with cold wash buffer and twice with BSA-free wash buffer, and then incubated at 37°C for 10-30 minutes with 1 u-g/ml buffer, and used in sterol efflux and translocation experiments. Cells were treated identically for the 37°C pulse incubation except that they were maintained at room temperature during the last wash, labeling media were prewarmed to 37°C, and cells were incubated in a 37°C water bath for 6 hours, chilled on ice, washed five times with ice-cold buffer, and used in sterol efflux and translocation experiments. Cells were treated identically for the 37°C pulse incubation except that they were maintained at room temperature during the last wash, labeling media were prewarmed to 37°C, and cells were incubated in a 37°C water bath for 3 hours.

To enrich plasma membranes of cholesterol-loaded fibroblasts with [3H]cholesterol, cells were washed at room temperature five times with wash buffer and incubated at 37°C with HEPES-buffered DMEM containing 1 mg/ml BSA, 2 μg/ml acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor 58.035, and the indicated amounts of [3H]mevalonolactone (10 μCi/ml). Cells were incubated in a 15°C water bath for 6 hours, chilled on ice, washed five times with ice-cold buffer, and used in sterol efflux and translocation experiments. Cells were treated identically for the 37°C pulse incubation except that they were maintained at room temperature during the last wash, labeling media were prewarmed to 37°C, and cells were incubated in a 37°C water bath for 3 hours.

Sterol Efflux From Cells
Washed cell monolayers were extracted with hexane/isopropanol (3:2, vol/vol) as previously described. Lipids were extracted from efflux medium by the method of Polch et al. Sterol species were separated on silica gel G thin-layer chromatography plates (PE sil G, Whatman, Clifton, N.J.), developed in heptane/diethyl ether/methanol/acetic acid (80:30:10:2, vol/vol/vol/vol) and were detected with I. For isotope measurements, individual spots corresponding to cholesterol esters, free cholesterol, and cholestene were scraped into scintillation vials and counted. The biosynthetically labeled sterols that migrate with the cholesterol in this system represent 27-carbon sterol intermediates, which include zymosterol, desmosterol, and cholesterol but exclude lanosterol and its precursors. When fibroblasts were pulsed with [3H]mevalonolactone, greater than 85% of the newly synthesized 27-carbon sterols were oxidized and comigrated with cholestene after extraction from silica gel with isopropanol, treatment with cholesterol oxidase, and rechromatography. Thus, since cholesterol oxidase appeared to react with all 27-carbon sterols, it was necessary to treat them as a homogeneous pool when using the cholesterol oxidase assay to measure cellular distribution of labeled sterol. To determine sterol mass, free and esterified cholesterol spots were scraped, extracted, and assayed by the cholesterol oxidase procedure described previously.

Sterol Translocation Between Cellular Pools
Translocation of newly synthesized [3H]sterol between cellular pools was measured by a modification of the method described by Lange and Ramos. After the efflux medium was collected and cell monolayers were incubated at 37°C with [3H]cholesterol, cells were washed twice with cold buffer, and extracted in hexane/ isopropanol. Lipid subclasses were separated by thin-layer chromatography, and incorporation of [3H]cholesterol into esterified and unesterified cholesterol was measured as described previously. Incorporation of radiolabel into unesterified sterol represents sterol esterification by ACAT. Incorporation of radiolabel into unesterified sterol represents biosynthe-
untreated

Pronase

Trypsin

untreated

Pronase

Trypsin

Apo A-I

Apo A-II

Figure 1. Photograph of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of native and protease-modified high density lipoprotein 3 (HDL). HDL particles were treated and subjected to electrophoresis as described in “Methods.” Identical amounts of Lowry-reacting proteins were added to each lane (20 μg), which were equivalent to 15, 48, and 36 nmoles phospholipid for lanes 1, 2, and 3, respectively. Apoprotein (apo) bands were identified by Coomassie Blue staining, and positions of apo A-I and A-II are indicated.

Results

Characterization of Protease-Modified High Density Lipoprotein 3

To degrade intact HDL apoproteins, lipoprotein particles were treated with the proteolytic enzymes trypsin or pronase as described in “Methods.” These treatment protocols reduced the Lowry-reacting proteins in the HDL particles by 40–50% (trypsin) and 50–70% (pronase). SDS-PAGE showed that both trypsin-modified HDL3 (TrHDL3) and pronase-modified HDL3 (PrHDL3) contained no detectable intact apo A-II but retained a small amount of intact apo A-I (Figure 1). Pronase treatment consistently caused a more extensive degradation of apo A-I than did trypsin treatment. Most of the assayable protein that remained associated with the protease-modified particles was in the form of low-molecular-weight peptides. For the gel shown in Figure 1, the same amount of total protein was added for each sample. Thus, the lanes with protease-modified HDL3 contained protein from two to three times more particles than did the lane for native HDL3. Based on densitometric scanning of this and other gels, it was estimated that protease treatment completely or partially digested greater than 80% of the HDL3 apoproteins.

Modified and native particles had the same ratios of unesterified and esterified cholesterol to phospholipid, indicating that the relative composition of the major HDL3 lipid components was unchanged by treatment with either enzyme (data not shown), as also reported by others. Nondenaturing gradient gel electrophoresis indicated that the size distribution of HDL3 particles was unaffected by protease treatment. To normalize values for particle concentration and surface area, lipoproteins were quantified according to phospholipid content.

Recent studies from our laboratory have identified a 110-kd membrane protein that specifically binds HDL and phospholipid vesicles containing apo A-I and apo A-II. To test the effects of proteolytic degradation of HDL apo on lipoprotein binding to this candidate receptor protein, we performed competitive binding studies using the ligand-blotting protocol described in “Methods.” The choice of cell type for these studies was cultured bovine aortic endothelial cells, since membranes from these cells have a high abundance of HDL-binding protein. The same protein appears to be present in cultured fibroblast membranes.

When endothelial cell membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, autoradiographs revealed a 110-kd protein that interacted with [125I]-HDL3 (Figure 2). The addition of excess unlabeled HDL3 to the binding medium reduced the signal by more than 80%, indicating that [125I]-HDL3 binding to this protein is saturable and can be reduced by competition with unlabeled ligand. However, addition of excess unlabeled TrHDL3 or PrHDL3 had little effect on [125I]-HDL3 binding to the 110-kd protein, indicating that protease-modified lipoproteins interact poorly with the HDL-binding protein.

Promotion of Cholesterol Efflux From Different Cellular Pools by High Density Lipoprotein 3 and Protease-Modified High Density Lipoprotein 3

Previous results from our laboratory have suggested that binding of HDL to its high-affinity bind-
ing sites promotes removal of sterol from intracellular pools, whereas transport of cholesterol directly from plasma membranes to HDL occurs independently of HDL binding.\textsuperscript{10,11} Since cholesterol loading of cells increases the number of high-affinity sites, we compared the effects of protease treatment of HDL\textsubscript{3} on its ability to promote efflux of labeled sterol from cholesterol-depleted and cholesterol-loaded cells.

To prelabel intracellular pools of unesterified sterol without increasing sterol mass, cells were pulsed with the biosynthetic precursor \textsuperscript{3H} mevalonolactone in the presence of an ACAT inhibitor. With this procedure, less than 50 ng/mg cell protein of newly synthesized sterol mass was introduced into cellular pools. \textsuperscript{3H} mevalonolactone was chosen as a precursor because it bypasses hydroxymethylglutaryl coenzyme A reductase, the enzyme in the biosynthetic pathway that undergoes the largest degree of regulation in response to changes in cell cholesterol content.

Before the efflux studies, experiments were conducted to test the effects of cholesterol loading on biosynthetic labeling of sterol and its cellular distribution. To assess the relative distribution of sterol between cellular pools, cells were fixed with glutaraldehyde and treated with cholesterol oxidase (see "Methods"). When fibroblasts were preincubated with lipoprotein-deficient serum to deplete cells of cholesterol, most of the biosynthetically labeled sterol appeared in cholesterol oxidase-accessible pools (Figure 3), presumably because of rapid translocation to the plasma membrane. Addition of nonlipoprotein cholesterol to this medium led to a reduction in both sterol synthesis and the relative proportion of labeled sterol appearing in cholesterol oxidase-accessible pools. Removal of serum from either the cholesterol-free or cholesterol-enriched
medium caused a further suppression of sterol synthesis and cholesterol oxidase accessibility, probably because serum growth factors were removed. Previous studies have suggested that inhibition of cell proliferation increases the pool size of the intracellular sterol that regulates biochemical processes. The lowest rates of sterol synthesis and incorporation of newly synthesized sterol into cholesterol oxidase-accessible pools occurred when cells were loaded with cholesterol in the absence of serum and in the presence of an ACAT inhibitor. Thus, as cells accumulate more unesterified cholesterol, there are progressive decreases in both the rates of sterol synthesis from mevalonolactone and relative cholesterol oxidase accessibility.

The decrease in cholesterol oxidase accessibility caused by cholesterol loading of cells could not be attributed to reduced biosynthesis of radiolabeled sterol. A time course revealed that the distribution of newly synthesized [3H]sterol between cholesterol oxidase-accessible and -inaccessible pools in cholesterol-loaded cells reached equilibrium within 2 hours (Figure 4B), even though the cellular content of [3H]mevalonolactone-derived sterol continued to increase after 2 hours. Thus, translocation of [3H]sterol between these cellular pools is not a function of the amount of [3H]sterol tracer that accumulates within the cell.

The above results suggest that cholesterol loading of cells traps newly synthesized radiolabeled sterol within intracellular pools of cholesterol that are inaccessible to cholesterol oxidase. These pools appear to be in rapid equilibrium with the substrate pool for the microsomal esterifying enzyme ACAT. When the ACAT inhibitor was omitted during the 3-hour pulse-labeling incubations, more than 75% of the newly synthesized sterol was esterified (Table 1). In contrast, less than 10% of the newly synthesized sterol was esterified in cholesterol-depleted cells. The efficient conversion to esters in cholesterol-loaded cells indicates that newly synthesized sterol tracer rapidly enters the ACAT substrate pool before exchange with the much larger cellular pool of unlaabeled cholesterol. To eliminate the cholesteryl-ester cycle as a variable, an ACAT inhibitor was included in all pulse-chase incubations using the biosynthetic labeling protocol.

When sterol-loaded cells were pulsed with [3H]mevalonolactone and chased with medium containing unlabeled mevalonolactone plus either native HDL3 or TrHDL3, the unmodified particles were much more effective than modified HDL3 in promoting efflux of labeled sterol (Figure 5A). In contrast, when cells were depleted of cholesterol before the pulse-chase incubations (Figure 5B), TrHDL3 had a slightly greater ability than native HDL3 to promote sterol efflux. Moreover, HDL3 removed a larger percentage of newly synthesized sterol from cholesterol-loaded cells than from cholesterol-depleted cells (Figure 5), even though the loaded cells incorporated most of the labeled sterol into cholesterol oxidase-inaccessible pools. Thus, it appears that HDL apoproteins facilitate efflux of biosynthetically labeled sterol only when cells are loaded with cholesterol, and most of the labeled sterol initially resides in intracellular pools that are inaccessible to cholesterol oxidase. In contrast, HDL apoproteins do not appear to play a role in promoting sterol efflux from cholesterol-depleted cells when most of the label resides within the plasma membrane.

These studies raise the possibility that HDL apoproteins selectively mediate removal of intracellular sterol from cholesterol-loaded cells. To further test this possibility, [3H]sterol efflux was measured after choles-

![Figure 4](http://atvb.ahajournals.org/)

**FIGURE 4.** Line plots of time course for sterol synthesis and translocation in cholesterol-loaded fibroblasts. Cultured fibroblasts were loaded with cholesterol by pretreatment with serum-free medium plus cholesterol plus ACAT inhibitor 58.035 and pulsed with [3H]mevalonolactone as described in Figure 3 legend. After the indicated time (hours, h), cells were fixed and treated with cholesterol oxidase (CO) in hypotonic buffer for 30 minutes, and incorporation of label in CO-accessible (○) and CO-inaccessible (■) sterol was measured as described in "Methods." Results are mean of three incubations expressed as either radioactivity counts ([3H]-sterol, cpm/dish; panel A) or percent of total [3H]-sterol (panel B). Standard error bars are included in panel A unless within the dimension of the symbols. ACAT, acyl coenzyme A:cholesterol acyltransferase.
TABLE 1. Esterification of [3H]Mevalonolactone-Derived Sterols in Cholesterol-Loaded and -Depleted Fibroblasts

<table>
<thead>
<tr>
<th>Sterol (cpm/dish)</th>
<th>Fibroblast type</th>
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<tr>
<td></td>
<td>Unesterified</td>
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<tr>
<td></td>
<td>Cholesterol loaded</td>
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<td>Cholesterol depleted</td>
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Cultured fibroblasts were either cholesterol loaded (serum-free medium plus cholesterol) or cholesterol depleted (10% lipoprotein-deficient serum) and incubated for 3 hours at 37°C with [3H]mevalonolactone as described in Figure 3 legend except that acyl coenzyme A:cholesterol acyltransferase inhibitor was omitted during the labeling incubation. Cell lipids were extracted and radiolabeled, and unesterified and esterified cholesterol was measured as described in “Methods.” Each value represents mean±SEM of 12 incubations. Values in parentheses represent percent total counts.

Label plasma membranes. Again, native HDL₃ was more effective than either TrHDL₃ or PrHDL₃ in promoting labeled sterol efflux derived from a biosynthetic precursor (Figure 6A). However, similar to what was observed for efflux of biosynthetically labeled sterol from cholesterol-depleted cells (Figure 5B), modified forms of HDL₃ were slightly better acceptors than were native particles for [3H]cholesterol associated with the plasma membrane of cholesterol-loaded cells (Figure 6B). These results provide additional evidence that HDL apo E mediate removal of sterol from intracellular pools of cholesterol-loaded cells but not from the plasma membrane.

Addition of native HDL₃ to the chase medium increased efflux of biosynthetically labeled sterol from cholesterol-loaded fibroblasts during the first 2 hours of incubation, after which efflux subsided (Figure 7). This early rapid-efflux phase was not evident when cells were exposed to an equal concentration of TrHDL₃.

Lipoprotein-Mediated Translocation of Cholesterol Between Cellular Pools

Our previous studies suggested that HDL₃ selectively removes intracellular sterol by stimulating translocation of sterol from intracellular pools to the cell surface where it becomes accessible to HDL₃ particles. To assess the role of HDL apo E in this translocation process, we measured the effects of native or TrHDL₃ on the distribution of endogenously synthesized [3H]sterol between cellular pools
that are inaccessible or accessible to cholesterol oxidase.

As shown earlier (Figure 3), when sterol pools of cholesterol-loaded fibroblasts are labeled with $[^3H]$mevalonolactone, most of the $[^3H]$sterol is incorporated into pools that are inaccessible to cholesterol oxidase. This distribution of $[^3H]$sterol was maintained even when cells were chased for 3 hours at 37°C with lipoprotein-free medium containing the unlabeled precursor (data not shown). Addition of native HDL$_3$ to the chase medium led to an increase in cholesterol oxidase-accessible $[^3H]$sterol associated with a decrease in cholesterol oxidase-inaccessible $[^3H]$sterol (Figure 8A). In contrast to native HDL$_3$, TrHDL$_3$ had little effect on the redistribution of biosynthetically labeled $[^3H]$sterol between cellular pools (Figure 8B). These results are consistent with our previous studies$^{10,11}$ showing that HDL$_3$ stimulates translocation of sterol from intracellular sites that are inaccessible to cholesterol oxidase to sites that are accessible to this enzyme. This stimulatory process appears to be mediated by HDL apos.

**Effects of Native and Trypsin-Modified High Density Lipoprotein on Depletion of Sterol Mass Within Intracellular Pools**

To test whether the HDL$_3$-stimulated depletion of biosynthetically labeled sterol within cholesterol oxidase-inaccessible pools represents net mass movement of intracellular sterol, we measured the effects of native and TrHDL$_3$ on the activities of two biochemical processes known to be regulated reciprocally by changes in the size of microsomal pools of sterol: cholesterol esterification (ACAT activity) and sterol biosynthesis. The relative activities of these two processes were assayed by pulse incubating cells for 1 hour with $[^14C]$oleate and measuring the incorporation of radiolabel into free and esterified cholesterol (see "Methods"). When cholesterol-loaded fibroblasts were incubated with lipoprotein-free
incubation with cholesterol-free medium to allow equilibration of cellular sterol pools, more than 80% of the isotope was found to be accessible to cholesterol oxidase (data not shown) and thus, mostly resided in pools other than those radiolabeled with biosynthetic tracer. Efflux of \(^{3}H\)cholesterol was increased by addition to the medium of both forms of HDL<sub>3</sub> particles (Figure 9C). These results further indicate that depletion of intracellular regulatory sterol pools but not other pools of cholesterol is mediated by HDL apoproteins.

HDL apoproteins also mediate depletion of intracellular sterol pools derived from the uptake and degradation of LDL particles. When HDL<sub>3</sub> was added to medium containing LDL during 24-hour incubations, there was a dose-dependent decrease in the ACAT activity (Figure 10A) in parallel with a decrease in the cellular cholesteryl ester content (Figure 10B). These data are consistent with previous results showing that HDL<sub>3</sub> removes intracellular cholesterol at a rate that exceeds delivery of LDL cholesterol, even though receptor-mediated uptake of LDL is enhanced by addition of HDL<sub>3</sub> (apparently in response to depletion of the intracellular sterol regulatory pool). In contrast to native HDL<sub>3</sub>, addition of TrHDL<sub>3</sub> or PrHDL<sub>3</sub> to the LDL medium had little effect on either ACAT activity or cellular cholesteryl ester mass. The much larger pool of unesterified cholesterol, most of which presumably resides in plasma membranes, decreased only slightly in the presence of increasing concentrations of either unmodified or modified HDL<sub>3</sub> (Figure 10C). These results are consistent with the conclusion that HDL<sub>3</sub> apoproteins, rather than lipids, mediate the selective removal of intracellular pools of cholesterol.

**Discussion**

The current study demonstrates that digestion of HDL<sub>3</sub> apoproteins with proteolytic enzymes reduces the ability of HDL<sub>3</sub> to remove sterol from cholesterol-loaded fibroblasts. Exposure of cholesterol-loaded cells to HDL<sub>3</sub> suppressed ACAT activity, decreased cholesteryl ester content, and stimulated efflux of labeled sterol derived from intracellular pools. All these effects were markedly reduced by treatment of HDL<sub>3</sub> with trypsin or pronase. In contrast, protease treatment did not reduce the ability of HDL<sub>3</sub> to promote efflux of labeled sterol associated with the plasma membrane. These results indicate that HDL<sub>3</sub> apoproteins, rather than lipids, mediate the selective removal of cholesterol from intracellular pools of sterol-laden cells.

For some of the selective-removal assays used in this study, unesterified cholesterol pools were radiolabeled by incubating cells with the biosynthetic precursor \(^{3}H\)mevalonolactone in the presence of an ACAT inhibitor. This procedure has the advantage over labeling with lipoprotein-derived cholesterol in that microsomal pools are labeled rapidly without the delay imposed by the endocytotic/lysosomal pathway. Of the 27-carbon sterols synthesized by fibroblasts during short-term incubations with \(^{3}H\)mevalonolactone,...
valonolactone in the absence of an ACAT inhibitor, appear to behave as a homogeneous pool with re-
tracer into the substrate pool for ACAT. Thus, this biosynthetic labeling procedure demonstrated results (SEM bars described in “Methods.” Each value represents mean±SEM of four incubations (SEM bars were omitted from panel C since there was no significant difference between groups.)

tone, only approximately 20% of the label is associated with authentic cholesterol (J.F. Oram, unpublished results). The remainder probably represents labeled desmosterol and zymosterol. However, most of these labeled 27-carbon sterols rapidly equilibrate with intracellular pools of cholesterol. This conclusion was based on results showing that when cholesterol-loaded cells were incubated with [3H]me-
valonolactone in the absence of an ACAT inhibitor, more than 70% of the newly synthesized sterol was esterified. Thus, this biosynthetic labeling procedure provides an efficient means of introducing sterol tracer into the substrate pool for ACAT.

The biosynthetically labeled 27-carbon sterols also appear to behave as a homogeneous pool with respect to sterol trafficking. Twenty-seven-carbon ste-
rols excreted from cells in the presence of HDL₃ have a subspecies composition similar to that retained by cells, indicating that the different subspecies are transported within and from cells at similar rates. These findings are consistent with a recent study by Echevarria et al showing that endogenously synthe-
sized zymosterol and cholesterol are transported from cells with the same kinetics. Therefore, it was assumed that labeling sterol pools with a biosynthetic precursor in the presence of an ACAT inhibitor was a valid procedure for studying efflux of cholesterol derived from intracellular pools.

Treatment of cells with cholesterol oxidase was used to monitor movement of labeled sterol between cellular pools. Many studies have shown that plasma membrane cholesterol is converted to cholestenone by treatment of cells with cholesterol oxidase. In contrast, at least some pools of intracellular cholesterol are inaccessible to oxidase treatment. Tabas et al reported that incubation of macrophages with ACAT-stimulating lipoproteins or 25-hydroxycholesterol increased translocation of ra-
diolabeled cholesterol from cholesterol oxidase-accessible to cholesterol oxidase–inaccessible pools, concomitant with increased cholesterol esterification. Similar results were obtained when fibroblasts were treated with an enzyme that degrades plasma mem-
brane sphingomyelin. Similar studies suggest that plasma membrane–derived cholesterol is transported to a cholesterol oxidase–inaccessible pool before being esterified. Other investigators have demonstrated that newly synthesized sterol first enters a cholesterol oxidase–inaccessible pool before transport to the plasma membrane. The current study extends our previous reports by showing that loading cells with cholesterol and inhibiting cell proliferation suppress the rate at which newly syn-
thetized sterol tracer is translocated from oxidase-inaccessible to oxidase-accessible pools. The most straightforward explanation for these results is that as cells accumulate cholesterol or require less for new membrane synthesis, the newly synthesized ste-
rol becomes trapped within intracellular pools of cholesterol, which are translocated very slowly to the plasma membrane.

Exposure of cholesterol-loaded cells to native HDL₃ but not to TrHDL₃ decreased the amount of labeled sterol within cholesterol oxidase–inaccessible pools and increased the amount within oxidase-accessible pools. These data suggest that the interaction of HDL₃ apos with cholesterol-loaded cells stimulates translocation of sterol from one intracel-

FIGURE 10. Line plots of effects of native and protease-modified (Pr, Tr) high density lipoprotein 3 (HDL₃) on ACAT activity and sterol mass in fibroblasts incubated with low density lipoprotein (LDL). Fibroblasts were incubated for 24 hours with 10% lipoprotein-deficient serum to induce LDL receptors, followed by 24-hour incubation with serum-free medium containing 2 mg/ml bovine serum albumin, 40 μg/ml LDL protein, plus the indicated concentration of either HDL₃, trypsin-modified (Tr) HDL₃, or protease-modified (Pr) HDL₃. Cells were then washed and pulsed for 1 hour at 37°C with serum-free medium containing 18 μM [14C]oleate bound to 0.03% bovine serum albumin. Lipids were extracted from cells and assayed for incorporation of [14C]oleate into cholesteryl esters (panel A), cholesteryl ester mass (panel B), and free cholesterol mass (panel C) as described in “Methods.” Each value represents mean±SEM of four incubations (SEM bars were omitted from panel C since there was no significant difference between groups.)
are unknown, they are likely to represent intermediates in the excretory pathway, since the HDL-stimulated translocation of labeled sterol from cholesterol oxidase-inaccessible to -accessible pools was associated with a parallel increase in cholesterol efflux.

Stimulation of radiolabeled sterol translocation and efflux by HDL_{3} reflects net mass transport of cholesterol. Exposure of cells to HDL_{3} suppressed ACAT activity and reduced the cellular cholesteryl ester content, implying that HDL_{3} depleted the intracellular cholesterol mass that was a substrate for ACAT. Protease treatment of HDL_{3} almost completely abolished the ability of HDL_{3} to deplete this intracellular pool, indicating that this process is mediated by HDL apos. Taken together, these different assays provide evidence that HDL_{3} apos activate one or more steps that translocate unesterified cholesterol from ACAT-accessible intracellular pools into an excretory pathway.

It is unlikely that the stimulatory effect of HDL_{3} on sterol translocation is secondary to removal of cholesterol from the plasma membrane. HDL_{3} treated with either TNM or proteases (this study) has the same or greater ability than untreated HDL_{3} to promote efflux of plasma membrane sterol, and yet the modified particles do not stimulate sterol translocation. Incubation of LDL-treated cells with native or modified HDL_{3} caused similar small decreases in unesterified cholesterol mass, most of which presumably resides in the plasma membrane, indicating that both types of particles promote net transport of cholesterol from the plasma membrane. Thus, our data demonstrate that flux of sterol between the plasma membrane and HDL particles is not mediated by the interaction of HDL apos with cells, in agreement with results reported by others.

The protease treatment protocols used in the current study do not provide information about the nature of the apos involved in stimulating intracellular sterol translocation. When HDL_{3} was treated with either trypsin or pronase, most of the apos were degraded to peptide fragments. Studies using isolated HDL apos are currently being conducted in our laboratory. Preliminary results indicate that lipid-free apo A-I can stimulate translocation of intracellular sterol to the plasma membrane under conditions where sterol efflux does not occur, suggesting that apo A-I alone can modulate the sterol translocation pathway. It is not yet known if other HDL apos have any direct effect on sterol trafficking in cells.

Although there are several possible explanations for the stimulatory effect of HDL apos on translocation and efflux of intracellular sterol, one possibility is that HDL conveys a signal to cells to initiate translocation of excess intracellular sterol to the plasma membrane, a process that may be mediated by receptor binding of HDL apos. We have identified a 110-kd membrane protein that has many features predicted for an HDL receptor, including specificity for HDL apos and regulation by changes in cell cholesterol content. The current study shows that binding of HDL to this candidate receptor protein is reduced by protease treatment of the lipoprotein particles. The HDL receptor hypothesis is consistent with earlier studies showing that some but not all of the cholesterol efflux from sterol-loaded cells correlates with binding of HDL apos to high-affinity sites on the cell surface. It is now apparent that sterol efflux from cells has at least two components: sterol translocation and efflux from intracellular pools, and transport of plasma membrane sterol to extracellular acceptor particles. It is only the first component that is mediated by HDL apos, possibly through their interaction with cell-surface receptors.

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