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 sterol 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.

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.1 The cellular binding sites and the isolated protein interact with HDL3 and phospholipid vesicles containing either apoprotein (apo) A-I or apo A-III1-7 but do not appear to interact with low density lipoprotein (LDL)14-14 acetylated LDL14 or vesicles containing apo E15. Moreover, the binding of HDL to intact cells and the isolated 110-kd protein is enhanced when cells are loaded with cholesterol1,3,4,8 or when the rate of cell proliferation is inhibited.9 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.10,11 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 cells12,13 and to stimulate translocation and efflux of intracellular sterol.10,11 Because TNM treatment causes extensive covalent cross-linking of apos to other apos and phospholipids,12,13 it could not be determined from these studies whether modification of apoproteins, lipids, or particle conformations accounted for the reduction in either cell-surface binding or sterol transport. Evidence that lipids rather than apoproteins mediate cell-surface binding of HDL particles was provided by Tabas and Tall14 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 apoA-I in HDL receptor-mediated transport of excess cholesterol from cells. To remove intact apoA-I from HDL₃ under conditions that preserve the native lipid composition of the particles, apoA-I were digested by mild treatment with proteolytic enzymes. We then tested these protease-modified particles for their abilities 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 apoA-I 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 10⁶ cells/dish and grown to confluence (7–9 days). For preparation of cell membranes used in ligand-blotting studies, endothelial cells were plated and grown to confluence 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 5 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 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; HDL₂, d=1.125–1.210 g/ml). Lipoproteins were iodinated by the modified Hahm et al. 17 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 CaCl₂, 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 HDL₃ 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 HDL₃ (“native” HDL₃) was subjected to the same protocol as trypsin-treated HDL₃ 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 HDL₃ particle size was performed by nondenaturing 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 2 hours at room temperature in the same buffer containing 5 µg/ml ³²P-HDL₃ (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 [³H]sterol under conditions that minimize sterol translocation to the plasma membrane, cells were pulsed with [³H]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 incubated in a 37°C water bath for 3 hours. Medium and cells were stored frozen until extraction for lipid and protein analyses, or cells were washed three times with cold wash buffer and twice with BSA-free wash 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 (Sandoz, East Hanover, N.J.), and 0.4 mM [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 monolayers of cholesterol-loaded and radiolabeled cells were incubated at 37°C with HEPES-buffered DMEM containing 1 mg/ml BSA, 2 µg/ml compound 58.035, and the indicated amounts of native and protease-modified HDL₃. After the times indicated, cells were chilled on ice, washed five times with ice-cold buffer, and used in sterol efflux studies. This procedure specifically radiolabels plasma membrane pools of cholesterol, since short-term incubation with trace quantities of [3H]cholesterol leads to incorporation of isotope into plasma membranes without significant transfer to intracellular membranes. 

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 cells were washed three times with cold wash buffer and twice with BSA-free wash buffer. Medium and cells were stored frozen until extraction for lipid and protein analyses, or cells were assayed immediately for plasma membrane-associated sterol as described below.

Cholesterol Esterification and Sterol Biosynthesis

To assess the relative activity of ACAT and the sterol biosynthetic pathway, cells were washed and incubated for 1 hour at 37°C with serum-free medium containing [14C]oleate (20 µM) bound to albumin (0.3 mg/ml). Cells were then chilled on ice, washed twice with cold buffer, and extracted in hexane/isopropanol. Lipid subclasses were separated by thin-layer chromatography, and incorporation of 14C radioactivity into esterified and unesterified cholesterol was measured as described previously. Incorporation of radiolabel into esterified sterol represents sterol esterification by ACAT. Incorporation of radiolabel into unesterified sterol represents biosynthe-
Untreated

Pronase treated

Trypsin treated

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.

sis of sterol from [14C]acetate units liberated from the β-oxidation of [14C]oleate. Results

Characterization of Protease-Modified High Density Lipoprotein 3

To degrade intact HDL apo, 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 HDL (TrHDL) and pronase-modified HDL (PrHDL) 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 HDL contained protein from two to three times more particles than did the lane for native HDL. Based on densitometric scanning of this and other gels, it was estimated that protease treatment completely or partially digested greater than 80% of the HDL apo.

Modified and native particles had the same ratios of unesterified and esterified cholesterol to phospholipid, indicating that the relative composition of the major HDL 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 HDL 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-HDL (Figure 2). The addition of excess unlabeled HDL to the binding medium reduced the signal by more than 80%, indicating that 125I-HDL binding to this protein is saturable and can be reduced by competition with unlabeled ligand. However, addition of excess unlabeled TrHDL or PrHDL had little effect on 125I-HDL 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{3}H\textsubscript{2}O\textsubscript{2} 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{3}H\textsubscript{2}O\textsubscript{2} mevalonolactone was chosen as a precur-

Figure 3. Bar graph showing effect of cholesterol (CHOL) loading of fibroblasts on rates of sterol synthesis (% cell \textsuperscript{3}H-sterol) from mevalonolactone and distribution of newly synthesized sterol between cellular pools. Cultured fibroblasts were incubated for 48 hours with medium containing 10% lipoprotein-deficient human serum (LPDS), 10% LPDS+50 \textmu g/ml CHOL, serum-free medium (SFM), SFM+CHOL, or SFM+CHOL and 2 \textmu g/ml ACAT inhibitor (58-035). Cells were then pulsed for 3 hours at 37°C with \textsuperscript{3}H\textsubscript{2}O\textsubscript{2} mevalonolactone (plus ACAT inhibitor) and treated with cholesterol oxidase (CO) in hypotonic buffer for 30 minutes, and incorporation of label into total, CO-accessible, and CO-inaccessible sterol was measured as described in "Methods." Mean relative rates of sterol synthesis are indicated in parentheses and are presented as percent of the maximum mean value. Values are mean±SEM of four incubations. Percentage of newly synthesized sterol that was inaccessible to CO treatment. ACAT, acyl coenzyme A: cholesterol acyltransferase.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Bar graph showing effect of cholesterol (CHOL) loading of fibroblasts on rates of sterol synthesis (% cell \textsuperscript{3}H-sterol) from mevalonolactone and distribution of newly synthesized sterol between cellular pools. Cultured fibroblasts were incubated for 48 hours with medium containing 10% lipoprotein-deficient human serum (LPDS), 10% LPDS+50 \textmu g/ml CHOL, serum-free medium (SFM), SFM+CHOL, or SFM+CHOL and 2 \textmu g/ml ACAT inhibitor (58-035). Cells were then pulsed for 3 hours at 37°C with \textsuperscript{3}H\textsubscript{2}O\textsubscript{2} mevalonolactone (plus ACAT inhibitor) and treated with cholesterol oxidase (CO) in hypotonic buffer for 30 minutes, and incorporation of label into total, CO-accessible, and CO-inaccessible sterol was measured as described in "Methods." Mean relative rates of sterol synthesis are indicated in parentheses and are presented as percent of the maximum mean value. Values are mean±SEM of four incubations. Percentage of newly synthesized sterol that was inaccessible to CO treatment. ACAT, acyl coenzyme A: cholesterol acyltransferase.}
\end{figure}
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.\(^9\)

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 \(^{1}H\)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 \(^{1}H\)mevalonolactone-derived sterol continued to increase after 2 hours. Thus, translocation of \(^{1}H\)sterol between these cellular pools is not a function of the amount of \(^{1}H\)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 73% 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 unla-bered 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 \(^{1}H\)mevalonolactone and chased with medium containing unlabeled mevalonolactone plus either native HDL\(_3\) or TrHDL\(_3\), the unmodified particles were much more effective than modified HDL\(_3\) in promoting efflux of labeled sterol (Figure 5A). In contrast, when cells were depleted of cholesterol before the pulse-chase incubations (Figure 5B), TrHDL\(_3\) had a slightly greater ability than native HDL\(_3\) to promote sterol efflux. Moreover, HDL\(_3\) 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-inaccessi-

![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 \(^{1}H\)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 \((^{1}H\)sterol, cpm/dish; panel A) or percent of total \(^{1}H\)-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 \[^{3}H\]Mevalonolactone-Derived Sterols in Cholesterol-Loaded and -Depleted Fibroblasts

<table>
<thead>
<tr>
<th>Sterol (cpm/dish)</th>
<th>Fibroblast type</th>
<th>Unesterified</th>
<th>Esterified</th>
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<tr>
<td></td>
<td>Cholesterol loaded</td>
<td>144 ± 24</td>
<td>383 ± 27</td>
</tr>
<tr>
<td></td>
<td>(27%)</td>
<td>(73%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cholesterol depleted</td>
<td>892 ± 107</td>
<td>91 ± 12</td>
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<tr>
<td></td>
<td>(91%)</td>
<td>(9%)</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 \[^{3}H\]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 HDL\(_3\) was more effective than either TrHDL\(_3\) or PrHDL\(_3\) 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 HDL\(_3\) were slightly better acceptors than were native particles for \[^{3}H\]cholesterol associated with the plasma membrane of cholesterol-loaded cells (Figure 6B). These results provide additional evidence that HDL apolipoproteins mediate removal of sterol from intracellular pools of cholesterol-loaded cells but not from the plasma membrane.

Addition of native HDL\(_3\) 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 TrHDL\(_3\).

Lipoprotein-Mediated Translocation of Cholesterol Between Cellular Pools

Our previous studies\(^{10,11}\) suggested that HDL\(_3\) selectively removes intracellular sterol by stimulating translocation of sterol from intracellular pools to the cell surface where it becomes accessible to HDL\(_3\) particles. To assess the role of HDL apolipoproteins in this translocation process, we measured the effects of native or TrHDL\(_3\) on the distribution of endogenously synthesized \[^{3}H\]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 HDL3 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 HDL3, TrHDL3 had little effect on the redistribution of biosynthetically labeled [3H]sterol between cellular pools (Figure 8B). These results are consistent with our previous studies10,11 showing that HDL3 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 apol.

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

To test whether the HDL3-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 TrHDL3 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
medium, the relative activities of ACAT and the sterol biosynthetic pathway remained constant for up to 8 hours (Figures 9A and 9B). Addition of HDL3 to the medium led to a decrease in ACAT activity (Figure 9A) in association with an increase in the rate of sterol synthesis (Figure 9B), indicating that HDL3 depleted cells of intracellular sterol pools. In contrast, TrHDL3 had no effect on either ACAT activity or the rate of sterol biosynthesis.

To monitor efflux of plasma membrane-derived cholesterol in the same experiment, plasma membranes were radiolabeled by incubating with [3H]cholesterol in the sterol-loading medium. After an 18-hour 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 [3H]cholesterol was increased by addition to the medium of both forms of HDL3 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 HDL3 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 cholesterol ester content (Figure 10B). These data are consistent with previous results showing that HDL3 removes intracellular sterol at a rate that exceeds delivery of LDL sterol, even though receptor-mediated uptake of LDL is enhanced by addition of HDL3 (apparently in response to depletion of the intracellular sterol regulatory pool). In contrast to native HDL3, addition of TrHDL3 or PrHDL3 to the LDL medium had little effect on either ACAT activity or cellular cholesterol 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 HDL3 (Figure 10C). These results are consistent with the conclusion that HDL3 apoproteins, rather than lipids, mediate the selective removal of intracellular pools of cholesterol.

**Discussion**

The current study demonstrates that digestion of HDL3 apoproteins by proteolytic enzymes reduces the ability of HDL3 to remove sterol from cholesterol-loaded fibroblasts. Exposure of cholesterol-loaded cells to HDL3 suppressed ACAT activity, decreased cholesterol ester mass, and stimulated efflux of labeled sterol derived from intracellular pools. All these effects were markedly reduced by treatment of HDL3 with trypsin or pronase. In contrast, protease treatment did not reduce the ability of HDL3 to promote efflux of labeled sterol associated with the plasma membrane. These results indicate that HDL3 apoproteins mediate selective removal of excess 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 with the biosynthetic precursor [3H]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 endocytic/lysosomal pathway. Of the 27-carbon sterols synthesized by fibroblasts during short-term incubations with [3H]mevalonolactone...
Valonolactone in the absence of an ACAT inhibitor, appear to behave as a homogeneous pool with respect to sterol trafficking. Twenty-seven-carbon sterols 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 synthesized 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 radio-labeled 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 membrane sphingomyelin. These 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 synthesized 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 sterol 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 apoproteins with cholesterol-loaded cells stimulates translocation of sterol from one intracellular pool to other cellular pools that are accessible to cholesterol oxidase. It cannot be concluded that the intracellular sterol is translocated exclusively to the plasma membrane, since recent studies have shown that cholesterol oxidase treatment may damage cell membranes and expose some intracellular pools to the enzyme. Although the precise cellular locations of the cholesterol oxidase-accessible pools

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 receptor expression, 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 pronase-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.)

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]mevalonolactone 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 sterols 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 synthesized 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 radio-labeled 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 membrane sphingomyelin. These 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 synthesized 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 sterol 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 apoproteins with cholesterol-loaded cells stimulates translocation of sterol from one intracellular pool to other cellular pools that are accessible to cholesterol oxidase. It cannot be concluded that the intracellular sterol is translocated exclusively to the plasma membrane, since recent studies have shown that cholesterol oxidase treatment may damage cell membranes and expose some intracellular pools to the enzyme. Although the precise cellular locations of the cholesterol oxidase-accessible pools
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 HDL₃ reflects net mass transport of cholesterol. Exposure of cells to HDL₃ suppressed ACAT activity and reduced the cellular cholesterol ester content, implying that HDL₃ depleted the intracellular cholesterol mass that was a substrate for ACAT. Protease treatment of HDL₃ almost completely abolished the ability of HDL₃ to deplete this intracellular pool, indicating that this process is mediated by HDL apoproteins. Taken together, these different assays provide evidence that HDL₃ apoproteins 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 HDL₃ on sterol translocation is secondary to removal of cholesterol from the plasma membrane. HDL₃ treated with either TNM₁₀,₁¹ or proteases (this study) has the same or greater ability than untreated HDL₃ 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 HDL₃ caused similar small decreases in unesterified cholesterol mass, most of which presumably resides in the plasma membrane,₂₂,₂₇ indicating that both types of particles promoted 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 apoproteins with cells, in agreement with results reported by others.₃₆–₃₈

The protease treatment protocols used in the current study do not provide information about the nature of the apoproteins involved in stimulating intracellular sterol translocation. When HDL₃ was treated with either trypsin or pronase, most of the apoproteins were degraded to peptide fragments. Studies using isolated HDL₃ apoproteins are currently being conducted in our laboratory. Preliminary results indicate that lipoprotein-A-I can stimulate translocation of intracellular sterol to the plasma membrane under conditions where sterol efflux does not occur,₃⁹ suggesting that apoprotein A-I alone can mediate the sterol translocation pathway. It is not yet known if other HDL apoproteins have any direct effect on sterol trafficking in cells.

Although there are several possible explanations for the stimulatory effect of HDL apoproteins 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 apoproteins. We have identified a 110-kd membrane protein that has many features predicted for an HDL receptor, including specificity for HDL apoproteins and regulation by changes in cell cholesterol content.₁⁹ 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 by Schouten et al.,₃,₁₂ showing that some but not all of the cholesterol efflux from sterol-loaded cells correlates with binding of HDL₃ 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 apoproteins, possibly through their interaction with cell-surface receptors.

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