High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages

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Abstract Binding of high density lipoprotein (HDL) to its receptor on cultured fibroblasts and aortic endothelial cells was previously shown to facilitate sterol efflux by initiation of translocation of intracellular sterol to the plasma membrane. After cholesterol-loaded human monocyte-derived macrophages were incubated with either [^{3}H]mevalonolactone or lipoprotein-associated [^{3}H]cholesteryl ester to radiolabel intracellular pools of sterol, incubation with HDL_3 led to stimulation of ^{3}H-labeled sterol translocation from intracellular sites to the cell surface which preceeded maximum ^{3}H-labeled sterol efflux. A similar pattern was demonstrated for macrophages that were preloaded with cholesterol derived from either low density lipoprotein (LDL), acetyl-LDL, or phospholipase C-modified LDL. However, in macrophages that were not loaded with cholesterol, HDL_3 stimulated net movement of ^{3}H-labeled sterol from the plasma membrane into intracellular compartments, the opposite direction from that seen for cholesterol-loaded cells. A similar influx pattern was found in nonloaded macrophages and fibroblasts that were labeled with trace amounts of exogenous [^{3}H]cholesterol. Cholesterol translocation from intracellular pools to the cell surface of cholesterol-loaded macrophages appeared to be stimulated by receptor binding of HDL, since chemical modification of HDL with tetranitromethane (TNM), which abolishes its receptor binding, reduced its ability to stimulate ^{3}H-labeled sterol translocation and efflux. In nonloaded cells, however, the ability of HDL_3 to stimulate sterol efflux and movement of sterol from the plasma membrane into intracellular pools was unaffected by TNM modification. Thus, binding of HDL to its receptor on cholesterol-loaded macrophages appears to promote translocation of intracellular cholesterol to the plasma membrane followed by cholesterol efflux into the medium. However, in nonloaded macrophages, HDL stimulates sterol movement from the plasma membrane into intracellular pools by a receptor-independent process.—**Aviram, M., E. L. Bierman, and J. F. Oram**. High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages. *J. Lipid Res.* 1989. **30:** 65–76.

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It is widely believed that high density lipoprotein (HDL) plays an important role in transport of cholesterol from extrahepatic cells to the liver. The first step of this reverse cholesterol transport pathway may involve the interaction of HDL with specific cell surface receptors that facilitate transport of cholesterol from cells to HDL particles (1–4). Recent studies (5) with cultured fibroblasts and aortic endothelial cells have suggested that this receptor-mediated transport is a two-step process, whereby intracellular pools of excess sterol are first translocated to the plasma membrane prior to removal from cells by HDL or other acceptor particles. It is only the sterol translocation step that appears to be facilitated by binding of HDL to its receptor (5). Transport of sterol directly from plasma membrane does not require cellular binding of acceptor particles (5, 6). These studies support the hypothesis that receptor binding of HDL stimulates selective removal of excess intracellular sterol, thus preventing formation of foam cells in the face of continuous uptake of sterol-rich lipoproteins.

Cholesterol accumulation in monocyte-derived macrophages is a feature of foam cell formation in the developing atherosclerotic lesion (7). In cell culture studies, massive sterol loading of macrophages can be achieved following incubations with modified forms of low density lipoprotein (LDL), such as acetylated LDL, that are taken up by the scavenger receptor pathway (7). Recently, it was demonstrated that phospholipase C-modified LDL can also produce massive accumulation of sterol in macrophages by a novel mechanism that is independent of scavenger recep-

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; PLC, phospholipase C; CO, cholesterol oxidase; TNM, tetranitromethane; ACAT, acyl CoA:cholesterol acyltransferase.

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tors (8). Studies from several laboratories have demonstrated that HDL has the ability to deplete cholesterol that accumulates in sterol-laden macrophages (4, 7, 9). The purpose of the present study was to characterize the mechanism by which HDL promotes removal of excess cholesterol from human monocyte-derived macrophages.

MATERIALS AND METHODS

Cells

Human monocytes were isolated by density gradient centrifugation from blood derived from fasting normolipidemic subjects (10). Twenty ml of blood (anti-coagulated with 10 U/ml heparin) was layered over 15 ml of Ficoll-Paque and centrifuged at 500 *g* for 30 min at 23°C. The mixed mononuclear cell band was removed by aspiration and the cells were washed twice in RPMI-1640 culture medium containing 100 U/ml penicillin and 100 μg/ml streptomycin (serum-free medium). The cells were plated in the same medium at 3×10^5 monocytes per 16-mm dish or 10^6 cells per 35-mm dish (Primaria brand, Falcon Labware, Becton Dickinson and Company, Oxnard, CA). After 2 hr of incubation at 37°C in 5% CO_2, 95% air, nonadherent cells were removed by three washes with serum-free medium. The cells were then given fresh medium containing 20% autologous serum and were fed twice weekly with the same medium. Monocyte-derived macrophages were used within 7–10 days of plating.

Lipoproteins

Low density lipoprotein (LDL, d 1.019–1.063 g/ml) and high density lipoprotein$_3$ (HDL_3, d 1.125–1.210 g/ml) were isolated by standard sequential ultracentrifugation. HDL_3 was then subjected to heparin-agarose affinity chromatography to remove apoE (11) and thus to prevent interaction of the HDL with the apoB,E receptor. Acetyl LDL (Ac-LDL) was prepared using acetic anhydride according to the method of Goldstein et al. (12). Phospholipase C (PLC)-modified LDL (PLC-LDL) was prepared by incubation of 5 mg/ml LDL with 1 U/ml of PLC at 37°C for 50 min followed by reisolation of the LDL on Sephadex G-100 minicolumns (2 × 10 cm). LDL was radiolabeled with [^{3}H]cholesteryl ester by incubation of HDL_3 containing [^{3}H]cholesteryl linoleate with LDL in the presence of partially purified cholesteryl ester transfer protein (13), followed by ultracentrifugal reisolation of the LDL. The final preparation of labeled LDL contained 99% of the ^{3}H-radioactivity in cholesteryl ester and had a specific activity of 350–450 cpm/ng of protein. The labeled LDL was then treated with PLC as described above. HDL_3 was treated with tetranitromethane (TNM) using 20 μl of 0.6 M TNM in absolute ethanol and 2 ml of HDL_3 (2 mg/ml) and incubating in the dark at room temperature for 60 min (3). TNM-HDL_3 was then separated on a Sephadex G-25 minicolumn.

Cholesterol loading

Macrophages were incubated in serum-free medium with no additions (control, nonloaded cells), or with 500 μg/ml of LDL, Ac-LDL, or PLC-LDL (cholesterol-loaded cells) at 37°C for 18 hr. An inhibitor of acyl CoA:cholesterol acyltransferase (ACAT), Sandoz compound 58.035, was added to the medium where indicated.

Labeling of endogenously synthesized cholesterol

After the cholesterol loading incubations, cells were washed 3 times with phosphate-buffered saline (PBS) and pulse-incubated for 3 hr at 15°C or 37°C with serum-free medium containing 1 mg/ml bovine serum albumin, 10 mM HEPES (pH 7.4), 5 μg/ml ACAT inhibitor 58.035, and [^{3}H]mevalonolactone (DuPont, NEN) (10 μCi/ml, 0.4 mM). At 15°C, newly synthesized ^{3}H-labeled sterol is translocated to the plasma membrane at a slow rate (14), thus allowing for selective labeling of intracellular pools (5). However, for these studies using macrophages, even at 37°C the rate of sterol translocation was slow. Thus for some experiments, pulse-labeling was performed at 37°C. [^{3}H]Mevalonolactone was used as a precursor of cholesterol because it bypasses the hydroxymethylglutaryl CoA reductase step in cholesterol biosynthesis, the activity of which is suppressed in cholesterol-loaded cells. After pulse incubation, the cells were chilled, washed 5 times with cold PBS, and used for the efflux experiments.

Labeling of plasma membrane

Cells were incubated for 2 hr at 15°C or 37°C with serum-free medium containing trace amounts of [^{3}H]cholesterol (Amersham Corp., 0.5 μCi/ml, 55 Ci/mmol added in ethanol). With this procedure [^{3}H]cholesterol is selectively incorporated into the plasma membrane (5, 15).

Cellular labeling with radioactive PLC-modified LDL.

Macrophages were incubated with serum-free medium containing 5 μg/ml ACAT inhibitor 58.035 plus 50 μg/ml PLC-LDL labeled with [^{3}H]cholesteryl linoleate for 18 hr at 37°C after an 8-hr incubation with medium containing ACAT inhibitor plus 500 μg/ml unlabeled PLC-LDL.

Measurement of [^{3}H]cholesterol efflux and intracellular movement

Cells were labeled with ^{3}H-labeled sterol derived from either [^{3}H]mevalonolactone, [^{3}H]cholesterol, or [^{3}H]cholesteryl linoleate-labeled PLC-LDL. Cells were then chilled on ice and washed 5 times with cold PBS. Each dish (35 mm, 10^6 cells) received 1 ml of serum free medium containing 1 mg/ml albumin, 10 mM HEPES (pH 7.4), 5 μg/ml ACAT inhibitor 58.035, 0.4 mM mevalonolactone,

and either no additions or HDL_3 or TNM-HDL_3. Cells were then incubated at 37°C, and efflux media were collected at the indicated time. The media were centrifuged (800 *g*, 20 min) to remove detached cells and transferred to conical glass stoppered tubes for extraction of ^{3}H-labeled sterol. The cells were chilled and washed 5 times with cold PBS followed by addition of 1 ml of cold 1% glutaraldehyde to fix the cells (10 min, 0°C). Cells were washed 5 times, transferred to a 37°C water bath, and incubated for 30 min with 1 ml PBS containing 1 U of cholesterol oxidase (CO) (Behring Diagnostics, 228250). Cells were chilled, washed with PBS, and analyzed for their radioactive sterols. This CO treatment protocol converts 70–80% of plasma membrane [^{3}H]cholesterol to [^{3}H]cholestenone (5, 15). In the absence of cholesterol oxidase treatment, no measurable [^{3}H]cholestenone could be found.

The ^{3}H-labeled sterols that corresponded to unesterified cholesterol (cells and medium) and to cholestenone (cells) were separated on Silica Gel G by conventional thin-layer chromatography (TLC) plates developed in hexane–diethyl ether–acetic acid 130:40:1.5 (v/v/v) and detected with iodine vapors. The individual spots were cut into scintillation vials and counted in Aquasol 2 (DuPont, NEN). To further separate sterol subspecies, sterol extracted from the TLC spots corresponding to unesterified cholesterol and cholestenone were spotted onto reversed-phase TLC plates impregnated with long-chain hydrocarbons (Analabs, Norwalk, CT). After development in acetonitrile–methanol 8:1 (v/v), adjacent 1-cm-wide strips of silica were scraped from the plates and counted. With this procedure, sterol standards were cleanly separated as follows: stigmasterol, R_f 0.1–0.3; lanosterol, R_f 0.4–0.5; cholesterol, R_f 0.55–0.75; and desmosterol, R_f 0.8–0.9.

Since cells were fixed in glutaraldehyde for the cholesterol oxidase treatment procedure which renders them unextractable for protein determination, results were expressed as cpm per dish or as percent of total ^{3}H-labeled sterol.

Sterol mass and cholesteryl ester synthesis

To determine relative activity of acyl-CoA:cholesterol acyltransferase (ACAT), cells were pulse-incubated for 1–2 hr with [^{14}C]oleate (10 μCi/ml, 0.2 mM) in the presence of albumin (0.07 mM), and incorporation of label into cellular cholesteryl ester was measured (16). To measure cellular sterol mass and radioactivity, cells were extracted in hexane–isopropanol, and lipid subclasses were separated by conventional TLC as described earlier. Unesterified and esterified cholesterol mass were assayed by the cholesterol oxidase method described previously (17).

RESULTS

Human monocyte-derived macrophages were incubated with modified forms of lipoproteins, such as acetylated LDL (Ac-LDL) or phospholipase C-modified LDL (PLC-LDL), to produce cholesterol accumulation (**Table 1**). When high concentrations (500 μg/ml) of both forms of modified lipoprotein were added to the medium, cells accumulated cholesteryl esters in association with an increase in the rate of cholesteryl ester synthesis. Addition of unmodified LDL to the medium at high concentrations also caused an increase in cellular cholesteryl esters, but to a lesser extent than that observed with modified LDL. When an ACAT inhibitor (Sandoz compound 58-035) was included in the medium, cholesteryl ester formation was inhibited and cells

TABLE 1. Macrophage cholesterol mass and esterification rate following incubation with various LDL preparations

Additions				
Lipoproteins	ACAT Inhibitor	Unesterified Cholesterol Mass	Esterified Cholesterol Mass	Cholesterol Esterification
		μg/mg cell protein		*nmol/mg cell protein*
None	–	24.7	2.1	0.3
None	+	26.5	0.9	0.2
LDL	–	34.3	10.2	0.9
LDL	+	41.1	1.9	0.2
PLC-LDL	–	38.2	45.2	7.8
PLC-LDL	+	81.1	2.9	0.4
AcLDL	–	42.1	50.2	4.0
AcLDL	+	84.2	3.5	0.4

Human monocyte-derived macrophages were incubated at 37°C for 18 hr in serum-free medium containing no addition or 500 μg/ml of LDL, phospholipase C-modified LDL (PLC-LDL), or acetyl-LDL (Ac-LDL) in the presence or absence of 5 μg/ml ACAT inhibitor Sandoz 58-035. Cellular content of unesterified and esterified cholesterol mass and cholesteryl ester synthesis from [^{14}C]oleate was determined as described in Materials and Methods. Results are the mean of duplicate incubation representative of four similar experiments.

accumulated sterol in the form of unesterified cholesterol. Since accumulation of cellular unesterified rather than esterified cholesterol is associated with an increase in HDL binding to cells (1, 4), ACAT inhibitor was included in the medium for most subsequent experiments.

To study the effects of HDL on movement of sterol between cellular pools of cholesterol-loaded macrophages and the incubation medium, intracellular pools of sterol were radiolabeled with a synthetic precursor by pulse incubating cells with [^{3}H]mevalonolactone. The appearance of newly synthesized sterol at the cell surface was monitored by treatment of fixed cells with cholesterol oxidase (CO) as described under Materials and Methods. When macrophages were incubated with PLC-LDL and then pulsed with [^{3}H]mevalonolactone, most of the newly synthesized ^{3}H-labeled sterol resided in cellular pools resistant to CO treatment (compare 0 time values for panels A and B, **Fig. 1**). Four-hour chase incubations with lipoprotein-free medium containing unlabeled mevalonolactone led to only a modest shift in the distribution of ^{3}H-labeled sterol between CO-sensitive and CO-resistant cellular pools. A small proportion of the newly synthesized ^{3}H-labeled sterol appeared in the lipoprotein-free medium as a function of time (Fig. 1C). Addition of HDL_3 to the chase medium caused an increase in the amount of CO-sensitive ^{3}H-labeled sterol (Fig. 1A) and an attendant decrease in CO-resistant ^{3}H-labeled sterol (Fig. 1B). Addition of HDL_3 also increased the rate of appearance of ^{3}H-labeled sterol in the medium (Fig. 1C), with the fastest rate of ^{3}H-labeled sterol efflux occurring after the redistribution of cellular ^{3}H-labeled sterol had equilibrated (2 hr). These results suggest that, with cholesterol-loaded macrophages, HDL_3 stimulated translocation of cellular ^{3}H-labeled sterol from a CO-resistant pool to plasma membrane domains that were accessible to both CO treatment and removal from cells by HDL particles.

Redistribution of cellular ^{3}H-labeled sterol was also observed when cells were radiolabeled with lipoprotein-derived cholesterol prior to exposure to HDL_3. When macrophages were incubated with PLC-LDL to load them with cholesterol and then incubated with PLC-LDL containing [^{3}H]cholesteryl linoleate to radiolabel cellular pools of cholesterol, the amount of cellular [^{3}H]cholesterol that was sensitive to CO treatment remained constant throughout a 4-hr chase period with lipoprotein-free medium (**Fig. 2A**). Addition of HDL_3 to the chase medium led to a time-dependent increase in CO-sensitive [^{3}H]cholesterol that reached a maximum after 2.5 hr (Fig. 2A). HDL_3 also stimulated [^{3}H]cholesterol efflux from cells (Fig. 2B) following a lag period of 2 hr. Thus, in the presence of HDL_3, lipoprotein-derived cholesterol that accumulated in cholesterol-loaded macrophages was translocated first to CO-sensitive plasma membrane domains and then into the medium. Similar effects of HDL_3 on ^{3}H-labeled sterol redistribution were observed when cellular sterols were

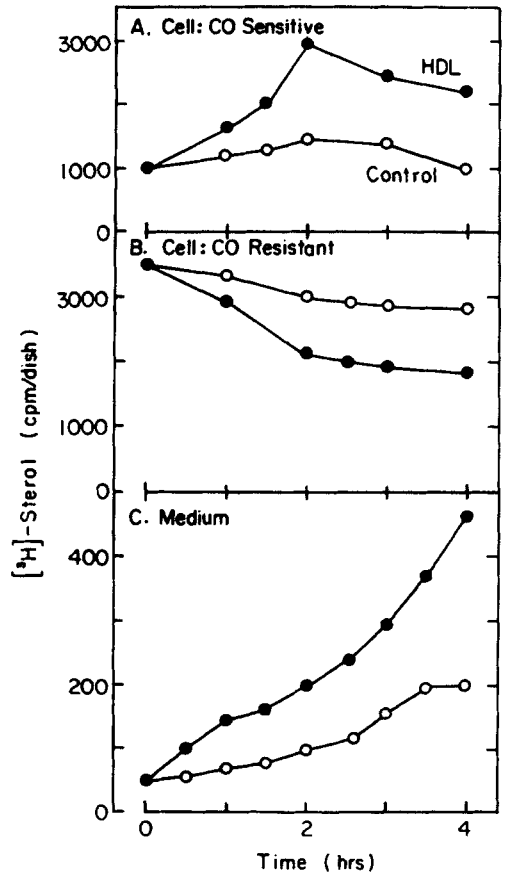

Fig. 1. Time course of HDL-mediated movement of endogenously synthesized sterol between cellular pools and the medium in cholesterol-loaded macrophages. Human monocyte-derived macrophages were loaded with free cholesterol by pre-incubation with 500 μg/ml PLC-LDL in the presence of 5 μg/ml ACAT inhibitor, 58-035, for 18 hr at 37°C. After washing and pulse-labeling with [^{3}H]mevalonolactone for 3 hr at 37°C, cells were washed and chase-incubated at 37°C without (control, ○) or with 20 μg/ml HDL_3 (●). At the indicated times cells were chilled on ice, fixed with glutaraldehyde, and treated with cholesterol oxidase (CO) as described in Materials and Methods. CO-sensitive cellular ^{3}H-labeled sterol (panel A) represents sterol converted to cholestenone whereas CO-resistant cellular ^{3}H-labeled sterol (panel B) represents sterol comigrating with unesterified cholesterol on TLC plates after the CO reaction (see Methods). Results are the mean of duplicate incubations representative of three similar experiments.

radiolabeled from either a biosynthetic precursor (Fig. 1) or an exogenous lipoprotein (Fig. 2).

To test whether HDL_3 stimulates net mass movement of cholesterol from intracellular pools, changes in cholesterol mass were measured after cholesterol-loaded macrophages were incubated for 6 hr in the presence or absence of HDL_3. As an index of the relative size of intracellular pools of unesterified cholesterol, rates of cholesteryl ester synthesis were measured at the end of the efflux incubations. Cholesterol esterification rates reflect the relative activity of ACAT which is a direct function of intracellular substrate pool size (1–3, 7, 9, 12). Because cholesterol esterification ws measured, ACAT inhibitor was omitted from the media. Addition of HDL_3 to the medium caused a small

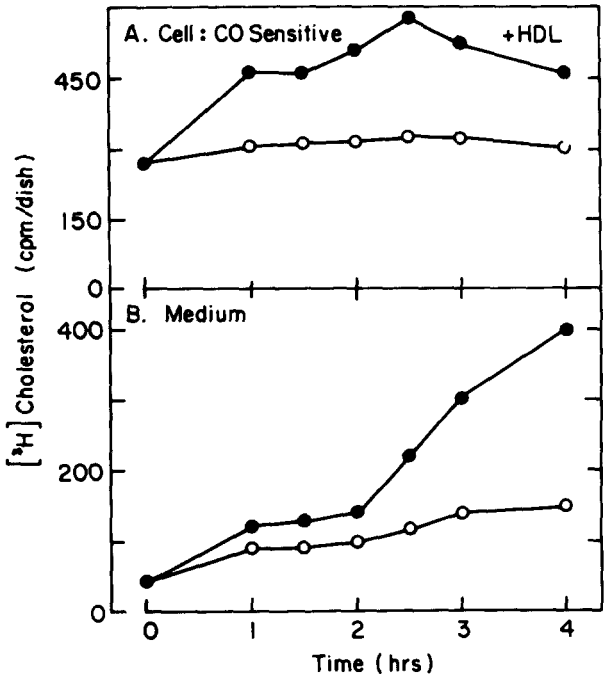

Fig. 2. Time course of HDL-mediated movement of lipoprotein-delivered cholesterol between cellular pools and incubation medium in cholesterol-loaded macrophages. Experimental procedures were as described for Fig. 1 except that the cholesterol loading was for 8 hr followed by the addition of [^{3}H]cholesteryl linoleate-labeled PLC-LDL for 18 hr at 37°C. As described in Materials and Methods, the efflux study was performed without (○) or with 20 μg/ml HDL_3 (●). Results are the mean of duplicate incubations representative of three experiments.

but significant reduction in total cell cholesterol content (**Table 2**). This reduction was almost exclusively due to a decrease in the cholesteryl ester content. Addition of HDL_3 to the medium also led to a significant decrease in the rate of cholesteryl ester synthesis. These results indicate that HDL_3 selectively depleted cells of cholesterol that was in equilibrium with the substrate pool for ACAT, suggesting that translocation of intracellular ^{3}H-labeled sterol to the cell surface and into the medium represents a net outward movement of sterol mass.

When macrophages were cholesterol-loaded by incubation with either LDL, PLC-LDL, or Ac-LDL and then pulsed with [^{3}H]mevalonolactone to radiolabel intracellular sterol, HDL_3 caused a redistribution of cellular ^{3}H-labeled sterol between CO-sensitive and CO-resistant pools in a dose-dependent but saturable manner (**Fig. 3A and B**). Maximum effect of HDL_3 were observed at 20 μg/ml HDL_3, indicating that the redistribution was mediated by a high-affinity process. This same saturability was observed when cells were loaded with cholesterol derived from any of the three forms of LDL. In contrast, ^{3}H-labeled sterol efflux showed only partial saturability (Fig. 3C), suggesting that both high- and low-affinity processes were involved in efflux. However, the incubation times were chosen on the basis of the maximum redistribution of cellular ^{3}H-labeled sterol (2.5 hr), a time that preceded the maximum rate of sterol efflux (see Figs. 1 and 2). Thus, efflux that was dependent on redistribution of ^{3}H-labeled sterol between cellular pools would be under-emphasized in these experiments.

When macrophages were incubated with lipoprotein-free medium prior to radiolabeling intracellular sterols with [^{3}H]mevalonolactone (nonloaded cells), chase incubations with medium containing HDL_3 produced redistribution of ^{3}H-labeled sterol in the opposite direction than that observed with cholesterol-loaded cells. In the absence of HDL_3, proportionately more of the ^{3}H-labeled sterol synthesized by nonloaded cells was sensitive to CO treatment when compared to the ^{3}H-labeled sterol synthesized by cholesterol-loaded cells (Fig. 3A, 3B). Exposure of nonloaded cells to HDL_3 led to a dose-dependent reduction in CO-sensitive ^{3}H-labeled sterol (Fig. 3A, control). Although some of this reduction was due to removal of membrane cholesterol by HDL_3 (Fig. 3C), most of it was accounted for by a shift in the proportion of radiolabel appearing as CO-resistant cellular sterol (Fig. 3B).

To examine the role of cellular binding of HDL_3 in stimulation of ^{3}H-labeled sterol translocation between cellular pools and the medium, redistribution of endogenously la-

TABLE 2. Effects of HDL_3 on cholesterol mass and esterification rate in cholesterol-loaded macrophages

Additions	Cholesterol Mass			Cholesterol Esterification
	Total	Unesterified	Esterified	
	μg/mg cell protein			*nmol/mg cell protein*
None	42.3 ± 1.4	22.8 ± 0.5	19.5 ± 1.1	2.50 ± 0.05
HDL_3	38.1 ± 0.6	21.8 ± 0.4	16.3 ± 0.5	2.00 ± 0.07
	$P < 0.05$	N.S.	$P < 0.02$	$P < 0.001$

Macrophages were incubated at 37°C for 24 hr in serum-free medium containing 250 μg/ml AcLDL. To allow for complete degradation of intracellular AcLDL that accumulated in lysosomal and prelysosomal pools, cells were incubated with serum-free medium containing no lipoprotein for an additional 16 hr. Cells were then washed and incubated at 37°C in the absence or presence of 20 μg/ml HDL_3. After 6 hr, cells were pulse-incubated with [^{14}C]oleate, and cellular content of unesterified cholesterol, esterified cholesterol, and cholesteryl [^{14}C]oleate was measured as described in Materials and Methods. Since the rate of cholesteryl esterification was measured, ACAT inhibitor was excluded from all media. Results represent the mean ± SEM of six incubations; N.S., not significant.

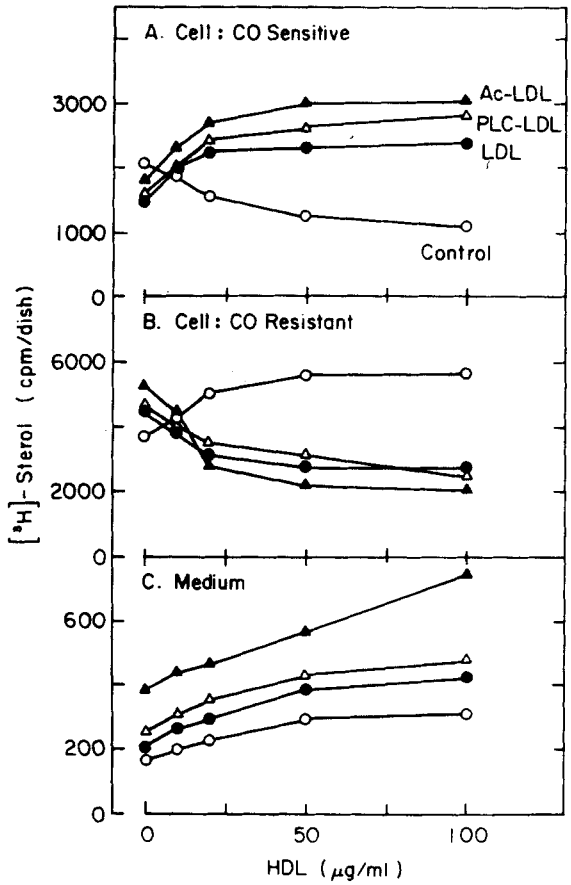

Fig. 3. Effect of HDL_3 concentration on distribution of cellular ^3H-labeled sterol pools and on ^3H-labeled sterol efflux in cholesterol-loaded and nonloaded macrophages. Human monocyte-derived macrophages were loaded with cholesterol by preincubation with 500 μg/ml of LDL (●), acetyl LDL (▲, Ac-LDL) or phospholipase C-modified LDL (△, PLC-LDL) at 37°C for 18 hr in the presence of 5 μg/ml ACAT inhibitor 58-035. Control cells (○) were incubated in the absence of loading lipoproteins. Cells were pulsed for 3 hr at 15°C with [^3H]mevalonolactone, and chased for 2.5 hr at 37°C with increasing concentrations of HDL_3. Distribution of cellular ^3H-labeled sterol between CO-sensitive (panel A) and CO-resistant (panel B) cellular pools and in the medium (panel C) was measured as described in Fig. 1. Results are mean of duplicate incubations representative of three experiments.

beled sterols was measured after exposure of cells to either native HDL_3 or to tetranitromethane (TNM)-treated HDL_3, a modified form of lipoprotein that does not bind to high-affinity HDL receptors (3, 18). When cells were loaded with cholesterol derived from PLC-LDL or LDL prior to pulse incubations with [^3H]mevalonolactone, subsequent chase incubations with native HDL_3 caused a significant increase in the proportion of cellular ^3H-labeled sterol that appeared both in CO-sensitive domains of the plasma membrane and in the medium (**Table 3**). In contrast, at the same concentration as native HDL_3 (20 μg/ml), TNM-HDL_3 had no effect on the distribution of ^3H-labeled sterol between cellular and medium pools. However, with macrophages that were not loaded with cholesterol, native and TNM-treated HDL_3 had similar abilities to stimulate movement of CO-sensitive ^3H-labeled sterol into CO-resistant and medium pools. Thus, enhancement of sterol movement by HDL_3 involved separate mechanisms for nonloaded and cholesterol-loaded macrophages. For loaded cells, HDL_3 stimulated translocation of newly synthesized sterol from CO-resistant pools to the cell-surface CO-sensitive pools and into the culture medium by a process that appeared to involve cellular binding of HDL_3. Conversely, with nonloaded cells, HDL_3 stimulated net movement of ^3H-labeled sterol from CO-sensitive membrane domains to both extracellular and CO-resistant intracellular compartments by a process that does not require cellular binding.

To determine whether the HDL-mediated redistribution of cellular ^3H-labeled sterol observed for nonloaded cells was limited only to newly synthesized sterol, plasma membrane cholesterol was directly radiolabeled by incubating cells with medium containing trace quantities of [^3H]cholesterol. In the absence of HDL_3, exposure of nonloaded cells to [^3H]cholesterol led to incorporation of isotope into CO-sensitive plasma membrane domains that reached a maximum within 2 hr (**Fig. 4**). In contrast, incorporation of isotope into CO-resistant pools did not saturate even after 4 hr of incubation. However, after only 2 hr of incubation, over half the cell-associated [^3H]cholesterol was resistant to CO treatment, suggesting extensive transport of exogenous cholesterol into intracellular compartments of macrophages.

When nonloaded macrophages were pulsed for 2 hr with [^3H]cholesterol and then chased with lipoprotein-free medium, there was a time-dependent decrease in the amount of cellular [^3H]cholesterol that resided in CO-sensitive pools (**Fig. 5A**). This decrease was associated with an increase in the amount of [^3H]cholesterol appearing both in CO-resistant cellular pools (Fig. 5B) and in the medium (Fig. 5C). Addition of HDL_3 to the chase medium enhanced the rate of disappearance of [^3H]cholesterol from cellular CO-sensitive pools and increased the appearance of radiolabel in cellular CO-resistant pools and in the medium. Thus, with nonloaded macrophages, HDL_3 stimulated redistribution of exogenously derived cellular [^3H]cholesterol in a manner similar to that observed for ^3H-labeled sterol derived from a biosynthetic precursor.

As with endogenously labeled sterol, the redistribution of exogenously labeled cellular cholesterol in nonloaded macrophages does not appear to involve the interaction of HDL_3 with cellular receptors, since both native and TNM-treated HDL_3 had the same effects (**Table 4**). Moreover, the HDL-mediated redistribution of cellular pools of cholesterol seen with cells not loaded with sterol is not confined to macrophages. Similar results were observed with nonloaded human skin fibroblasts (Table 4). Compared to macrophages, fibroblasts incorporated markedly less [^3H]cholesterol into CO-resistant pools during the 2-hr pulse incubations. However when either native or TNM-treated HDL_3 were added to the chase medium, there was a decrease in isotope within cellular CO-sensitive cholesterol

TABLE 3. Effect of HDL_3 and TNM-HDL_3 on cholesterol efflux and intracellular movement in cholesterol-loaded and nonloaded macrophages

Additions		Distribution of ^{3}H-labeled Sterol	
Loading	Chase	Cell: CO-Sensitive	Medium
		% of total ^{3}H-labeled sterol	
LDL	None	9.7 ± 2.1	4.2 ± 0.4
LDL	HDL_3	20.7 ± 2.1*	9.2 ± 0.9*
LDL	TNM-HDL_3	10.9 ± 2.2	4.9 ± 0.5
PLC-LDL	None	6.3 ± 1.3	5.2 ± 0.5
PLC-LDL	HDL_3	15.0 ± 2.5*	10.1 ± 1.2*
PLC-LDL	TNM-HDL_3	7.1 ± 2.0	5.6 ± 0.5
None	None	31.1 ± 4.2	4.5 ± 0.4
None	HDL_3	9.8 ± 1.1*	7.8 ± 0.7*
None	TNM-HDL_3	9.6 ± 1.7*	6.1 ± 0.4*

Nonloaded cells or cells loaded with sterol derived from LDL (500 μg/ml) or PLC-LDL (500 μg/ml) were pulse-incubated with [^{3}H]mevalonolactone at 15°C and chased for 2.5 hr with medium containing the indicated lipoprotein (none or 20 μg/ml). ^{3}H-Labeled sterol efflux or CO-sensitive ^{3}H-labeled sterol was measured. Results are expressed as % of total ^{3}H-labeled sterol per dish and represent the mean ± SD from three incubations. Total ^{3}H-labeled sterol per dish was not affected significantly by addition of HDL_3 or TNM-HDL_3.

*$P < 0.01$ compared to no additive.

and increases in isotope appearing in CO-resistant and medium cholesterol pools for both cell types (Table 4).

Previous studies (19, 20) have demonstrated that only a small fraction of ^{3}H-labeled sterol synthesized from radiolabeled precursors during short-term pulse incubations represents authentic cholesterol. By using a two-step thin-layer chromatography procedure to first isolate sterols from other cellular lipids and to then separate sterol subclasses (see Materials and Methods), we found that most of the ^{3}H-labeled sterol synthesized by macrophages during pulse-incubations with [^{3}H]mevalonolactone chromatographed in the area of desmosterol. When cholesterol-loaded macrophages were pulsed with [^{3}H]mevalonolactone and chased for 2.5 hr with lipoprotein-free medium, most of the ^{3}H-labeled sterol remaining in CO-resistant cellular pools (**Fig. 6A**) and nearly all of the ^{3}H-labeled sterol that was secreted from cells (Fig. 6B) was associated with desmosterol after separation of sterol subspecies by reverse-phase thin-layer chromatography. Addition of HDL_3 to the chase medium markedly reduced the amount of radioactivity in the noncholesterol sterol fraction. Although there was a modest increase in ^{3}H-labeled sterol efflux (Fig. 6B), most of the decreased CO-resistant ^{3}H-labeled sterol was accompanied by an increase in CO-sensitive ^{3}H-labeled sterol (data not shown).[3] Thus, a substantial proportion of this noncholesterol ^{3}H-labeled sterol was secreted from cells and translocated from CO-resistant to CO-sensitive pools when HDL_3 was added to the medium, similar to

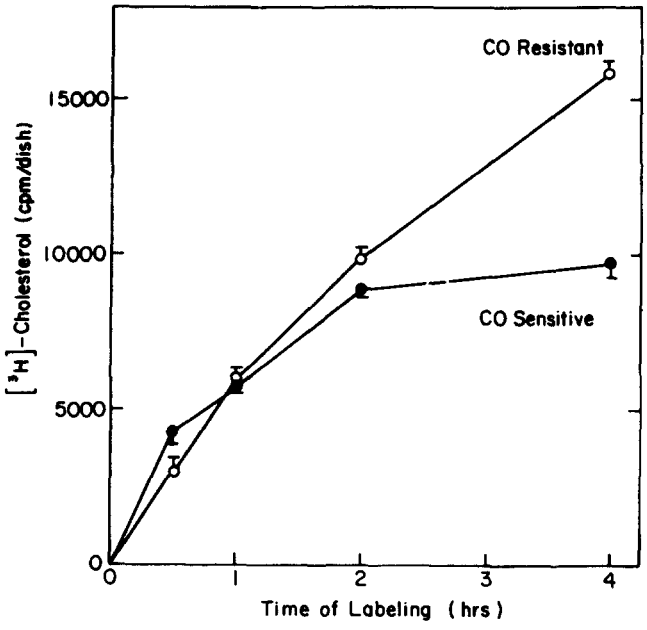

Fig. 4. Rate of incorporation of [^{3}H]cholesterol into cholesterol oxidase (CO)-sensitive and -resistant pools of nonloaded macrophages. Nonloaded macrophages were incubated with trace amounts of [^{3}H]cholesterol (1 μCi/ml) at 15°C. At the time indicated, cells were washed and cellular CO-sensitive (●) and -resistant (○) [^{3}H]cholesterol was analyzed as described under Materials and Methods.

[3]When lipids from cholesterol oxidase-treated cells were separated by conventional TLC, the radioactivity that corresponded to cholestenone migrated as a single peak that corresponded to cholestenone when the sterol was rechromatographed on reversed-phase plates. Thus, we were unable to distinguish sterol subspecies among the products of the cholesterol oxidase reaction with this method. That cholesterol and noncholesterol sterol subspecies interacted with cholesterol oxidase was deduced from data that demonstrated that the HDL_3-mediated changes in the amount of radiolabel within the different sterol subspecies were largely accounted for by a reciprocal change in the amount of radiolabel in the single cholestenone peak.

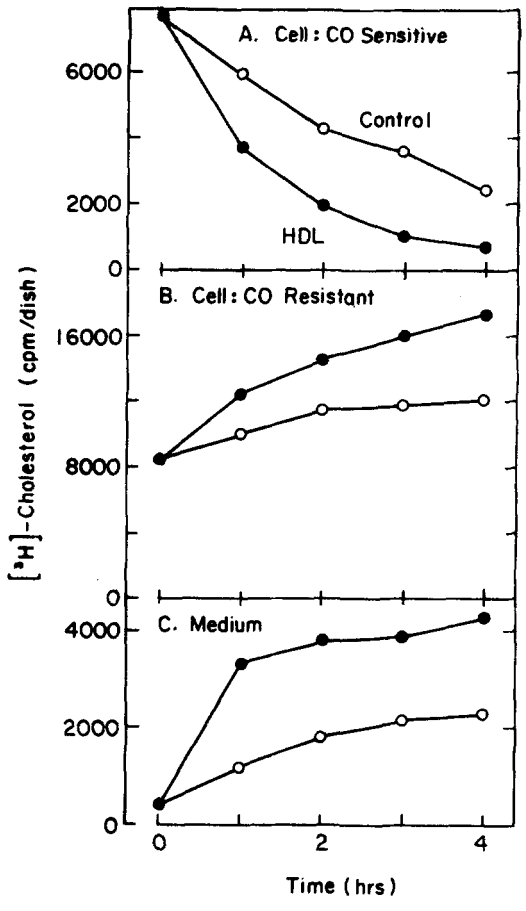

Fig. 5. The effect of HDL_3 on redistribution of [^{3}H]cholesterol between cellular pools and the medium in nonloaded macrophages. Nonloaded cells were pulse-labeled with 1 μCi/ml [^{3}H]cholesterol for 2 hr at 15°C. Radiolabeled cells were then incubated without (control ○) or with 20 μg/ml HDL_3 (●) for 2 hr at 37°C. CO-sensitive (A) and CO-resistant (B) cellular [^{3}H]cholesterol and medium [^{3}H]cholesterol (C) were analyzed as measured under Materials and Methods.

what was observed for cells radiolabeled with lipoprotein-derived [^{3}H]cholesterol (Fig. 2).

As with cholesterol-loaded macrophages, sterol synthesized from [^{3}H]mevalonolactone in nonloaded cells co-migrated with desmosterol on reverse phase thin-layer chromatography plates, although proportionately more of the radiolabel co-migrated with authentic cholesterol than in cholesterol-loaded cells (**Fig. 7A**). Addition of HDL_3 to the chase medium enhanced the amount of radiolabel that appeared in cellular pools resistant to CO treatment (Fig. 7A) and in the culture medium (Fig. 7B), and this increase was observed for all sterol subspecies. The increase in sterol in these two pools was accompanied by a decrease in CO-sensitive ^{3}H-labeled sterol (data not shown).[3] Taken together, the results in Figs. 6 and 7 indicate that the non-cholesterol sterol subspecies derived from biosynthetic precursors are transported within cellular pools and into the medium in response to HDL_3 treatment by processes similar to those that transport authentic cholesterol.

DISCUSSION

Studies from several laboratories have shown that newly synthesized sterol is translocated vectorially from microsomal membranes to the plasma membrane by an energy-dependent process that may involve vesicular transport (14, 15, 21). Recent results from our laboratory (5) have demonstrated that the interaction of HDL with sterol-laden cultured fibroblasts and aortic endothelial cells stimulates translocation of intracellular sterol to the plasma membrane where it becomes accessible for removal from cells by HDL or other acceptor particles. Results in the current study indicate that this same stimulatory process occurs in cholesterol-loaded human monocyte-derived macrophages,

TABLE 4. Effect of HDL_3 and TNM-HDL_3 on [^{3}H]cholesterol efflux and movement between cellular pools in macrophages and fibroblasts not loaded with cholesterol

		[^{3}H]Cholesterol		
Cell Type	Chase (2.5 hr)	Cell: CO-Sensitive	Cell: CO-Resistant	Medium
			cpm/dish	
Macrophages	None	5820	9678	2056
Macrophages	HDL_3	2992	11276	3982
Macrophages	TNM-HDL_3	2915	10635	3961
Fibroblasts	None	5454	1811	1191
Fibroblasts	HDL_3	2799	3454	2114
Fibroblasts	TNM-HDL_3	2495	3293	2241

Nonloaded cells were labeled with [^{3}H]cholesterol (1 μCi/ml) for 2 hr at 37°C followed by washing and incubation with either no lipoprotein or 20 μg/ml HDL_3 or TNM-HDL_3. After 2.5 hr at 37°C, medium [^{3}H]cholesterol and cellular CO-sensitive and CO-resistant [^{3}H]cholesterol were measured as described under Methods. Each value is the mean of duplicate incubations representative of three similar experiments.

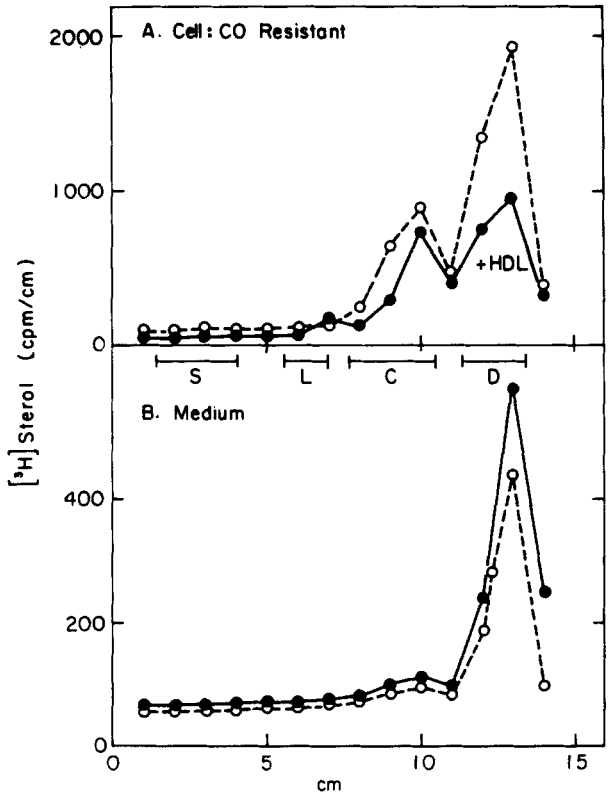

Fig. 6. HDL-mediated changes in cellular CO-resistant and in medium ^{3}H-labeled sterol subspecies in cholesterol-loaded macrophages. Macrophages were cholesterol-loaded by incubation with 500 μg/ml PLC-LDL for 18 hr at 37°C. Cells were labeled with [^{3}H]mevalonolactone (10 μCi/ml) for 3 hr at 37°C. Cells were then incubated without (○) or with (●) 20 μg/ml HDL_3 for 2.5 hr at 37°C. After washing, fixation, CO treatment, and extraction, the cellular lipids were subjected to TLC and reversed-phase TLC analysis as described in Materials and Methods. One-cm strips of the reversed-phase TLC plates were scraped and assayed for ^{3}H radioactivity. Location of sterol standards is indicated: stigmasterol (S), lanosterol (L), cholesterol (C), and desmosterol (D).

the major cell type believed to become foam cells in early atherosclerotic lesions.

To study sterol translocation, it was necessary to specifically radiolabel intracellular pools of sterol after macrophages were loaded with cholesterol. The protocol of choice was to radiolabel intracellular pools biosynthetically by pulse incubation with [^{3}H]mevalonolactone. This sterol precursor enters the biosynthetic pathway at a step beyond hydroxymethylglutaryl-CoA reductase, which is repressed in cholesterol-loaded cells. Moreover, during short-term incubations, especially at lower temperatures (15°C), the basal rate of translocation of newly synthesized sterol to the plasma membrane is slow (5, 14), thus favoring a relative enrichment of microsomal membranes prior to incubation with HDL_3. With this biosynthetic labeling procedure, the rate of appearance of newly synthesized sterol into the culture medium tended to exhibit a lag phase of about 2 hr, consistent with the concept that most of the radiolabel resides within intracellular membranes and must undergo transport to the cell surface prior to release from cells. Labeled sterol that resides in the plasma membrane is released rapidly into the medium without a lag phase (see Fig. 5).

An alternative protocol for enriching microsomal membranes with labeled cholesterol was to expose cells to PLC-LDL containing [^{3}H]choesteryl linoleate during the final hours of the cholesterol-loading incubations with PLC-LDL. In this case, lysosomal degradation of incoming lipoprotein delivers unesterified cholesterol to microsomal pools prior to translocation to the plasma membrane. As with the biosynthetic labeling protocol, the appearance of [^{3}H]cholesterol in the medium during subsequent chase incubations with HDL_3 had a 2-hr lag phase, suggesting that most of the lipoprotein-derived labeled cholesterol secreted from cells was initially located within intracellular membranes.

To directly monitor movement of radiolabeled sterol between intracellular and cell surface pools, we used the cholesterol oxidase (CO) treatment protocol originally described by Lange and Ramos (15). In our hands, CO treatment of fibroblasts that had been briefly exposed to trace quantities of [^{3}H]cholesterol converted 70–80% of the total cell-associated [^{3}H]cholesterol to [^{3}H]cholestenone, suggesting that this assay readily detects cholesterol that

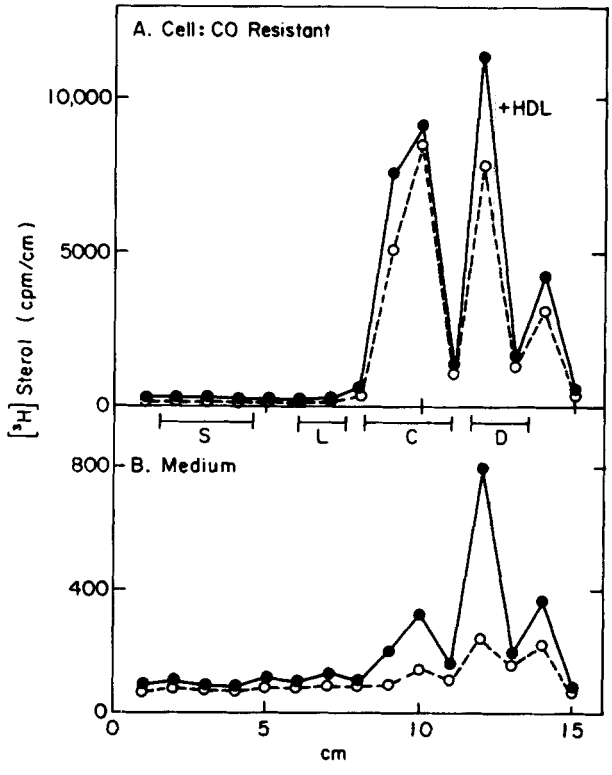

Fig. 7. HDL-mediated changes in CO-resistant and in medium ^{3}H-labeled sterol subspecies in noncholesterol-loaded macrophages. Experimental procedure was as described in the legend to Fig. 6 except that PLC-LDL was omitted from the preincubation medium.

is associated with the plasma membrane. With our intracellular membrane-labeling protocols, only 20–40% of the total cell-associated labeled sterol was sensitive to CO treatment, providing additional support for the assumption that most of this label was intracellular.

Net movement of intracellular pools of ^{3}H-labeled sterol to the plasma membrane in cholesterol-loaded macrophages was a very slow process in the absence of lipoprotein in the medium. This slow transport was observed whether intracellular pools were radiolabeled with a biosynthetic precursor or an exogenous lipoprotein. This is in contrast to previous reports demonstrating that the rate of transport of newly synthesized cholesterol to the plasma membrane of Chinese hamster ovary cells (14), fibroblasts (15, 21), and Leydig tumor cells (22) is rapid with half-times ranging between 10 and 60 min. It is possible that cholesterol-loading of cells dampens flux of intracellular sterol to the plasma membrane. In support of this explanation are results showing that the rate of translocation of newly synthesized sterol to the plasma membrane is more rapid in cholesterol-depleted macrophages than in cholesterol-loaded cells. It should be noted that for these experiments, macrophages were loaded with cholesterol in the presence of an ACAT inhibitor and thus had an abnormally expanded pool of unesterified cholesterol. However, slow rates of sterol translocation were observed previously when fibroblasts and aortic endothelial cells were loaded with cholesterol in the absence of ACAT inhibitor (5).

When HDL_3 was added to the serum-free and lipoprotein-free culture medium, there was a rapid translocation of intracellular sterol to the plasma membrane that preceded its appearance in the medium. It is likely that this HDL_3-stimulated translocation required binding of HDL_3 to high-affinity receptors, since HDL_3 that was treated with tetranitromethane (TNM) to destroy receptor binding gave no stimulatory response. When cells that had not been loaded with cholesterol were used (which have proportionately more of the radiolabeled cholesterol residing within plasma membranes), native and TNM-modified HDL_3 had the same ability to remove [^{3}H]cholesterol from cells, indicating that both forms of lipoprotein can act as acceptors for plasma membrane cholesterol. This observation also supports the assumption that HDL_3 is not acting on cholesterol-loaded macrophages by first depleting plasma membranes of cholesterol and thus initiating diffusion of sterol from intracellular pools down a concentration gradient to the plasma membrane. If this were the case, then it would be expected that TNM-modified HDL_3 would have the same effect as native HDL_3. It is more likely that the interaction of HDL_3 with cells stimulates vectorial translocation of intracellular sterol to the plasma membrane where it becomes accessible for removal by HDL_3 particles.

Since most of the sterol synthesized from a precursor such as mevalonolactone may represent noncholesterol sterol intermediates (19, 20), we considered the possibility that HDL_3 may be modulating the interconversion of sterol subspecies that have different rates of intracellular transport. Although most of the newly synthesized sterol migrated as desmosterol rather than cholesterol on reversed-phase thin-layer chromatography plates, macrophages appeared to transport this sterol derivative as if it were authentic cholesterol. Movement of the newly synthesized noncholesterol sterol between cellular pools and into the medium exhibited a pattern similar to the movement of cellular radiolabeled cholesterol derived from exogenous sources. Moreover, the noncholesterol sterol was freely reactive with cholesterol oxidase.[3] Thus, it is likely that HDL_3 is stimulating movement of cellular pools of sterol rather than affecting the interconversion of sterol subspecies. These results also support the validity of using biosynthetic labeling protocols to study cellular cholesterol transport processes.

The HDL_3-mediated translocation of ^{3}H-labeled sterol from intracellular pools to the plasma membrane and into the medium appears to represent a net outward movement of sterol mass rather than exchange of cellular labeled sterol for unlabeled HDL_3 sterol. When macrophages loaded with cholesterol in the absence of ACAT inhibitor were exposed to HDL_3, they lost a small but significant proportion of their cholesteryl ester mass within 6 hr. This occurred even though there was no significant loss of unesterified cholesterol mass, most of which presumably resides in the plasma membrane (15). Since only unesterified cholesterol is secreted from cells in the presence of HDL_3, these results suggest that pools of excess intracellular sterol that represent substrate for ACAT are selectively removed by HDL_3. In support of this conclusion are results showing that HDL_3 suppresses the activity of this enzyme. Similar suppression of ACAT activity by HDL_3 was observed in previous studies using cholesterol-loaded fibroblasts (3, 23). Apparently, the sterol translocation pathway serves to transport excess cholesterol from intracellular sites, where it would be esterified by microsomal enzymes and accumulate as cholesteryl esters, to the cell surface, where it can be removed from cells by HDL_3 or other acceptor particles.

A surprising finding in this study was the effect of HDL_3 on movement of cellular sterol in macrophages that were not loaded with cholesterol. When sterol pools of nonloaded cells were radiolabeled with either a biosynthetic precursor or exogenous cholesterol, addition of HDL_3 to the medium not only promoted efflux of labeled sterol, it stimulated movement of sterol from the plasma membrane into intracellular pools. Thus, movement of sterol between cellular pools was in the opposite direction in nonloaded cells than in cholesterol-loaded cells. This phenomenon did not appear to involve receptor binding of HDL, since native and TNM-treated HDL_3 were equally effective in stimulating influx of plasma membrane cholesterol. Stimulation of sterol influx by HDL_3 was also observed for nonloaded fibroblasts, indicating that it is not unique to macrophages.

Recently Tabas, Rosoff, and Boykow (24) reported that exposure of J-774 macrophages to LDL and AcLDL stimulated movement of plasma membrane cholesterol into intracellular sites that were accessible to ACAT. Taken together, these results suggest that increased flux of plasma membrane cholesterol into intracellular pools is a general response to exposure of cells to lipoproteins.

The results from the current and previous (5) studies help clarify some of the apparent discrepancies reported from different laboratories about the role of cellular binding of HDL in cholesterol transport between cells and HDL particles. For the early studies from our laboratory (1–3, 23, 25, 26), net transport of cholesterol from cells was assessed as either efflux of [^{3}H]cholesterol from cells previously loaded in the presence of high concentrations of isotope, which probably accumulates in intracellular as well as plasma membrane pools, or as changes in cellular biochemical processes that are regulated by the level of sterol within intracellular pools, such as ACAT activity. With these assays, translocation of cholesterol from intracellular pools to the plasma membrane and into the medium would be detected as either [^{3}H]cholesterol efflux or decreased ACAT activity. It was concluded from our early studies that receptor binding of HDL_3 facilitates net transport of cholesterol from cells to HDL_3 particles. In contrast, Karlin et al. (6) concluded that cellular binding of HDL_3 is not required for transport of cholesterol between cells and HDL_3 particles. However, these investigators measured cholesterol efflux from cells that had been radiolabeled by exposure to medium containing trace quantities of [^{3}H]cholesterol. Under these conditions, cells were in a relatively sterol-depleted state and most of the isotope was probably associated with plasma membranes. Our current and recent (5) studies also demonstrated that cholesterol efflux directly from plasma membranes does not require receptor binding. It now appears likely that receptor binding of HDL facilitates cholesterol efflux selectively from intracellular pools.

In summary, the results from the current study provide additional support for the existence of HDL receptors on macrophages that have a specific biological function: to initiate translocation of intracellular sterol to the plasma membrane in response to binding of HDL. Once in the plasma membrane, the cholesterol can be readily removed from cells by HDL or other acceptor particles in the extracellular environment. It is possible that receptor binding of HDL invokes a signal transduction process that stimulates transport of cholesterol-laden vesicles to the cell surface. Studies designed to test this hypothesis are currently being pursued.

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