Quantitation of Preβ-HDL-Dependent and Nonspecific Components of the Total Efflux of Cellular Cholesterol and Phospholipid†

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ABSTRACT: Both receptor-mediated and diffusional processes have been proposed as mechanisms for the efflux of cellular cholesterol to plasma. The depletion of a minor high-density lipoprotein subfraction (preβ-1-HDL) from plasma by incubation was associated with a proportional reduction in up to 58% of cholesterol and lecithin efflux from cultured fibroblasts. Preβ-HDL-dependent efflux was blocked by protease pretreatment of the cells, while residual ("nonspecific") efflux was protease-insensitive. The whole of cholesterol efflux from blood erythrocytes was both preβ-1-HDL- and protease-independent. These data suggest that two distinct pathways contribute to total efflux from fibroblast monolayers; one of these is directly proportional to plasma preβ-1-HDL concentration and may involve a cell-surface protein.

Unesterified cholesterol transfers spontaneously between lipid surfaces. Studies with synthetic vesicle populations and other models, such as blood erythrocytes, have suggested that the mechanism of this transfer is diffusional, and probably limited by the desorption of molecular cholesterol from the interface (McLean & Phillips, 1981; Lange et al., 1983). On the other hand, in several cell lines, including fibroblasts, endothelial and smooth muscle cells, hepatocytes, and macrophages, evidence has been obtained that plasma lipoprotein particles, particularly high-density lipoproteins (HDL), can bind effectively to the cell surface and promote cholesterol efflux (Biesbroeck et al., 1983; Havekes et al., 1984; Hoeg et al., 1985; Schmitz et al., 1985). HDL binding proteins have also been demonstrated by ligand blotting of cell membrane preparations (Graham & Oram, 1987; Tosuka & Fidge, 1989). One such HDL binding protein has recently been cloned and sequenced (McKnight et al., 1992). At the same time, other investigators have reported data indicating that diffusional mechanisms alone could account for the transfer observed, even in cells with HDL binding sites (Karlin et al., 1987).

Whatever the mechanism of cholesterol transfer, there is evidence suggesting that a minor component of HDL, of low molecular weight and with preβ electrophoretic mobility, plays a special role as the initial acceptor of cellular cholesterol. When plasma was incubated with cells isotopically labeled with cholesterol, the earliest labeling occurred in the preβ-1-HDL fraction (Castro & Fielding, 1988). When macrophages were incubated with apolipoprotein A-I, the major protein of HDL, small preβ-HDL particles were generated (Hara & Yokoyama, 1991). Finally, cell-derived cholesterol could be traced through to HDL particles where cellular cholesterol was sequestrated in esterified form following the action of lecithin: cholesterol acyltransferase (LCAT) (Francone et al., 1989).

In this research, we show that efflux of cellular cholesterol from cultured fibroblasts is the sum of two distinct components: one nonspecific or preβ-HDL-independent, and the other dependent on the concentration of preβ-1-HDL. The preβ-HDL-dependent component of efflux depends on the integrity of a cell-surface protein.

EXPERIMENTAL PROCEDURES

Blood was obtained from fasting normolipemic volunteers and collected into ice-cooled plastic tubes containing streptokinase (150 IU/mL final concentration) as anticoagulant. Plasma was obtained by centrifugation (2000*g*, 0 °C, 30 min). The mean unesterified cholesterol content of plasma used in these studies was 428 ± 49 μg mL^{-1}. In some experiments, the washed blood erythrocytes were also utilized, as described below. These contained 1.04 ± 0.04 mg mL^{-1} cholesterol per milliliter of packed cells.

Cell Experiments. Normal human skin fibroblasts were cultured as previously described (Fielding & Fielding, 1981). For individual experiments, cells were grown to near-confluence in 3.5-cm plastic dishes and incubated for 48 h with 10% fetal calf serum in Dulbecco's modified Eagle's medium containing [1,2-^{3}H]cholesterol (New England Nuclear, Boston, MA) (0.1 mCi mL^{-1} medium) or [^{3}H]choline (New England Nuclear) (0.2 mCi mL^{-1} medium). The cell monolayers (containing 2.2 ± 0.6 μg of cholesterol) were washed (4 times) with phosphate-buffered saline. The washed monolayers were incubated with freshly isolated native plasma (1.0 mL) at 37 °C for 0.5–5 min ([^{3}H]cholesterol label) or up to 60 min ([^{3}H]choline label) on an orbital shaker (1.5 cycles s^{-1}). The mean mass ratio of plasma to fibroblast cholesterol was 195 (428/2.2). Samples were taken into ice-cooled tubes and centrifuged at 8800*g* for 10 min. Aliquots were then taken for determination of radioactivity or for further analysis of lipoprotein subfractions as described below.

Blood erythrocytes were labeled as follows. 0.25 mCi of [^{3}H]cholesterol complexed with human serum albumin (Fielding & Fielding, 1981) was incubated with 12 volumes of whole blood in the presence of 1.5 mM dithiobis(2-nitrobenzoic acid) to inhibit in vitro esterification. After 4-h incubation at 37 °C, the plasma was removed following centrifugation (2000*g*, 30 min, 0 °C), and the blood cells were washed (5 times) with 4 volumes of Ca^{2+},Mg^{2+}-free phosphate-buffered saline. The mean free cholesterol content of the packed red cells was 1.1 ± 0.1 mg mL^{-1}.

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Six-tenths milliliter of packed cells was incubated with native or preincubated plasma (0.9 mL) as described above, that is, a final hematocrit of 40% v/v. The preparation contained >99.9% erythrocytes as determined by differential cytometry. The mean mass ratio of plasma to red cell cholesterol was $(428 \times 0.9)/(1040 \times 0.6) = 0.62$.

Blood cell and fibroblast cholesterol mass was measured enzymatically with cholesterol oxidase (Fielding, 1985), and radioactivity was determined by liquid scintillation spectrometry. Cholesterol specific activity was (1–2.5) $\times 10^6$ cpm μg^{-1}. The mass of individual choline-containing phospholipids (lecithin, lysolecithin, sphingomyelin) was determined following the extraction of total cellular lipids with chloroform and methanol (1/1 v/v) followed by thin-layer chromatography on glass plates (Whatman LK-6) developed in chloroform/methanol/2-propanol/0.25% w/v aqueous KCl/triethylamine, 30/9/25/6/18 v/v (Fielding & Fielding, 1986). Phospholipid mass was determined as phosphate and phospholipid radioactivity by liquid scintillation spectrometry. Phospholipid specific activity varied between 0.6×10^6 and 1.2×10^6 cpm μg^{-1} for different choline-containing phospholipids.

In some experiments, [^{3}H]cholesterol-labeled fibroblasts or blood cells were pretreated with proteinase K (Sigma Chemical Co., St. Louis, Mo). The cells were incubated for 45 min at 37 °C with protease (9–10 μg). The cells were then washed with phosphate-buffered saline, and cholesterol efflux was then determined as described above.

Electrophoresis and Immunoassays. Analysis of plasma HDL subspecies before and after incubation at 37 °C was carried out by nondenaturing two-dimensional electrophoresis (Castro & Fielding, 1988). Briefly, a 20-μL plasma sample was first separated by electrophoresis in 0.75% agarose in 50 mM barbital buffer (pH 8.6). In the second dimension, polyacrylamide gradient electrophoresis was carried out in a 2–15% w/v gradient developed in 0.025 Tris/glycine buffer (pH 8.3). Following electrophoresis, the separated plasma proteins were electrotransferred to a nitrocellulose membrane (Sartorius, 0.45 μM). HDL were identified by immunoblotting with ^{125}I-labeled goat anti-human apo A-I IgG followed by autoradiography with Kodax X-OMAT AR film at −70 °C. The autoradiograph of the nitrocellulose membrane was used as a template to identify areas in the membrane containing radiolabeled complexes of apo A-I and specific ^{125}I-labeled anti-apo A-I antibody. These areas were then cut from the membranes. Quantitation of individual HDL species was determined by γ radiation spectrometry. An internal standard of a known mass of pure human apo A-I (Sigma) carried through the procedure served as internal standard (Ishida et al., 1987). The proportion of apo A-I in each species was determined from the sum of radioactivity in individual fractions. The equivalence of the signal from different preβ- and α-migrating HDL species was previously documented (Ishida et al., 1987). Intraassay variation of multiple analyses carried out on the same plasma sample was 5% of the mean.

RESULTS

Dependence of Cholesterol Efflux upon Preβ-1-HDL. The efflux of cholesterol into native plasma from [^{3}H]cholesterol-labeled fibroblasts or blood cells was linear with time. Plasma was preincubated (30–90 min at 37 °C) to deplete the level of preβ-1-HDL (Miida et al., 1992) and then tested for its ability to accept labeled cholesterol from cultured cells compared to unincubated plasma. The rate of cholesterol efflux into preincubated plasma was significantly lowered

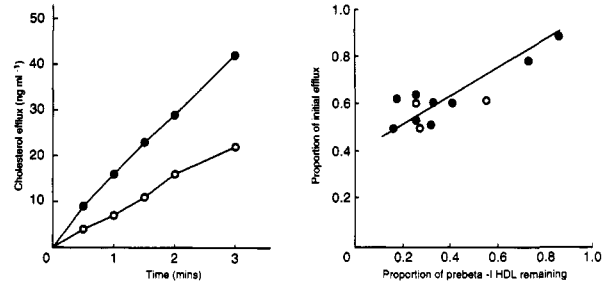

FIGURE 1: Effects of preβ-1-HDL level on cholesterol efflux. Left panel: time course of efflux from labeled fibroblasts into native plasma (closed circles) or plasma preincubated (90 min, 37 °C) to reduce preβ-1-HDL (open circles). Initial and final preβ-1-HDL concentrations determined by quantitative two-dimensional electrophoresis (Castro & Fielding, 1988) were 7.3 and 2.2 μg mL^{-1}, respectively. Right panel: comparison of the decline in preβ-1-HDL concentration and efflux of cholesterol (closed circles) and lecithin (open circles). Each data point represents an individual experiment.

(Figure 1, left panel). In all experiments, a mean decrease of 42 ± 11% below efflux rates with nonincubated plasma was obtained following 90 min of preincubation. Preincubation was also associated with a decrease of 75 ± 8% in the level of the small preβ-migrating HDL fraction (preβ-1-HDL), as previously reported (Miida et al., 1992). There was a linear relationship between these effects, which when extrapolated to the total absence of preβ-1-HDL predicts that efflux would then be about 42% of its initial value (Figure 1, right panel). This was equivalent to 5.9 ng of cholesterol min^{-1} from the fibroblast monolayer. When this basal ("preβ-HDL independent") rate was subtracted from the total rate of efflux, a linear relationship was found between the remaining ("preβ-HDL specific") efflux and the concentration of preβ-1-HDL whose slope was 1.4 ng of cholesterol min^{-1} (μg of preβ-1-HDL)$^{-1}$ ($r = 0.87$).

It was previously shown that in the presence of DTNB the decrease in preβ-HDL otherwise observed during incubation was blocked (Miida et al., 1992). Under these conditions, the decrease in efflux that otherwise occurred was also completely prevented. In three experiments, the decrease in efflux was 42 ± 4% in the absence of DTNB and 4 ± 4% in the presence of 1.5 mM DTNB. During incubation in the presence of DTNB, there was also no change in the levels of other HDL fractions as determined after nondenaturing two-dimensional electrophoresis.

The effect of the loss of preβ-1-HDL on cholesterol efflux was also determined with washed red blood cells prelabeled with [^{3}H]cholesterol. These cells, unlike fibroblasts, did not support the maintenance of preβ-1-HDL levels in plasma (Miida et al., 1992), even though the concentration of cell cholesterol in the assay, relative to plasma free cholesterol, was approximately 300-fold greater for red cells than fibroblasts. As shown in Figure 2, cholesterol efflux from blood cells was also independent of the level of preβ-HDL in plasma. As a result, even plasma from which preβ-1-HDL had been greatly reduced by preincubation in the absence of fibroblasts remained as effective as native plasma in accepting labeled cholesterol derived from blood cells.

Dependence of Phospholipid Efflux upon Preβ-1-HDL. The transfer of cellular free cholesterol to HDL had been shown earlier to be associated with a simultaneous transfer of cellular phospholipids (Hara et al., 1991). In the present study, the total rate of efflux of cellular choline phospholipids was considerably slower than that of cholesterol (mean 4.9 ± 1.0 ng mL^{-1} h^{-1}) but, like that of cholesterol, was affected by the level of preβ-1-HDL in plasma. As shown in Table I,

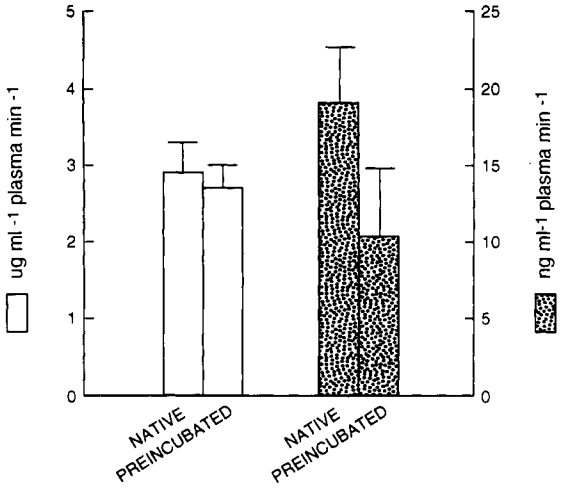

FIGURE 2: Cholesterol efflux from cultured fibroblast monolayers and red blood cells to native and preincubated plasma. Cholesterol efflux was determined during a 5-min incubation at 37 °C. Open bars are the data for blood cells, and dotted bars are for fibroblasts. Values are means ± 1 SD for three experiments in each case.

Table I: Phospholipid Efflux from Fibroblast Monolayers[a]

lipid	efflux to plasma (ng min^{-1}) unincubated	preincubated
lecithin	2.5 ± 0.7	1.1 ± 0.1[b]
sphingomyelin	1.0 ± 0.1	0.7 ± 0.2
lysolecithin	1.4 ± 1.1	1.1 ± 1.0

[a] Fibroblast monolayers cultured as described under Experimental Procedures were incubated with native plasma or with plasma preincubated for 90 min at 37 °C to deplete preβ-1-HDL content. Lecithin mass was calculated on the basis of MW 750, sphingomyelin as MW 817, and lysolecithin as MW 468. Values are means ± 1 SD for three experiments. [b] $p < 0.05$ compared to nonincubated value.

when plasma was incubated with [^{3}H]choline-labeled fibroblasts, phospholipid radioactivity was recovered in the medium. There was a greater transfer of lecithin than of sphingomyelin or lysolecithin, and the transfer of lecithin was significantly dependent upon plasma preβ-1-HDL concentration. While sphingomyelin and lysolecithin were transferred at somewhat lower rates into preincubated plasma, the difference did not reach significance ($p > 0.05$). As shown in Figure 1, right panel, the relationship between the decrease of efflux and preβ-HDL concentration for lecithin was consistent with that for cholesterol. These data suggest that the transfer of cellular lecithin to plasma, like that of cholesterol, is made up of two separate mechanisms: one dependent on the specific acceptor pathway mediated by preβ-1-HDL and the other independent. In contrast, the transfers of sphingomyelin and lysolecithin were mainly or exclusively nonspecific.

Effects of Protease Treatment. To determine whether preβ-HDL-dependent efflux relied upon the integrity of the fibroblast membrane, the rate of cholesterol efflux into native and preβ-depleted plasma was determined with cultured fibroblast monolayers, and with the same cells pretreated with protease to digest cell-surface proteins (Goldstein & Brown, 1974). As shown in Table II, with untreated cells the expected decrease in cholesterol efflux was observed when plasma was depleted of preβ-1-HDL. However, with the protease-treated cells, not only was efflux with native plasma reduced, but also there was no effect of the presence of preβ-1-HDL on the rate of appearance of cholesterol in plasma. As also shown in Table II, when blood cells were treated with protease under the same conditions, there was no effect on efflux.

Table II: Effect of Protease Digestion on the Efflux of Cholesterol from Cultured Fibroblasts and Red Blood Cells[a]

	unincubated	preincubated
fibroblasts (ng min^{-1})		
control	20.8 ± 15.7	12.5 ± 9.3[b]
+protease	9.2 ± 2.9	10.7 ± 6.1
blood cells (μg min^{-1})		
control	1.8 ± 0.4	
+protease	1.7 ± 0.5	

[a] Values are the means ± 1 SD for four experiments. Statistical significance was calculated by paired t-test. [b] $p < 0.05$ compared to the nonincubated value.

After protease-treated cells were returned to the original culture medium containing 10% serum, and incubated overnight at 37 °C, efflux was 104 ± 12% ($n = 3$) of that found in cells cultured over the same period without protease. These data suggest that a cell-surface protein is involved, either directly or indirectly (for example, in influencing the distribution of cholesterol in the cell surface), in the part of total cholesterol efflux involving preβ-1-HDL.

DISCUSSION

This research provides new evidence that cholesterol efflux from cultured fibroblasts may reflect the combination of two distinct pathways: one preβ-HDL-dependent and the other preβ-HDL-independent ("nonspecific"). It was previously shown that cell-derived free cholesterol was rapidly incorporated into preβ-HDL (Castro & Fielding, 1988). A mean of about 55% of label was recovered after 1 min in preβ-HDL, most in the small preβ-1 species. A second estimate was made from data obtained with anti-apo A-I affinity chromatography, when only about half the total efflux of cellular cholesterol to native plasma could be replaced by albumin, a nonspecific acceptor of cell cholesterol (Fielding & Moser, 1982). These values are reasonably consistent with the estimate of the proportion of total efflux (58%) ascribed to the specific or preβ-HDL pathway, based on the data in the present study. As discussed earlier (Fielding et al., 1982), the proportion of cholesterol utilized by HDL which is contributed by cell membranes depends on the surface area per milliliter of plasma, a parameter more favorable for cellular cholesterol transport in small than in large diameter vessels.

The rate of specific efflux was found to be proportional to preβ-HDL concentration over the whole range of concentrations studied. This suggests that preβ-1-HDL concentration may be rate-limiting, at least in normal plasma, and could directly reflect the rate at which cellular cholesterol is being transferred from the cell membrane to preβ-HDL. Because cholesterol does not normally accumulate in tissues, cholesterol efflux must balance cholesterol influx (corrected for local synthesis or utilization).

The efflux of both lecithin and cholesterol is preβ-1-HDL-dependent. However, the rate of mass efflux for cholesterol is about 5-fold greater (equivalent to a 10-fold greater efflux in molar terms). This is clearly disproportionate to the composition of the plasma membrane, which contains an approximately equimolar mixture of cholesterol and phospholipid, about half of the latter being lecithin (Renkonen et al., 1972). As a result, it is unlikely that preβ-1-specific efflux involves the transfer of intact lipid complexes from the membrane, and more likely that this pathway involves molecular transfer, by a pathway showing a relatively high specificity for cholesterol, but some activity with membrane lecithin.

The specific efflux pathway depends on the integrity of the cell surface. This is indicated by the inhibition of preβ-HDL-dependent (but not nonspecific) efflux by protease digestion. Although a fibroblast cell-surface HDL binding protein has been described and characterized (Havekes et al., 1984; Graham & Oram, 1987), its properties seem to differ significantly from those predicted by the pathway described here. In terms of specificity, the HDL-dependent component of cholesterol efflux into native plasma appears to involve mainly the preβ-1-HDL fraction, while the HDL receptor/binding protein reacted rather nonspecifically with a wide range of native and synthetic recombinant particles containing apo A-I or several other HDL proteins (Graham & Oram, 1987). The HDL binding protein was detectable only in cholesterol-loaded fibroblasts (Graham & Oram, 1987) while the preβ-HDL pathway makes up about half of efflux from normal, unloaded cells. The effect of protease digestion may well be only indirect, affecting the distribution or local concentration of lipids on the outer leaflet of the plasma membrane. More detailed information on cell-surface architecture will be required to distinguish these possibilities, but the effect of protease provides an additional criterion for distinguishing two components of cholesterol efflux.

The remaining ("nonspecific") component of efflux is most likely to represent simple diffusion, limited by the desorption of cholesterol from the interfacial surface (McLean & Phillips, 1981). Consistent with this interpretation, erythrocyte cholesterol acts as a single pool whose efflux rate and thermodynamics are consistent with diffusion (Lange et al., 1983). Efflux from blood cells was preβ-HDL-independent, and protease-resistant. The preβ-HDL-independent efflux component from fibroblasts appears to have very similar properties to that of the whole transport process from erythrocytes.

In summary, the evidence supporting a major role for preβ-1-HDL consists of the very early labeling of this fraction after incubation of plasma with cholesterol-labeled cells (Castro & Fielding, 1988); the loss of preβ-1-HDL by preincubation of plasma (Miida et al., 1992) and part of cholesterol efflux when plasma cholesterol metabolism proceeds in vitro, and the retention of both when it is inhibited (this research); the proportionality between the loss of preβ-1-HDL and cholesterol efflux also first shown here; and evidence that preβ-HDL-associated cholesterol may be more effectively esterified via the LCAT reaction (Castro & Fielding, 1988; Francone et al., 1989). These data do not rule out additional roles in efflux by other, as yet undescribed HDL fractions.

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