Oxidative Tyrosylation of HDL Enhances the Depletion of Cellular Cholesteryl Esters by a Mechanism Independent of Passive Sterol Desorption†

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ABSTRACT: It is believed that HDL protects against atherosclerosis by removing excess cholesteryl esters from cells of the artery wall. Previous studies have suggested that HDL depletes cells of cholesteryl esters both by stimulating cholesterol efflux from the plasma membrane and by activating transport processes that divert cholesterol from the cholesteryl ester cycle, but it is unknown if these are independent processes. We previously found that HDL oxidized by tyrosyl radical has a markedly enhanced ability to promote the removal of cholesterol from cultured cells [Francis, G. A., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6631−6635]. Here we show that incubation of cholesterol-loaded human fibroblasts with low concentrations of tyrosylated HDL depleted cells of cholesteryl esters and increased cellular free cholesterol without increasing efflux of cholesterol into the medium as compared to incubation with untreated HDL. Cells preincubated with tyrosylated HDL and then exposed to a variety of cholesterol acceptors exhibited significantly higher rates of free cholesterol efflux than did cells preincubated with HDL. This effect was observed in the presence or absence of an inhibitor of acyl CoA:cholesterol acyltransferase (ACAT) and was independent of cholesteryl ester hydrolysis, suggesting that alterations in cholesteryl ester cycle enzymes were not responsible for the loss of cholesteryl esters. In contrast to the reduction of cholesteryl esters, the rates of cholesterol and phospholipid efflux from the plasma membranes of cells exposed to tyrosylated HDL and HDL were identical. These results suggest for the first time that a mechanism exists to deplete cellular cholesteryl esters and the cholesterol substrate pool for esterification by ACAT prior to the removal of cholesterol from the plasma membrane. Identification of products in tyrosylated HDL responsible for this redistribution of cellular cholesterol may provide important insights into mechanisms of intracellular cholesterol trafficking and the ability of modified forms of HDL to protect the artery against wall pathological cholesterol accumulation.

The striking inverse relationship between levels of high-density lipoprotein (HDL)[1] and atherosclerotic disease is felt to be due in part to the ability of HDL to promote the removal of cholesterol from peripheral tissues, the first step in a pathway referred to as reverse cholesterol transport (Glomset, 1968). The mechanisms by which HDL removes cholesterol from cells are not fully understood. Two principal models have been proposed. The first is the nonspecific passive exchange of free cholesterol through the aqueous phase separating the donor plasma membrane and acceptor particles (Hagerman & Gould, 1951), with net excretion occurring when the concentration of free cholesterol and the free cholesterol:phospholipid ratio in the acceptor particle are lower than those in the plasma membrane (Phillips et al., 1980; Rothblat & Phillips, 1982; Johnson et al., 1991). The second model proposes an additional specific interaction between HDL and the cell surface that actively promotes cellular cholesterol excretion (Oram et al., 1983; Theret et al., 1990; Mendez et al., 1991). This interaction depletes cells of the substrate pool of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (the ACAT substrate pool or "ACAT-accessible" cholesterol), and is dependent on the presence of intact HDL apolipoproteins (Oram et al., 1991). This effect can also be induced by synthetic amphipathic helical peptides that mimic the secondary structure of apolipoprotein A-1 (apo A-1) (Mendez et al., 1994). The ability of free apolipoproteins and lipid-poor HDL particles to promote cellular cholesterol excretion and generate nascent HDL particles has been demonstrated by several investigators (Barbaras et al., 1987; Castro & Fielding, 1988; Hara & Yokoyama, 1991; Bielicki et al., 1992; Forte et al., 1993; Kawano et al., 1993). This apolipoprotein-mediated pathway has been shown to be defective in Tangier disease (Francis et al., 1995).

It is still unclear how HDL selectively depletes cells of ACAT-accessible cholesterol, but this may occur by two possible mechanisms. First, HDL could remove plasma membrane pools of cholesterol that feed into the cholesteryl ester cycle, thus diverting cholesterol substrate from ACAT

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[1] Abbreviations: HDL, high-density lipoprotein; apo A-1, apolipoprotein A-1; ACAT, acyl-CoA:cholesterol acyltransferase; LDL, low-density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, essentially fatty acid free bovine serum albumin; FC, free cholesterol; CE, cholesteryl ester; NCEH, neutral cholesteryl ester hydrolase; SFM, serum-free medium.

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which is localized to the endoplasmic reticulum (ER). This concept is supported by studies suggesting that the actual substrate pool for ACAT is derived from the plasma membrane (Tabas et al., 1988; Mazzone et al., 1995). In this case, depletion of cholesteryl esters and translocation of intracellular cholesterol to the plasma membrane may occur simply to replenish plasma membrane pools of cholesterol removed by HDL. Second, the interaction of HDL with cells may stimulate active transport of cholesterol from the ER to the plasma membrane. In support of this possibility are studies showing that HDL stimulates translocation of newly synthesized sterols to the plasma membrane (Oram et al., 1991; Walter et al., 1994; Rogler et al., 1995) by a process that appears to require activation of protein kinase C (Theret et al., 1990; Mendez et al., 1991; Li & Yokoyama, 1995). By the second mechanism, transport of intracellular cholesterol to the plasma membrane would be triggered by signals independent of the initial removal of cell-surface cholesterol.

The possible existence of a regulated cholesterol excretory pathway has important biological and clinical implications. First, it suggests that the ability of HDL to remove cholesterol from cells may be modulated by yet unidentified molecules that directly affect the activity of this transport pathway. Second, it supports the feasibility of developing pharmaceutical agonists for this pathway that would enhance the efficacy of HDL in clearing excess cellular cholesterol without requiring an increased availability of HDL particles or an improved cholesterol acceptor capacity. Previous studies, however, have been unable to dissociate completely the ability of HDL and its components to stimulate cholesterol transport between cellular pools from its effect on cholesterol efflux from the plasma membrane. Treatment of HDL with tetranitromethane or proteases has been shown to markedly reduce the ability of HDL to deplete ACAT-accessible cholesterol from cells, but these treatments can also reduce HDL-mediated cholesterol efflux (Brinton et al., 1986; Francis et al., 1995). We have previously described an oxidative modification using peroxidase-generated tyrosyl radical that markedly enhances the ability of HDL to promote removal of cholesterol from cultured fibroblast and macrophage foam cells (Francis et al., 1993). We investigated whether this enhancement reflects an increased capacity of HDL to accept plasma membrane cholesterol or a greater ability to stimulate movement of cholesterol between cellular pools. Our results show that the increased clearance of cholesteryl esters by tyrosylated HDL was associated with a redistribution of radiolabeled cholesterol from ACAT-accessible pools to plasma membrane domains accessible for removal by extracellular cholesterol acceptors. Since this occurred under conditions where there was no increase in cholesterol efflux from the cell, these findings describe for the first time a modification of HDL that enhances its ability to divert cholesterol from the cholesteryl ester cycle into efflux-accessible pools independent of the initial removal of cholesterol from the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol, L-tyrosine, hydrogen peroxide (30%, ACS grade), diethylenetriaminepentaacetic acid, sodium phosphotungstic acid, and essentially fatty acid-free bovine serum albumin were purchased from Sigma. [1,2-^{3}H]Cholesterol (51 Ci/mmol) and [*methyl*-^{3}H]choline chloride (81 Ci/mmol) were purchased from DuPont NEN, and [1-^{14}C]oleate (55 mCi/mmol) and [1-^{14}C]oleoyl-coenzyme A (54 mCi/mmol) were from Amersham. [^{3}H]Cholesterol was subjected to thin-layer chromatography on silica gel G plates (Whatman) developed in chloroform/methanol (100:2 by volume) to remove potential polar impurities prior to use in labeling experiments. Tissue culture medium was purchased from Bio-Whitaker, and fetal bovine serum was from Hyclone.

Lipoproteins and Apolipoproteins. HDL_3 (d = 1.125−1.21 g/mL, hereafter referred to as HDL), LDL (d = 1.019−1.063 g/mL), and lipoprotein-deficient serum (d > 1.25 g/mL) were isolated by standard ultracentrifugation techniques from the pooled plasma of healthy male volunteers (Chung et al., 1980). HDL fractions were subjected to heparin−agarose affinity chromatography to remove apo E- and apo B-containing particles (Weisgraber & Mahley, 1980). Apo A-1 was isolated from HDL by delipidation and DEAE-cellulose chromatography as described previously (Yokoyama et al, 1982). Tyrosylation of HDL was carried out at 37 °C for 24 h in buffer A [66 mM potassium phosphate buffer, pH 8.0, which had been passed over Chelex 100 resin (Bio-Rad) to remove transition metal ions]. The reaction mixture contained a final concentration of 1 mg/mL HDL protein, 100 μM diethylenetriaminepentaacetic acid (to inhibit metal-ion-catalyzed oxidation), 100 nM horseradish peroxidase (Boehringer Mannheim, 250 units/mg), 100 μM H_2O_2, and 100 μM L-tyrosine. Prior to analysis or cell studies, the HDL was subjected to size-exclusion chromatography on a 10-DG column (Bio-Rad) equilibrated with buffer A, or Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM HEPES (pH 7.4), to remove free dityrosine and other reaction components. Residual horseradish peroxidase associated with the modified HDL was shown in control experiments to have no independent effect on measures of cholesterol efflux. Lipoprotein-associated dityrosine was measured by fluorescence using excitation and emission wavelengths of 328 nm and 410 nm, respectively (Anderson, 1966; Amado et al., 1984). HDL and LDL used to assess rates of passive sterol exchange were labeled with [^{3}H]-cholesterol by Celite exchange as described (Avigan, 1959). Copper oxidation of HDL was carried out in PBS containing 1 mg/mL HDL and 1 μM $CuSO_4$ incubated at 37 °C for 24 h. The reaction was terminated by addition of 2 mM ethylenediaminetetraacetic acid, overlaying with N_2(g), and cooling to 4 °C.

Cell Culture. Normal human skin fibroblasts were cultured in DMEM containing 10% fetal bovine serum as described (Brinton et al., 1986). Fibroblasts were plated at 15 000−20 000 cells/16 mm well or 70 000−100 000 cells/35 mm dish and grown to confluence (about 7 days). To cholesterol-load the cells, confluent cultures were washed twice with phosphate-buffered saline containing 2 mg/mL bovine serum albumin (essentially fatty acid free) (PBS−BSA) and incubated for 24−48 h in DMEM containing 2 mg/mL BSA and 30 μg/mL cholesterol (added from a 10 mg/mL solution in ethanol). To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS−BSA and incubated for a further 24 h in DMEM containing 1 mg/mL BSA (DMEM−BSA). In some cases, cholesteryl ester formation was blocked by including 2 μg/mL of the ACAT inhibitor compound 58-035 (a generous gift from Sandoz Pharmaceuticals) in the cholesterol-loading, equilibration, and

efflux media. Cells were depleted of cholesterol by incubating confluent cultures in DMEM containing 10% lipoprotein-deficient human serum for 48 h.

Labeling of Cellular Cholesterol Pools and Phospholipids. In the indicated experiments, rapidly growing cells (approximately 60% confluent) were switched to DMEM containing 10% fetal bovine serum and 0.2 μCi/mL [^{3}H]-cholesterol and grown until confluent (3 days). Cell layers were then rinsed twice with PBS−BSA prior to cholesterol loading and equilibration as described above. In some experiments, cells were labeled with [^{3}H]cholesterol after cholesterol loading during the 24 h equilibration step, with results in efflux studies being comparable to those where labeling was done before cholesterol loading. Cells were washed 3 times with PBS−BSA before addition of efflux media. To label cellular cholesteryl ester, confluent cell layers were washed twice with PBS−BSA and incubated with DMEM containing 30 μg/mL free cholesterol, 9 μM [^{14}C]oleate (0.5 μCi/mL, specific activity 17 850 dpm/nmol), and 5 mg of BSA/mL, for 30 h at 37 °C. Cell layers were rinsed twice with PBS−BSA, equilibrated 24 h in the same medium without free cholesterol, and rinsed 5 more times prior to addition of efflux media. Total ^{14}C radioactivity ranged from 1.6×10^5 to 1.9×10^5 cpm/mg of cell protein. To selectively label plasma membrane cholesterol of fibroblasts (Mendez et al., 1991), cholesterol-loaded cells were incubated for 2 h with DMEM−BSA containing 0.2 μCi/mL [^{3}H]cholesterol after the 24 h equilibration step. Cell layers were then washed 5 times with PBS−BSA prior to addition of efflux media. To radiolabel phospholipids in cholesterol-loaded cells, 1.0 μCi/mL [^{3}H]choline chloride was added to the DMEM−BSA during the 24 h equilibration incubations (Mendez et al, 1994). Cells were washed 5 times with PBS−BSA before efflux incubations.

Cholesterol and Phospholipid Efflux. After the appropriate labeling protocol, cells were incubated in DMEM−BSA and the indicated additions. After the indicated times, the efflux medium was collected and centrifuged to remove cell debris, and cell layers were rinsed twice with ice-cold PBS−BSA and twice with PBS. Cells were stored at −20 °C until extraction for lipid and protein content. Efflux media supernatants were either counted directly (for cells labeled with [^{3}H]cholesterol) or extracted by the method of Folch et al. (1957). Cellular lipids were extracted in hexane/2-propanol (3:2 by volume) as described (Hara & Radin, 1978). Sterol species were separated by thin layer chromatography on silica gel G plates developed in hexane/diethyl ether/acetic acid (130:40:1.5 by volume). Choline-containing phospholipids were separated by thin-layer chromatography on silica gel H plates developed in chloroform:methanol/acetic acid/water (100:60:16:8 by volume). Lipid spots were identified by staining with iodine vapor and comigration with standards. After allowing iodine stain to evaporate, appropriate spots were taken for determination of radioactivity and/or lipid mass (Heider & Boyett, 1978).

Determination of ACAT Activity. Fibroblasts grown to confluence were cholesterol-loaded and equilibrated as described above. Following incubation with 40 μg/mL HDL or tyrosylated HDL for 16 h, cells were rinsed twice with ice-cold PBS−BSA and twice with PBS and scraped from their dishes, and whole cell homogenates were prepared by sonication for 5 s × 2 in PBS with 0.5 mM EDTA. ACAT activity in the homogenates was determined by the formation of cholesteryl ester from excess exogenous cholesterol (200 μM) and [^{14}C]oleoyl-CoA (100 μM, specific activity 10 000 dpm/nmol) (Bilheimer et al., 1981).

Other Methods. The lipid composition of HDL and tyrosylated HDL was determined enzymatically (Warnick, 1986). For measurement of conjugated dienes, fatty acids of control and tyrosylated HDL were isolated by the method of Dole and Meinertz (1960), and the absorbance at 232 nm was determined. Cell protein was measured by the method of Lowry et al. (1951) using BSA as standard. Determination of HDL protein was determined by the method of Bradford (1976) to minimize potential errors in estimation due to alterations in tyrosine residues. Estimates of protein in HDL and tyrosylated HDL using this method compared closely to values obtained using interference spectroscopy in an analytical ultracentrifuge (Babul & Stellwagen, 1969).

RESULTS

We have previously demonstrated that tyrosylated HDL is more effective than HDL at reducing cellular stores of cholesteryl ester and (at higher concentrations) free cholesterol, and at inhibiting new cholesteryl ester synthesis (Francis et al., 1993). These effects might be interpreted in several ways. First, tyrosylated HDL might enhance the diversion of free cholesterol away from a pool accessible to ACAT to sites on the cell surface for desorption to extracellular acceptor particles. Second, tyrosylated HDL might inhibit ACAT directly or stimulate neutral cholesteryl ester hydrolase (NCEH). Third, tyrosylated HDL might be a more effective acceptor of cholesterol passively desorbed from the cell surface, with cholesteryl ester stores being depleted mainly to replenish plasma membrane cholesterol lost to the medium. To distinguish between these possibilities, we used the following experimental approach.

Time and Concentration Dependence of Cholesteryl Ester Turnover and Cholesterol Efflux by Tyrosylated HDL. Our previous results showed that compared to cells incubated with HDL, the reduction of cellular cholesteryl esters by tyrosylated HDL was most pronounced at low concentrations of HDL protein (2.5−10 μg/mL) in the efflux medium. To investigate further the turnover of cholesteryl ester (CE) and free cholesterol (FC) in cells exposed to a low concentration of tyrosylated HDL, fibroblasts were first labeled with [^{3}H]-cholesterol during the final phase of growth, and then cholesterol-loaded with nonlipoprotein cholesterol for 48 h. Subsequent incubations of up to 24 h with serum-free medium containing a low concentration (10 μg/mL) of HDL or tyrosylated HDL showed no difference in efflux of [^{3}H]-cholesterol into the medium (Figure 1A). In contrast, significant differences were seen in the level of [^{3}H]-cholesterol remaining in the CE and FC pools of cells incubated with these particles for more than 6 h. The loss of radiolabeled CE was accompanied by a simultaneous increase in radiolabeled FC in cells incubated with tyrosylated HDL (Figure 1B,C). Cells treated with serum-free medium alone had a further rise of label in CE during the incubation, suggesting continued movement of [^{3}H]cholesterol from a plasma membrane compartment to sites for esterification by ACAT. These results indicate that relatively low concentrations of tyrosylated HDL stimulate turnover of cholesteryl esters in cells, leading to accumulation of cholesterol in the free cholesterol pool, and that this

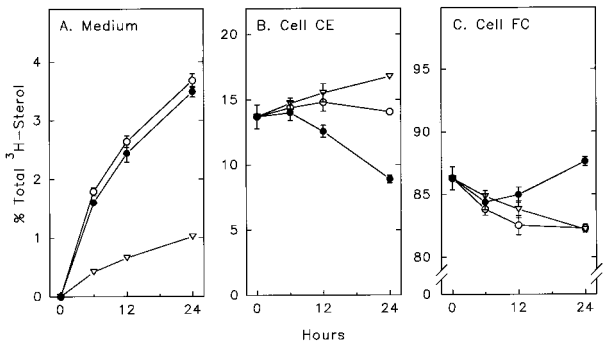

FIGURE 1: Time course of cholesteryl ester turnover and cholesterol efflux from cholesterol-loaded fibroblasts exposed to tyrosylated HDL. Human skin fibroblasts were labeled with [^{3}H]cholesterol during the last 40% of growth to confluence and then loaded with nonlipoprotein cholesterol for 48 h and equilibrated for 24 h in serum-free medium (SFM, DMEM containing 1 mg/mL BSA) to allow equilibration of cholesterol pools. The cells were then incubated with SFM alone (▽) or the same medium plus 10 μg/mL HDL (○) or tyrosylated HDL (●). After the indicated intervals, the medium was removed and centrifuged at 10000*g* for 10 min, and radiolabeled cholesterol in the supernatant was determined by scintillation counting. Cellular lipids were analyzed for free and esterified [^{3}H]cholesterol as described under Experimental Procedures. Results are expressed as percent of total (cell plus medium) [^{3}H]sterol in the medium (A), in cellular cholesteryl ester (CE) (B), and in cellular free cholesterol (FC) (C). Total [^{3}H]cholesterol was (2.3−2.6) × 10^5 cpm/mg of cell protein. Values are the mean ± SD of four determinations and are representative of two similar experiments. Error bars not shown are within the symbol dimensions. Differences in cellular cholesteryl ester and free cholesterol radioactivity between cells treated with HDL and tyrosylated HDL are significant, with $p < 0.01$ at 12 h and $p < 0.001$ at 24 h, as calculated by the Mann−Whitney rank sum test.

redistribution of cellular cholesterol occurs before any increase in efflux of cholesterol to the medium.

Our previous results also showed that concentrations of tyrosylated HDL higher than 20 μg/mL resulted in significant depletion of the total free cholesterol pool of cultured fibroblasts and macrophages as compared to cells incubated with HDL (Francis et al., 1993). To examine the concentration dependence of tyrosylated HDL-induced efflux of cellular cholesterol, cholesterol-loaded and labeled cells were incubated with 0−40 μg/mL HDL or tyrosylated HDL in serum-free medium for 24 h. We again observed a marked drop of [^{3}H]cholesterol in labeled CE together with a rise in labeled FC in cells incubated with up to 10 μg/mL tyrosylated HDL (Figure 2B,C). At higher concentrations of tyrosylated HDL, there was a leveling off of cellular CE label but a fall in cellular FC label. Similar results were also seen in cells incubated with higher concentrations of HDL. At concentrations higher than 10 μg/mL, tyrosylated HDL promoted significantly greater efflux of [^{3}H]cholesterol into the medium than did HDL (Figure 2A). Changes in cellular CE and FC mass (Figure 3) paralleled closely the changes in radiolabel in these two pools (Figure 2). This indicates that dilution of radiolabel by newly synthesized cholesterol during the course of the experiment was not significant in these cholesterol-loaded cells.

To confirm that increased efflux of radiolabeled cholesterol to medium from cells incubated with tyrosylated HDL represented increased net cholesterol efflux, cholesterol mass in the efflux medium was determined. After subtracting lipoprotein-associated FC in the medium from cell-free dishes, 40 μg/mL tyrosylated HDL protein caused about 50%

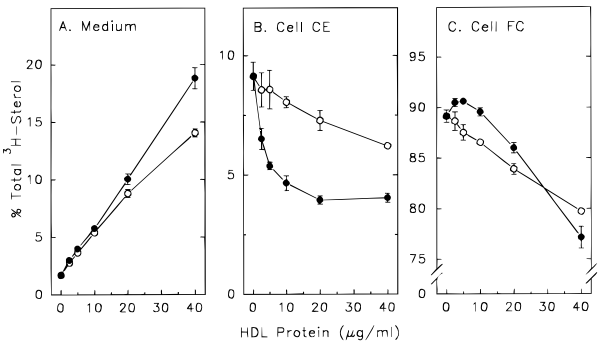

FIGURE 2: Cholesteryl ester turnover and cholesterol efflux from fibroblasts incubated with varying concentrations of HDL and tyrosylated HDL. Fibroblasts were cholesterol-loaded and labeled as described for Figure 1 except that [^{3}H]cholesterol labeling was done during the 24 h equilibration step following cholesterol loading. After extensive washing, the cells were then incubated with SFM and the indicated concentrations of HDL (○) or tyrosylated HDL (●) for 24 h. The medium (panel A), cellular cholesteryl ester (panel B), and cellular free cholesterol (panel C) were analyzed and the results expressed as in Figure 1. Total [^{3}H]cholesterol was (7.3−8.3) × 10^5 cpm/mg of cell protein. Values are the mean ± SD of four determinations and are representative of two similar experiments. In panel 2A, radioactivity in the medium containing tyrosylated HDL was significantly different with $p < 0.01$ at 20 and 40 μg/mL compared with HDL. All differences between HDL and tyrosylated HDL in panels B and C are significantly different ($p < 0.01$).

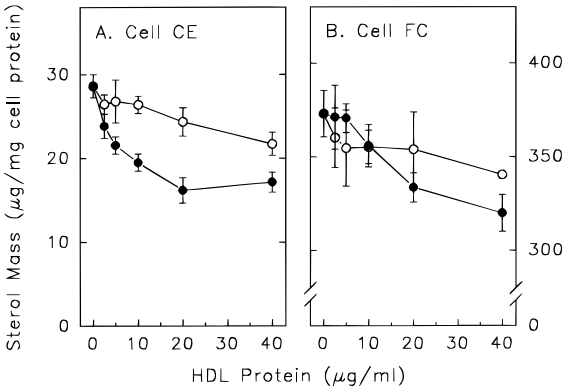

FIGURE 3: Tyrosylated HDL is more effective than HDL at depleting cellular cholesteryl esters. Cells from the experiment shown in Figure 2 were assessed for mass of cholesteryl ester (A) and free cholesterol (B) as described under Experimental Procedures. (○) HDL; (●) tyrosylated HDL.

more FC to move into the medium from cholesterol-loaded fibroblasts than did HDL during a 24 h incubation (differences of 7.90 ± 1.37 μg of FC/mg of cell protein for tyrosylated HDL versus 5.39 ± 1.44 μg for HDL, average ± SEM, three experiments). These results together suggested that tyrosylated HDL was more effective than HDL at inducing movement of FC to efflux-accessible regions of the plasma membrane, but that removal of this cholesterol was dependent on adequate concentrations of acceptor particle in the medium, in this case higher than 10 μg/mL.

Promotion of Cholesterol Transport to Efflux-Accessible Sites by Tyrosylated HDL. The apparent increase in translocation of ACAT-accessible cholesterol to efflux-accessible sites on the cell surface by tyrosylated HDL was explored further using chase incubations with various acceptor particles. Cholesterol-loaded fibroblasts labeled with [^{3}H]-cholesterol were again incubated with low concentrations (10 μg/mL) of HDL and tyrosylated HDL for up to 48 h. At various time points, the efflux medium was collected; the

Table 1: Efflux of Cholesterol to HDL, Free Apolipoprotein A-1, and LDL following Initial Incubation with HDL and Tyrosylated HDL[a]

initial acceptor	initial efflux	efflux to chase medium		
		HDL	apo A-1	LDL
no ACAT inhibitor				
HDL	13.52 ± 0.80	4.37 ± 0.22	3.33 ± 0.24	3.51 ± 0.47
tyrosylated HDL	14.60 ± 0.86	7.06 ± 0.28[d]	5.49 ± 1.32[b]	7.06 ± 0.76[d]
plus ACAT inhibitor				
HDL	38.25 ± 2.91	9.29 ± 1.76	8.03 ± 1.62	8.40 ± 2.64
tyrosylated HDL	40.77 ± 2.65	17.97 ± 5.27[b]	14.90 ± 1.51[c]	15.21 ± 3.13[b]

[a] Human fibroblasts labeled with [^{3}H]cholesterol and cholesterol-loaded as described were incubated with 10 μg/mL HDL or tyrosylated HDL for 48 h, washed, and then incubated with HDL (100 μg/mL), free apo A-1 (10 μg/mL), or LDL (50 μg/mL) for 2 h. Cholesterol loading and incubations were performed in the absence or presence of 2 μg/mL ACAT inhibitor Sandoz 58-035. Efflux to initial or chase medium is indicated as percent of total medium plus cellular [^{3}H]cholesterol counts. Total [^{3}H]cholesterol was (2.0−2.5) × 10^5 cpm/mg of cell protein. Values are mean ± SD for four incubations. Statistical significance for the difference from cells initially incubated with HDL: [b] $p < 0.05$; [c] $p < 0.01$; [d] $p < 0.001$.

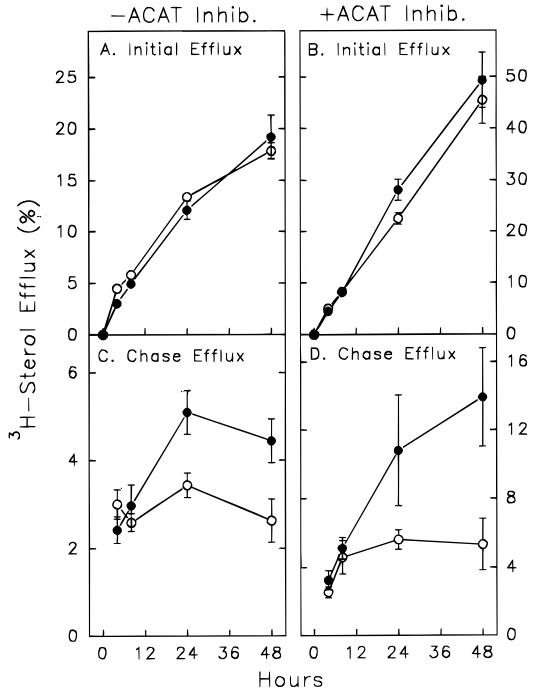

FIGURE 4: Promotion of cholesterol transport to efflux-accessible sites by HDL and tyrosylated HDL. Fibroblasts were labeled with [^{3}H]cholesterol and cholesterol-loaded as described in Figure 1 in the absence (panels A and C) or presence (panels B and D) of 2 μg/mL ACAT inhibitor 58-035. Cells were then incubated with SFM containing 10 μg/mL HDL (○) or tyrosylated HDL (●) for the indicated time, washed 2 times with PBS−BSA and 2 times with PBS, and chased with 100 μg/mL control HDL for 1 h. Media were collected after the initial incubations (panels A and B) and the 1 h chase incubations (panels C and D), and aliquots were counted for radioactivity. Results are expressed as the percent of total (cell plus medium) [^{3}H]sterol in the medium for each step. Total [^{3}H]cholesterol was (2.2−2.6) × 10^5 cpm/mg of cell protein. Values are the mean ±SD of four determinations and are representative of two similar experiments. Tyrosylated HDL chase efflux values at 48 h in the −ACAT condition and at 24 and 48 h in the +ACAT condition are significantly different with a p value <0.05 compared with efflux with HDL.

cells were then washed extensively and incubated for 1 h with higher levels of HDL (100 μg/mL) (Figure 4A,C). Parallel incubations were performed with [^{3}H]cholesterol-labeled cells loaded with cholesterol, equilibrated, and incubated with HDL or tyrosylated HDL in the presence of an ACAT inhibitor to prevent esterification of newly incorporated cholesterol or re-esterification of hydrolyzed cholesteryl ester (Figure 4B,D). [^{3}H]Cholesteryl ester levels in cells treated with ACAT inhibitor were less than 1% of total counts. As in Figure 1, [^{3}H]sterol efflux to medium containing 10 μg/mL tyrosylated HDL was similar to that to HDL for up to 48 h, whether or not an ACAT inhibitor was present (Figure 4A,B) . In the presence of ACAT inhibitor, a higher percentage of total cellular [^{3}H]sterol was released into the initial efflux medium, but this was due to higher specific activity rather than increased mass of cholesterol removed from these cells. Changes in non-lipoprotein free cholesterol mass in the medium after the 48 h initial incubation were 2.53 ± 0.28 and 2.09 ± 0.72 μg/dish for control and tyrosylated HDL respectively, in the absence of ACAT inhibitor, and 2.59 ± 0.51 and 2.21 ± 0.41 μg/dish in the presence of ACAT inhibitor (average ± SD, 4 determinations). Despite this similarity in efflux to HDL and tyrosylated HDL initially, efflux to the 1 h chase medium containing 100 μg/mL HDL was significantly higher when cells were initially incubated with tyrosylated HDL (Figure 4C,D). Experiments using HDL (100 μg/mL), free apo A-1 (10 μg/mL), or LDL (50 μg/mL) as the acceptor particle in the chase medium for 2 h similarly showed significantly increased efflux to all the acceptors from cells initially incubated with tyrosylated HDL, again in the presence or absence of an ACAT inhibitor (Table 1). These results provide further evidence that tyrosylated HDL enhances the movement of cholesterol to plasma membrane sites capable of releasing this cholesterol to acceptor particles in the medium.

Activity of Cholesteryl Ester Cycle Enzymes. The results shown in Figure 4 and Table 1 indicate similar effects of tyrosylated HDL in the presence or absence of an ACAT inhibitor, suggesting that the effect of tyrosylated HDL is by other than direct inhibition of ACAT. We previously found increased new cholesterol synthesis in cells exposed to tyrosylated HDL (Francis et al., 1993), an effect never seen in the presence of an ACAT inhibitor. The effect of preincubation with HDL and tyrosylated HDL on the activity of ACAT in cholesterol-loaded cells was also measured directly in cell homogenates using exogenous cholesterol as substrate. Formation of cholesteryl [^{14}C]oleate by homogenates of cells preincubated with serum-free medium alone for 24 h was 1170 pmol (mg of cell protein)$^{-1}$ 15 min^{-1}, compared to 796 pmol/mg for cells preincubated with 40 μg/mL HDL and 911 pmol/mg with tyrosylated HDL. These results suggest that the decreased rate of new cholesteryl ester synthesis by cells incubated with tyrosylated HDL is due to movement of free cholesterol out of the regulatory/substrate pool for ACAT rather than direct inhibition of the enzyme.

Table 2: Protein and Lipid Composition of HDL_3 and Tyrosylated HDL_3[a]

HDL preparation	protein	FC	CE[b]	PL	TG	total lipid/protein	FC/PL
HDL	52.0 ± 1.2	2.3 ± 0.0	18.0 ± 0.7	25.5 ± 0.6	2.2 ± 0.0	0.92 ± 0.04	0.09 ± 0.01
tyrosylated HDL	52.8 ± 1.2	2.2 ± 0.1	17.6 ± 0.4	25.3 ± 0.5	2.1 ± 0.1	0.90 ± 0.03	0.09 ± 0.01

[a] FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride. Values are mean ± SD for 4 preparations of HDL and represent the percent of total protein plus lipid mass, except total lipid/protein and FC/PL which are mass ratios. [b] CE calculated using the formula CE = (TC − FC) × 1.69 (Morton & Steinbrunner, 1990).

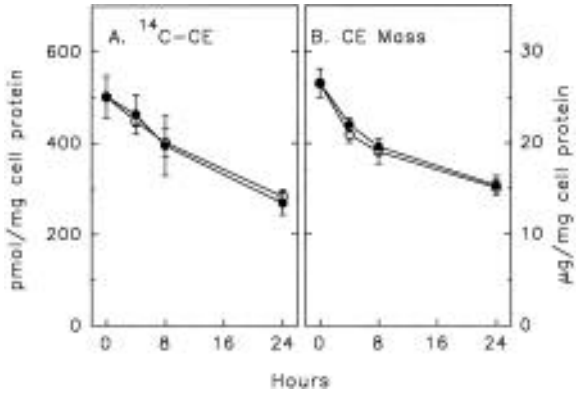

FIGURE 5: Neutral cholesterol ester hydrolase activity in the presence of HDL and tyrosylated HDL. Confluent fibroblasts were labeled with [^{14}C]oleate during cholesterol loading and equilibration as described under Experimental Procedures. Cells were then incubated with SFM containing 10 μg/mL HDL (○) or tyrosylated HDL (●) and 2 μg/mL ACAT inhibitor 58-035. After the indicated interval, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity (A) and mass (B). Values are the mean ± SD of three determinations and are representative of two similar experiments.

Stimulation of neutral cholesteryl ester hydrolase (NCEH) by tyrosylated HDL was unlikely to account for its enhanced effect on efflux, as this was also observed in cells cholesterol-loaded in the presence of an ACAT inhibitor where accumulation of cholesteryl esters was blocked. To confirm whether or not tyrosylated HDL increases hydrolysis of CE by NCEH, fibroblasts labeled in the ester moiety of CE with [^{14}C]oleate were incubated with equivalent concentrations of HDL and tyrosylated HDL for up to 24 h, and the remaining ^{14}C-labeled CE and CE mass were measured to assess the rate of hydrolysis of preformed CE. To measure the rate of cholesteryl ester hydrolysis directly, an ACAT inhibitor was added during the HDL incubation to prevent re-esterification of liberated [^{14}C]oleate and FC. With re-esterification blocked, there were no differences in the rates of decrease in [^{14}C]-labeled CE or in CE mass in cells incubated with 10 μg/mL HDL or tyrosylated HDL, both falling by about 50% over 24 h (Figure 5). These results are identical to rates of CE hydrolysis previously reported for macrophages incubated in the presence or absence of HDL (Brown et al., 1980). The lack of a difference in the rate of disappearance of CE together with the results shown in Figure 4 indicates that the increased FC excretion stimulated by tyrosylated HDL is not attributable to an increase in cholesteryl ester hydrolysis by NCEH.

Composition of Major Components of HDL and Tyrosylated HDL. Compositional differences between HDL and tyrosylated HDL might also explain the increased removal of cellular cholesterol by tyrosylated HDL. Previous reports [reviewed in Johnson et al. 1991)] demonstrated that sterol exchange between acceptor particles and cells is dependent on the content of free cholesterol in the donor and acceptor surfaces, with movement of free cholesterol down a concentration gradient, and on the ratio of free cholesterol to phospholipid in the acceptor particle. As shown in Table 2, no significant differences were seen in the percent content of protein or any of the major lipid classes between HDL and tyrosylated HDL. Mass ratios of free cholesterol to phospholipid were 0.09 for both types of HDL (equivalent to molar ratios of 0.18); total lipid:protein ratios were also similar for the two particles. These values contrast markedly to the results of Ghiselli et al. (1992), who reported a free cholesterol:phospholipid mass ratio of 0.18 and a total lipid: protein ratio of 0.70 for copper-oxidized HDL. However, levels of conjugated dienes, a marker for lipid peroxidation, were increased in tyrosylated HDL as previously reported for LDL oxidized by tyrosyl radical (Savenkova et al., 1994): an increase in the absorbance at 232 nm of 1.4 units/mg of tyrosylated HDL protein relative to HDL, as compared with 2.5 units/mg for copper-oxidized HDL. These results indicate that the differences in cholesterol efflux generated by HDL and tyrosylated HDL cannot be explained by alterations in the percent mass of the major HDL components, but may be explained by the formation of unique oxidation products in tyrosyl radical-modified HDL.

Exchange of Free Cholesterol between HDL or Tyrosylated HDL and the Plasma Membrane of Fibroblasts. The lack of differences in the content of free cholesterol and the free cholesterol:phospholipid ratio between these particles suggested that passive desorption of cell surface cholesterol to HDL and tyrosylated HDL should be the same. To address this question directly, sterol-loaded fibroblasts were briefly exposed to [^{3}H]cholesterol to specifically label the plasma membrane pool of free cholesterol, and the labeled cells were then incubated for 20 h with concentrations up to 40 μg/mL HDL and tyrosylated HDL. During these incubations, less than 2% of the total [^{3}H]cholesterol appeared in CE pools, indicating very little internalization of plasma membrane label (although movement of small amounts of labeled sterol to intracellular FC pools cannot be excluded). Determination of the percent of total [^{3}H]sterol in the medium revealed no differences in efflux of labeled cholesterol to HDL or tyrosylated HDL, even at higher concentrations (Figure 6). This result suggests that there is no difference in the acceptor capacity of these two particles for plasma membrane cholesterol passively released into the medium from cholesterol-loaded cells.

To examine further the exchange of free cholesterol between HDL and tyrosylated HDL and cells, we determined the rate of influx of [^{3}H]cholesterol from HDL particles labeled prior to tyrosylation to cholesterol-depleted and -loaded cells. Fibroblasts grown to confluence and exposed to either lipoprotein-deficient serum or free cholesterol were then incubated with equivalent concentrations of labeled HDL or tyrosylated HDL for up to 20 h, and the percent of total [^{3}H]sterol transferred to the cells was determined. As

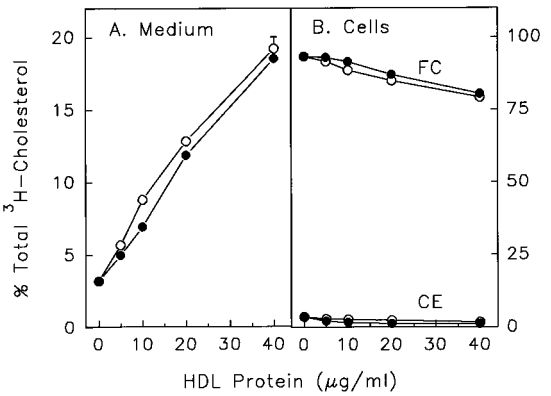

FIGURE 6: Efflux of plasma membrane cholesterol to HDL and tyrosylated HDL. Cholesterol-loaded fibroblasts were incubated with [^{3}H]cholesterol for 2 h as described under Experimental Procedures to specifically label plasma membrane cholesterol. Cells were then incubated with SFM containing the indicated concentration of HDL (○) or tyrosylated HDL (●) for 20 h. Media (panel A) and cellular (panel B) lipids were analyzed for [^{3}H]cholesterol and the results expressed as in Figure 1. Total [^{3}H]cholesterol was (3.7−4.7) × 10^5 cpm/mg of cell protein. Values are the mean ± SD of three determinations and are representative of three experiments.

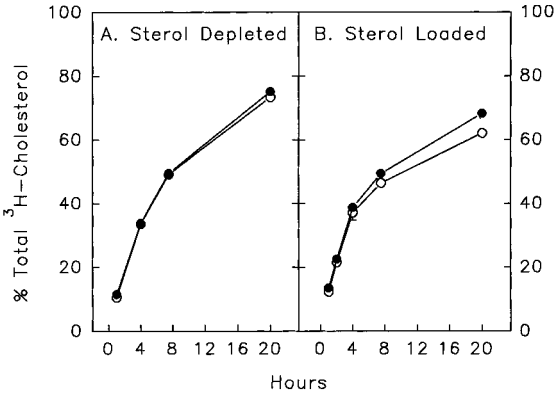

FIGURE 7: Influx of [^{3}H]cholesterol from HDL and tyrosylated HDL to cholesterol-depleted or cholesterol-loaded fibroblasts. Confluent fibroblasts were cholesterol-depleted or -loaded for 48 h by incubation with lipoprotein-deficient serum (panel A) or SFM containing cholesterol (panel B), respectively, as described under Experimental Procedures. Following overnight equilibration in SFM, cells were then incubated with 10 μg/mL [^{3}H]cholesterol-labeled HDL (○) or tyrosylated HDL (●) for the indicated interval. Media and cellular lipids were analyzed for [^{3}H]cholesterol and results expressed as the percent of total (media plus cell) [^{3}H]sterol present in cells. Total [^{3}H]cholesterol used in each incubation was 2.8 × 10^4 cpm. Values are the mean of duplicates and are representative of two experiments.

shown in Figure 7, there was little difference in the degree of influx of [^{3}H]cholesterol from HDL or tyrosylated HDL to either cholesterol-depleted or cholesterol-loaded cells. When cell-free dishes were used to assess the degree of nonspecific sticking of [^{3}H]cholesterol to plastic, equivalent uptake of label was seen for both particles, up to a maximum of ~30% of the total counts for both at 20 h. Cholesterol influx to cells was equivalent from both HDL and tyrosylated HDL even when results were corrected for this potential nonspecific association of radiolabeled cholesterol with plastic. Similarly, no differences were seen in the rate of exchange of labeled cholesterol between LDL particles and either HDL or tyrosylated HDL (data not shown). These results confirmed our findings with cells and suggest that the differences in cholesteryl ester turnover in cells treated

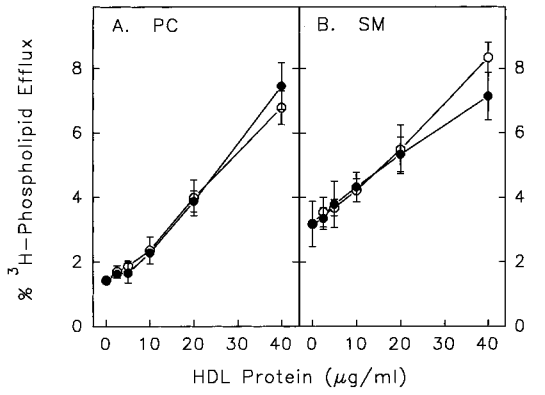

FIGURE 8: Phospholipid efflux to HDL and tyrosylated HDL. Cholesterol-loaded fibroblasts were labeled with [^{3}H]choline during the 24 h equilibration phase as described under Experimental Procedures. After extensive washing, cells were incubated with SFM containing HDL (○) or tyrosylated HDL (●) for 24 h, and medium and cellular phosphatidyl[^{3}H]choline (PC) and [^{3}H]sphingomyelin (SM) were measured. Values are the mean ± SD of quadruplicate incubations expressed as a percentage of the total cellular plus medium radiolabeled phospholipid appearing in the medium, and are representative of two experiments. Total [^{3}H]choline was (5.5−6.4) × 10^5 cpm/mg of cell protein.

with HDL or tyrosylated HDL were not due to differences in the rates of passive cholesterol transfer to or from the lipoproteins.

Phospholipid Efflux to HDL and Tyrosylated HDL. Phospholipid efflux to lipid acceptor particles including HDL and free apo A-1 is felt to be a prerequisite or concomitant event to cholesterol efflux (Hara & Yokoyama, 1991; Yancey et al., 1995). To test the possibility that differences in the removal of cellular cholesterol by HDL and tyrosylated HDL might be related to differential phospholipid efflux, we measured the ability of these two particles to stimulate efflux of radiolabeled phosphatidylcholine and sphingomyelin from cholesterol-loaded cells labeled with [^{3}H]choline. Efflux of radiolabeled phosphatidylcholine (Figure 8A) and sphingomyelin (Figure 8B) to media containing increasing concentrations of either HDL or tyrosylated HDL over 24 h were identical. These results show that the increased free cholesterol efflux mediated by tyrosylated HDL is not accompanied by an increase in phosphatidylcholine or sphingomyelin efflux.

DISCUSSION

The current studies suggest that oxidation of HDL by tyrosyl radical enhances its ability to deplete cells of cholesteryl esters by promoting the translocation of free cholesterol from an ACAT substrate pool to an efflux-accessible pool. This effect was independent of increased passive cholesterol desorption from the plasma membrane, inhibition of cholesterol esterification, or stimulation of cholesteryl ester hydrolysis. These results indicate for the first time that initial removal of cell surface cholesterol is not necessarily a prerequisite for depletion of cellular cholesteryl esters.

Tyrosylated HDL was more effective than HDL in depleting cellular cholesteryl esters at concentrations at or below 10 μg/mL. In contrast, at these concentrations, tyrosylated HDL was no more effective than HDL in promoting cholesterol efflux. Instead, the excess free cholesterol was not re-esterified and accumulated within cells. Thus, this

effect was not attributable to an enhanced ability of tyrosylated HDL to remove cholesterol from cells that would otherwise be re-esterified. Rather, this cholesterol appeared to be diverted from a pool accessible for esterification by ACAT. Moreover, tyrosylation of HDL did not alter its ratio of free cholesterol to phospholipid or its ability to act as either an acceptor or a donor of plasma membrane cholesterol. The enhanced cholesterol ester depletion in the presence of tyrosylated HDL could therefore not be explained by it simply being a better acceptor of cholesterol that passively desorbs from the plasma membrane.

With increasing concentrations of tyrosylated HDL, more of the free cholesterol that accumulated in cells was released into the medium, so that tyrosylated HDL promoted fractional cholesterol efflux to a greater extent than HDL at concentrations above 10 μg/mL. A plausible explanation for these findings is that tyrosylated HDL stimulated translocation of cholesterol to sites in the plasma membrane accessible for efflux but at a rate that exceeded desorption from the cell at low particle concentrations. At higher concentrations, where the availability of cholesterol acceptor particles was no longer rate-limiting, a greater fraction of this cholesterol was released into the medium. Thus, excess free cholesterol may accumulate within efflux-accessible domains of tyrosylated HDL-treated cells until the concentration of acceptor particles reaches a level high enough to remove it. In support of this concept, incubation of cells with a concentration of tyrosylated HDL too low to promote efflux to a greater extent than equivalent amounts of HDL caused a marked increase in cholesterol efflux when cells were subsequently incubated with higher levels of acceptor particles (HDL, apo A-I, or LDL). This increase was evident even when the cholesterol-loading and efflux media contained an ACAT inhibitor to prevent cholesteryl ester formation, indicating that the stimulatory effects of tyrosylated HDL were independent of the cholesteryl ester cycle. These results strongly suggest that oxidation of HDL by tyrosyl radical enhances its ability to divert excess cholesterol from cellular storage compartments to plasma membrane domains where it becomes accessible for removal from cells. The data also suggest that this is an active process occurring separate from passive removal of the translocated sterol from the plasma membrane. The apparent distinctness of these two processes suggests that conversion of efflux-resistant to efflux-accessible cholesterol does not occur simply to replenish cholesterol lost to passive desorption.

It is noteworthy that treatment of cells with an ACAT inhibitor during the cholesterol loading and subsequent incubations but after radiolabeling with cholesterol tracer led to a severalfold increase in fractional radiolabeled cholesterol efflux stimulated by both HDL and tyrosylated HDL. At 10 μg/mL, however, HDL and tyrosylated HDL removed the same amount of cholesterol mass from cells whether or not the cells were treated with an ACAT inhibitor, consistent with the conclusion that availability of cholesterol acceptor particles in the media limited net cholesterol transport from cells at this low lipoprotein concentration. These results indicate that the cholesterol released from ACAT inhibitor-treated cells had a much higher specific activity. Apparently, inhibition of cholesterol esterification during the loading incubations redistributed previously introduced tracer so as to increase the specific activity of efflux-accessible pools of free cholesterol.

Several additional lines of evidence support the conclusion that the tyrosylated HDL-mediated redistribution of cellular free cholesterol occurs independent of direct modulation of cholesteryl ester cycle enzymes. We found that the activity of ACAT in cell homogenates using exogenous cholesterol as substrate was no less after pretreatment of cells with tyrosylated HDL than with HDL, indicating that treatment of cells with tyrosylated HDL does not suppress the intrinsic activity of this enzyme. Moreover, we previously showed that the decrease in cholesteryl ester formation in cells incubated with tyrosylated HDL was accompanied by an increase in new cholesterol synthesis (Francis et al., 1993). Earlier studies indicated that the absence of ACAT activity or inhibition of ACAT by compound 58-035 is associated with either a decrease or no effect on endogenous cholesterol synthesis (Jamal et al., 1985; Tabas et al., 1986; Cadigan et al., 1988; Salter et al., 1989; Kam et al., 1989). The effect of tyrosylated HDL could also not be attributed to stimulating NCEH, as it did not increase turnover of cholesteryl esters in cells when the re-esterification branch of the cholesteryl ester cycle was blocked by an ACAT inhibitor. Simple amplification of hydrolysis by NCEH without removal of the liberated free cholesterol from the ACAT substrate pool would not be expected to enhance net cholesteryl ester turnover or cholesterol excretion from cells (Brown et al., 1980). Collectively, these results are consistent with the conclusion that tyrosylated HDL inhibits cholesteryl ester synthesis by depleting the substrate pool of free cholesterol available for esterification, and not by directly inhibiting ACAT or stimulating NCEH.

This study does not address the actual processes involved in diverting cholesterol from the cholesteryl ester cycle to efflux-accessible pools or the cellular localization of these pools. Previous studies have shown that the interaction of HDL with cholesterol-loaded cells increases the accessibility of biosynthetically-labeled sterols to exogenous cholesterol oxidase, suggesting that HDL stimulates translocation of intracellular sterols to the plasma membrane (Oram et al., 1991; Mendez et al., 1991; Walter et al., 1994; Rogler et al., 1995). Recently, Mendez (1995) reported that inhibitors of Golgi transport reduced HDL-mediated cholesterol efflux, suggesting that a fraction of the cholesterol removed from cells by HDL passes through the Golgi apparatus. Thus, tyrosylation of HDL may improve its ability to stimulate transport of intracellular pools of cholesterol to the plasma membrane. Alternatively, the effect of tyrosylated HDL may occur exclusively at the plasma membrane. The lipid composition of the plasma membrane is heterogeneous, and free cholesterol appears to be localized to several kinetically distinct domains (Schroeder et al., 1991; Rothblat et al., 1992). Several investigators have reported that the substrate pool for esterification by ACAT is derived directly from the plasma membrane (Tabas et al., 1988; Mazzone et al., 1995). It is possible that tyrosylated HDL stimulates the lateral movement of cholesterol between different plasma membrane domains or alters the properties of ACAT substrate domains so as to reduce inward flux of cholesterol. Methods are not currently available to distinguish between these possible mechanisms.

It is also unknown how tyrosylation of HDL increases its efficacy in mobilizing cholesterol from the cholesteryl ester cycle. Previous studies have shown that apolipoproteins are the major components of HDL that deplete cells of ACAT-

accessible cholesterol. We previously found that tyrosylation of HDL cross-links HDL apolipoproteins (Francis et al., 1993), raising the possibility that this modification may alter the structural properties of apolipoproteins so as to increase their activity or promote dissociation from the lipoprotein surface. Several lines of evidence, however, argue against these possibilities. First, preliminary experiments indicate that neither the apolipoproteins isolated from tyrosylated HDL nor tyrosylated free apo A-I is more active than nontyrosylated apo A-I in removal of cellular cholesterol whether incubated with cells in their free form or as reconstituted HDL particles. Second, tyrosylated HDL was no more effective than HDL in stimulating efflux of either radiolabeled cholesterol, added to plasma membranes during 2 h pulse incubations, or choline-labeled phospholipids. Since purified HDL apolipoproteins stimulate efflux of these two sources of radiolabeled lipids, alterations of apolipoproteins that increase their cholesterol transport activity would also be expected to enhance pulse-labeled cholesterol and phospholipid efflux. Third, the response time to tyrosylated HDL appears to be too slow to be attributable to more active apolipoproteins, in that the differential effects of tyrosylated HDL compared to HDL on cholesterol transport did not become apparent until after 6−8 h of incubation. These results are consistent with the possibility that the active components in tyrosylated HDL may need to accumulate in cells or be transported to specific cellular compartments before eliciting their stimulatory effects.

In addition to modifying apolipoproteins, tyrosylation of HDL increases lipid oxidation products, some of which may alter membrane lipid domains or elicit signals that modulate cholesterol trafficking. These components are likely to be different from the oxidation products generated in copper ion-oxidized HDL, which has a markedly diminished ability to deplete cells of cholesterol (Nagano et al., 1991; Sakai et al., 1992). We found no differences in the content of major lipid classes between HDL and tyrosylated HDL. The nature of the more subtle changes to protein or lipid components induced by tyrosyl radical oxidation and responsible for this effect is currently being investigated.

Generation of tyrosyl radical by myeloperoxidase represents one physiologically plausible mechanism for the oxidation of lipoproteins *in vivo* (Heinecke et al., 1993). The recent demonstration of high levels of myeloperoxidase, as well as protein-bound dityrosine, in human atherosclerotic lesions (Daugherty et al., 1994; Leeuwenburgh et al., 1996), and that tyrosyl radical can initiate lipid peroxidation in LDL (Savenkova et al., 1994), suggests that tyrosyl radicals may play an important role in the oxidation of lipoproteins in the artery wall. The balance between potentially detrimental effects on LDL and beneficial effects on HDL would depend on the relative concentration of each that is available for modification, and the relative susceptibility of HDL and LDL to oxidation. In this study, we have used a surrogate enzyme, horseradish peroxidase, as a means of generating tyrosyl radical. We have also found that myeloperoxidase isolated from human neutrophils can generate tyrosyl radicals and oxidize HDL to a form that promotes cholesterol excretion more effectively than HDL (G. A. Francis, unpublished results). The possibility that activated human monocytes and neutrophils can oxidize HDL by the myeloperoxidase-dependent generation of tyrosyl radical is currently under investigation.

In summary, this study demonstrates that oxidation by tyrosyl radical enhances the capacity of HDL to mobilize cholesterol from cellular pools that feed into the cholesteryl ester cycle to cellular surface domains where it becomes available for removal by acceptor particles. Mechanisms of intracellular cholesterol transport are in general poorly understood (Liscum & Underwood, 1995). The ability of tyrosylated HDL to promote cholesterol excretion *in vitro* makes it a useful tool for the study of intracellular cholesterol transport and the removal of excess cholesterol from cells. Identification of the events activated by tyrosylated HDL, and the component of tyrosylated HDL responsible for activation, may provide important insights into the molecular events underlying early steps in reverse cholesterol transport, and the ability of HDL to prevent vascular disease. Further understanding of these mechanisms might suggest ways of promoting this pathway to prevent atherosclerosis.

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