Enhanced Cholesterol Efflux by Tyrosyl Radical-oxidized High Density Lipoprotein Is Mediated by Apolipoprotein AI-AII Heterodimers*

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Myeloperoxidase secreted by phagocytes in the artery wall may be a catalyst for lipoprotein oxidation. High density lipoprotein (HDL) oxidized by peroxidase-generated tyrosyl radical has a markedly enhanced ability to deplete cultured cells of cholesterol. We have investigated the structural modifications in tyrosylated HDL responsible for this effect. Spherical reconstituted HDL (rHDL) containing the whole apolipoprotein (apo) fraction of tyrosylated HDL reproduced the ability of intact tyrosylated HDL to enhance cholesterol efflux from cholesterol-loaded human fibroblasts when reconstituted with the whole lipid fraction of either HDL or tyrosylated HDL. Free apoAI or apoAII showed no increased capacity to induce cholesterol efflux from cholesterol-loaded fibroblasts following oxidation by tyrosyl radical, either in their lipid-free forms or in rHDL. The product of oxidation of a mixture of apoAI and apoAII (1:1 molar ratio) by tyrosyl radical, however, reproduced the enhanced ability of tyrosylated HDL to induce cholesterol efflux when reconstituted with the whole lipid fraction of HDL. HDL containing only apoAI or apoAII showed no enhanced ability to promote cholesterol efflux following oxidation by tyrosyl radical, whereas HDL containing both apoAI and apoAII did. rHDL containing apoAI-apoAIImonomer and apoAI-(apoAII)2 heterodimers showed a markedly increased ability to prevent the accumulation of LDL-derived cholesterol mass by sterol-depleted fibroblasts compared with other apolipoprotein species of tyrosylated HDL. These results indicate a novel product of HDL oxidation, apoAI-apoAII heterodimers, with a markedly enhanced capacity to deplete cells of the regulatory pool of free cholesterol and total cholesterol mass. The recent observation of tyrosyl radical-oxidized LDL in vivo suggests that a similar modification of HDL would significantly enhance its ability to deplete peripheral cells of cholesterol in the first step of reverse cholesterol transport.

Lipoprotein oxidation is felt to be pivotal for the formation of macrophage foam cells in the developing atherosclerotic lesion (1). Low density lipoprotein (LDL) particles containing modifications consistent with in vivo oxidation have been isolated from human atheromatous lesions (2–5). Numerous potential mechanisms for the oxidation of lipoproteins in vivo have been proposed (6). One such pathway is the release of myeloperoxidase and oxygen radicals by activated phagocytes at sites of inflammation, such as the intima of the atherosclerotic artery (7). Myeloperoxidase employs hydrogen peroxide to catalyze the oxidation of substrates, including chloride ion and L-tyrosine, in the extracellular fluid (8). The likelihood of oxidation of lipoproteins by myeloperoxidase-generated tyrosyl radical in vivo is suggested by the presence of the active enzyme (9), micromolar concentrations of L-tyrosine (10), and tyrosyl radical-modified LDL (5) in the artery wall.

High density lipoprotein (HDL) is felt to protect against atherosclerosis in part by stimulating the removal of excess cholesterol from peripheral tissues, the initial step in a pathway referred to as reverse cholesterol transport (11). Numerous reports have suggested that HDL is at least or more susceptible to oxidation than LDL in vitro (12–15); however, oxidatively modified HDL isolated from in vivo sources has not been reported. We have previously reported that HDL can be readily oxidized by peroxidase-generated tyrosyl radical (16). In striking contrast to other reports of HDL oxidation (reviewed in 17), tyrosyl radical-oxidized or “tyrosylated” HDL has a markedly enhanced ability to promote the removal of cholesterol from cultured fibroblasts and macrophage foam cells (16). This effect is due specifically to an increased ability of tyrosylated HDL to promote the translocation of cellular cholesterol from sites available for esterification by acyl-CoA:cholesterol acyltransferase (the “ACAT-accessible” pool), to sites on the cell surface available for off-loading to acceptor particles (the “efflux-accessible” pool) (18). No differences are seen in the ability of HDL and tyrosylated HDL to promote diffusional efflux or to act as passive acceptors of cholesterol or phospholipids from the surface of cholesterol-loaded cells, nor in their ability to directly inhibit or stimulate cholesterol ester cycle enzymes (18). These results suggest that a mechanism activated by tyrosylated HDL exists that can deplete cellular cholesterol esters and the cholesterol substrate pool for esterification by ACAT prior to the depletion of cholesterol from the plasma membrane.

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1 The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; rHDL, reconstituted HDL; apo, apolipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; DTPA, diethylenetriaminepentaacetic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, essentially fatty acid-free bovine serum albumin; PC, phosphatidylcholine; SFM, serum-free medium; LCAT, lecithin-cholesterol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
existence of such a pathway has important implications for the development of pharmaceutical agents that could mimic this effect for the treatment or prevention of atherosclerosis.

The efficient depletion by HDL of the regulatory pool of cellular cholesterol available for esterification is felt to depend on the presence of intact HDL apolipoproteins (19, 20). This observation plus our finding that tyrosylated HDL shows a markedly increased ability to deplete this cellular cholesterol pool suggested that oxidative modifications to HDL apolipoproteins might be responsible for this enhanced effect. Tyrosyl radical oxidation of HDL results in the formation of cross-linked oligomers of its apoproteins, which by molecular weight and immunoblotting include apoAI-apoAII monomer and apoAI- (apoAII) heterodimers, apoAI dimers, and apoAI trimers (16). This form of oxidation also generates moderate levels of lipid peroxidation products on HDL, but no significant change in the percent content of major lipid classes compared with HDL (18).

In the current study, we have isolated the whole lipid and protein fractions of tyrosylated HDL to determine which of these modifications is responsible for its enhanced effect. Our results show that tyrosyl radical modification of HDL apolipoproteins mediates the enhanced cholesterol efflux by these particles, and that the specific modification responsible for this effect is the formation of apoAI-apoAII heterodimers.

**Experimental Procedures**

**Materials**—Cholesterol, 1-tyrosine, hydrogen peroxide (30%, ACS grade), diethylenetriaminepentaacetic acid (DTPA; free acid form), essentially fatty acid-free bovine serum albumin (BSA), and CAPS were purchased from Sigma. Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids. [1-14C]Oleate (55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Tissue culture medium was prepared by extraction of liver from rats housed under a 12:12 light-dark cycle, using a modification of the method of Bligh and Dyer (27). Some stock lipid fractions were prepared by extraction with ether:acetone (1:3, v/v) and purified apoAI and apoAII linked oligomers of its apolipoproteins, which by molecular weight and immunoblotting include apoAI-apoAII monomer and apoAI- (apoAII) heterodimers, apoAI dimers, and apoAI trimers (16).

To assess the ability of HDL, free apoproteins, and rHDL particles to deplete cellular free cholesterol available for esterification, cholesterol-loaded cells were incubated for 16 h in DMEM containing 2 mg/ml BSA and 30 μg/ml cholesterol (added from a 10 μg/ml solution in ethanol). To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS-BSA and incubated for another 24 h in DMEM containing 1 mg/ml BSA (DMEM-BSA). Cells used for cholesterol mass determination were initially depleted of cholesterol by growth from 60% to full confluence in DMEM containing 10% liver-derived-deficient serum.

**Cell Culture**—Normal human skin fibroblasts were cultured in DMEM containing 10% fetal bovine serum as described (34). Fibroblasts were plated at 15,000 cells/16-mm well or 70,000 cells/35-mm well and grown at 37 °C and 5% CO2 to confluence (about 7 days). To cholesterol-load the cells, confluent cultures were washed twice with PBS containing 2 mg/ml BSA (PBS-BSA) and incubated for 24 h in DMEM containing 2 mg/ml BSA and 30 μg/ml cholesterol (added from a 10 μg/ml solution in ethanol). To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS-BSA and incubated for another 24 h in DMEM containing 1 mg/ml BSA (DMEM-BSA). Cells used for cholesterol mass determination were initially depleted of cholesterol by growth from 60% to full confluence in DMEM containing 10% liver-derived-deficient serum.

**Cell Cholesterol Determination**—To assess the ability of HDL, free apoproteins, and rHDL particles to deplete cellular free cholesterol available for esterification, cholesterol-loaded cells were incubated for 16 h in DMEM-BSA and the indicated additions, washed with PBS, and incubated for 1 h at 37 °C with DMEM containing 9 μg [1-14C]oleate bound to 3 μg BSA (19). Cells were chilled on ice and rinsed twice with ice-cold PBS-BSA, twice with PBS, and stored at −20 °C until extraction. Cell layers were extracted with hexane/isopropanol (3:2, v/v) as described (35). Sterol species were separated by thin layer chromatography on PE SIL G plastic-backed plates (Whatman) developed in hexane/diethyl ether/acetic acid (130:40:1.5 v/v/v). Lipid spots were identified by staining with I2 vapor and comigration with standard. After allowing I2 stain to evaporate, cholesteryl ester spots were identified for determination of radioactive lipid content.

**Other Methods**—Protein content of intact and rHDL particles was determined by the method of Bligh and Dyer (27). Some water-soluble lipid-peroxidation products may be lost during this isolation, however; control experiments showed no difference in the ability of tyrosylated HDL isolated before or after cell filtration to remove these products to induce cholesterol efflux. Lypophilized HDL (10 mg) was mixed with 0.4 ml of water and 5 ml of methanol for 30 min; 2.5 ml of chloroform was added and after 30 min samples were centrifuged at 2000 rpm for 10 min. The supernatant plus two rinses of the residue (2 ml of methanol/chloroform (2:1 v/v) each) was evaporated under nitrogen and the dried extract dissolved in 10 ml of chloroform/methanol/0.1% KCl (8:4:3 v/v/v) (28). The mixture was vortexed and centrifuged at 2000 rpm for 10 min. The upper aqueous phase and any protein at the phase interface were discarded, and the organic phase was evaporated under nitrogen. This step was repeated as necessary (usually three times) until protein precipitate no longer appeared in the supernatant. The modified tyrosyl radical from 1-tyrosine. DTPA (100 μM) was added to 10 mg of apoAI- and apoAII-containing HDL, prepared as described (30). Briefly, HDL was incubated with lipid-free apoAI at a ratio of 1:1 (w/w) for 2 h at 25 °C. The mixture was then adjusted to d = 1.2 g/ml with KBr, centrifuged at 99,000 rpm for 18 h at 4 °C to remove free apoproteins, and the top fraction containing AII-HDL dialyzed against phosphate-buffered saline with 1 mM EDTA, pH 7.4 (buffer C). The absence of residual apoAI on the AII-HDL was confirmed by SDS-PAGE.

Reconstituted HDL (rHDL) particles were prepared using the cholate dialysis method (31). Discoidal HDL particles were prepared by removing free cholesterol, and delipidated apoproteins using an initial molar ratio of 80:80:1, PC:cholesterol:sodium cholate:apoproteins. Spherical rHDL were prepared using a molar ratio of total phospholipid in the isolated whole lipid fraction, sodium cholate, and isolated apoproteins of 80:80:1. Cholate was removed by extensive dialysis against 10 mM Tris HCl, 150 mM NaCl, 0.01% EDTA, pH 7.4 (buffer C), following which the density of the dialyzed sample was adjusted to d = 1.2 g/ml with KBr and a final volume of 3 ml, and samples centrifuged at 99,000 rpm for 18 h at 4 °C to isolate HDL free of unbound or loosely bound apoproteins. The top 1 ml fractions were collected and dialyzed for another 3 days against buffer C. HDL were stored at 4 °C under argon and used within 2 weeks of preparation. The incorporation of lipids into reconstituted particles was determined enzymatically (32), and the conformation and size of rHDL were assessed by negative stain electron microscopy (33).

Whole lipid fractions of HDL and tyrosylated HDL were obtained by extraction with ether/acetone (1:3, v/v) and purified apoAI and apoAI obtained as described (23). Individual apolipoprotein species of tyrosylated HDL were isolated by separation on a 12% SDS-PAGE gel and eluted with 60 mM Tris, 40 mM CAPS, pH 9.4 using a 30-channel Bio-Rad whole gel eluter (200 mA for 30 min) based on the method of Andersen and Heron (24). Eluted fractions were concentrated and their protein content identified on subsequent SDS-PAGE using a 7–20% acrylamide gradient gel. Fractions enriched in individual tyrosylated HDL apoprotein species were pooled and passed over a 5 × 1.5-cm AG-11 ion retardation column (Bio-Rad) to remove SDS (25). Residual SDS was determined by the method of Hayashi (26) and found to be in the range of 0.1–1 mol/mol of protein.
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**RESULTS**

Structural modifications to HDL induced by tyrosyl radical oxidation, including protein cross-linking and formation of moderate levels of lipid peroxidation products, accompany the marked increase in capacity of these HDL particles to deplete cellular cholesterol (16, 18). In the current studies, we isolated the protein components and total lipid fractions of HDL and tyrosylated HDL to determine which of these modifications is responsible for this enhanced activity.

**Determination of the Active Fraction of Tyrosylated HDL**

The whole apolipoprotein and lipid fractions of HDL and tyrosylated HDL were isolated and incubated with cholesterol-enriched human fibroblasts to determine their ability to deplete the cellular cholesterol pool available for esterification. Our previous demonstration of a direct correlation between depletion of this regulatory, ACAT-accessible pool and cellular cholesteryl ester mass in the absence of a direct inhibitory effect on ACAT indicated the cholesterol esterification assay is an accurate measure of cholesterol efflux capacity by these particles (16, 18).

As seen in Fig. 1A, no increase was seen in the capacity of the isolated total protein fraction of tyrosylated HDL compared with that of HDL to stimulate removal of the pool of free cholesterol available for esterification during a subsequent 1 h incubation with [14C]oleate. In fact, the lipid-free tyrosylated HDL apoprotein fraction showed a diminished capacity to promote efflux from this pool compared with the control HDL apoprotein fraction. To determine whether the apolipoprotein fraction of tyrosylated HDL needs to be in a lipid-bound form to demonstrate its enhanced efflux capacity, HDL and tyrosylated HDL proteins were presented to cholesterol-loaded cells on the surface of both discoidal and spherical rHDL particles. Discoidal rHDL particles containing tyrosylated HDL apoproteins plus commercial phosphatidylcholine and free cholesterol showed a modest but significant increase in ability to deplete ACAT-accessible cholesterol compared with particles containing the HDL protein fraction at concentrations lower than 10 μg of protein/ml (Fig. 1B). Particles generated using the total protein fraction of tyrosylated HDL and the whole lipid fraction of HDL or tyrosylated HDL, however, showed a markedly increased ability to deplete cholesterol available for esterification compared with particles generated with the HDL protein fraction and the whole lipid fraction of either HDL or tyrosylated HDL (Fig. 1C). These results mirror closely our results obtained using this assay with intact HDL and tyrosylated HDL (16), and provide evidence that modification of the apolipoprotein fraction of HDL by tyrosyl radical oxidation is responsible for the enhancement of cholesterol efflux by these particles. The whole lipid fraction of tyrosylated HDL showed no ability to alter cholesterol esterification by particles containing the whole apoprotein fraction of either HDL or tyrosylated HDL. In subsequent experiments, we used the whole lipid fraction of HDL plus the indicated apolipoproteins to generate rHDL for cell incubations.

Reconstituted HDL particles with a neutral lipid core and spheroidal shape have previously been generated using the cholate dialysis method only with subsequent treatment with lecithin:cholesterol acyltransferase (LCAT) (41). The sequential gradient ultracentrifugation method we have used to isolate HDLc depletes HDLc of most of their LCAT activity (42). We typically obtained, however, greater than 20% incorporation of cholesteryl esters and 80% of triglycerides into rHDL particles using cholate dialysis to combine the whole lipid fraction of HDL or tyrosylated HDL and apoprotein fractions, without the subsequent addition of LCAT. This level of incorporation suggested the presence of a neutral lipid core and therefore spherical rHDL particles. This was confirmed by electron microscopy (Fig. 2), which showed spheroidal particles mainly in the 11–33-nm range, equivalent to HDLc to small VLDL-sized parti-

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**Fig. 1. Reduction of cellular cholesterol esterification by the protein and lipid components of tyrosylated HDL.** Human fibroblasts loaded with nonlipoprotein cholesterol were incubated with DMEM containing 1 mg/ml BSA (SFM) plus the indicated concentration of apolipoproteins of HDL (○) or tyrosylated HDL (△) in either their lipid-free form (A), in rHDL complexed with PC and free cholesterol (B), or in rHDL complexed with the whole lipid fraction of HDL (open symbols) or tyrosylated HDL (closed symbols) (C) for 16 h. Cells were then washed, incubated for 1 h with [14C]oleate, and cellular cholesteryl [14C]oleate was measured as described under “Experimental Procedures.” Results are the mean ± S.D. of four determinations expressed as percentage of picomoles of [14C]oleate incorporated into cholesteryl ester/mg of cell protein/h in cells treated with SFM alone, and are representative of three separate experiments. Error bars not shown are within the symbol dimensions. Results for tyrosylated HDL apoproteins were significantly less than HDL apoproteins in A (p < 0.01 at all concentrations), but significantly greater for rHDL containing tyrosylated HDL apoproteins in B (p < 0.05 at all concentrations except 10 μg/ml) and C (p < 0.001 at all concentrations).
cles (43). Reconstituted HDL generated in the absence of neutral lipids had the expected discoidal appearance (11–17 nm) (Fig. 2, inset).

Determination of the Active Complex in the Tyrosylated Apolipoprotein Fraction—To determine the individual apolipoproteins required during tyrosyl radical oxidation to generate HDL particles with enhanced efflux capacity, we first produced rHDL containing control or tyrosylated apoAI or apoAII alone. As with the whole HDL and tyrosylated HDL apoprotein fractions, initial experiments showed no enhanced activity of lipid-free apoAI or apoAII oxidized by tyrosyl radical compared with control apoproteins (data not shown). SDS-PAGE of spherical rHDL generated using control or tyrosylated apoAI or apoAII and the whole lipid fraction of HDL indicated the presence of monomers and homooligomers of apoAI or apoAII (data not shown). Cell incubations with these rHDL also failed to show an enhanced capacity of the tyrosylated apoprotein-containing particles to deplete cells of cholesterol available for esterification (Fig. 3).

SDS-PAGE and immunoblot analysis had previously indicated the presence in tyrosylated HDL of cross-linked heterodimers containing both apoAI and apoAII, in addition to apoAI dimers and trimers (16). The lack of enhanced activity in particles containing homoooligomeric species of apoAI and apoAII suggested that these heterooligomeric species might be responsible for the enhanced efflux capacity of tyrosylated HDL. To test this hypothesis, rHDL containing various combinations of control and tyrosyl radical-oxidized apoAI or apoAII were generated and their ability to promote the removal of cellular cholesterol available for esterification assessed. As seen in Fig. 4, only rHDL containing apoAI and apoAII oxidized by tyrosyl radical in the presence of each other (Tyr(AI+AII)) reproduced the ability of intact tyrosylated HDL to promote cholesterol removal from cultured fibroblasts. Reconstituted HDL particles containing control apoAI or apoAII or the tyrosylated forms of these proteins, oxidized separately from each other (e.g. TyrAI/TyrAII), all had similar abilities to deplete ACAT-accessible cholesterol, which was midway between the activity of intact HDL and tyrosylated HDL. We typically find that, even after ultracentrifugation to remove loosely bound apoproteins from their surface, rHDL have enhanced efflux capacity compared with intact HDL. The data in Fig. 4, however, clearly showed an enhanced ability of rHDL containing the products of simultaneous oxidation of apoAI and AII by tyrosyl radical to deplete cholesterol available for esterification relative to the other rHDL particles. This strongly suggested that the increased ability of tyrosylated HDL to promote cholesterol excretion from cells is a result of the formation of cross-linked species containing both apoAI and apoAII.

Requirement of Both ApoAI and ApoAII for Generation of Tyrosylated HDL with Enhanced Cholesterol Efflux Capacity—To confirm the requirement of cross-linked apoAI-apoAII species for the enhanced activity of tyrosylated HDL, HDL particles containing apoAI only (LpAI) and particles containing both apoAI and apoAII (LpAI/AII) were isolated by immunoaf-finity chromatography and exposed to tyrosyl radical oxidation. Incubation of cholesterol-loaded fibroblasts with control LpAI and tyrosylated LpAI particles resulted in a similar degree of depletion of the cholesterol substrate pool available for esterification (Fig. 5A). The $K_m$ ($n = 2$ experiments) for half-maximal inhibition of cholesterol esterification was 10.5 and 12.7 μg/ml for LpAI, and 12.0 and 16.3 μg/ml for tyrosylated LpAI. Tyrosyl radical oxidation of LpAI/AII, however, resulted in particles

**Fig. 2.** Electron micrographs of rHDL. Negative staining electron micrograph of rHDL, prepared using a molar ratio of total phospholipid in the isolated whole lipid fraction of HDL, sodium cholate, and the isolated apoprotein fraction of tyrosylated HDL of 80:80:1. Inset shows rHDL prepared using a molar ratio of commercial egg PC, free cholesterol, sodium cholate, and the isolated whole apoprotein fraction of tyrosylated HDL of 80:80:1. Magnification, ×192,000. Bar represents 100 nm.

**Fig. 3.** Reduction in cholesterol esterification by reconstituted HDL containing control or tyrosylated apoAI or apoAII. Cholesterol-loaded fibroblasts were incubated with the indicated concentrations of rHDL prepared using control apoproteins (○) or apoproteins oxidized by tyrosyl radical (●) plus the whole lipid fraction of HDL, as described in Fig. 1. A, apoAI; B, apoAII. Results are expressed as in Fig. 1.
with enhanced efflux capacity as compared with unoxidized LpAI/AII (Fig. 5B), LpAI, or tyrosylated LpAI. Although the $V_{\text{max}}$ for inhibition of cholesterol esterification by control and tyrosylated LpAI/AII appeared to be the same, the $K_m$ for half-maximal inhibition of this reaction in two experiments was markedly lower for tyrosylated LpAI/AII particles, 2.2 and 2.4 $\mu$g/ml, compared with 15.8 and 20.0 $\mu$g/ml for control LpAI/ AII. The requirement for both apoAI and apoAII during the oxidation by tyrosyl radical for enhanced activity was further confirmed using particles containing apoAII only, oxidation of which had no effect on the particles ability to deplete fibroblasts of cholesterol available for esterification (data not shown).

Isolation and Activity of Individual Apolipoprotein Species in Tyrosylated HDL—To attempt to determine the stoichiometry of the active species of cross-linked apoAI-apoAII necessary for the enhanced biologic activity of tyrosylated HDL, we separated the monomeric and cross-linked apoprotein species in the whole delipidated protein fraction of tyrosylated HDL by preparative electrophoresis, and isolated individual protein bands by whole gel elution (24). The enrichment of pooled fractions in particular tyrosylated HDL apoprotein species was confirmed by SDS-PAGE (Fig. 6A), and the apoprotein contents of each of the bands was probed in immunoblots with anti-apoAI and anti-apoAII (Fig. 6, B and C). In addition to bands staining for either apoAI or apoAII alone, bands of ~45.8 and ~36.9 kDa consistent with apoAI-(apoAII)$_2$ and apoAI-apoAII$_{\text{monomer}}$ heterodimers staining with both anti-apoAI and anti-apoAII were identified. A series of higher molecular mass bands (~97 kDa) apparently staining for both apoAI and apoAII were seen in the parent tyrosylated HDL lanes (Tyr, Fig. 6, B and C); however, no significant staining of these bands was seen in the apoAII immunoblot of this isolated fraction (Fig. 6C, lane 7). The reason for this discrepancy is unclear, but has been seen on repeat immunoblots of the isolated apoprotein fractions.

To investigate the ability of the individual tyrosylated HDL apoprotein species to promote cholesterol efflux, we exposed human fibroblasts with up-regulated LDL receptor activity to both LDL and rHDL containing each of the isolated protein species for 24 h, and measured cholesterol mass accumulated by the cells. Under these conditions, LDL delivers cholesterol to cells by receptor-mediated endocytosis (44), whereas the rHDL would be expected to remove excess free cholesterol from a pool available for esterification by ACAT and deliver it to pools in the plasma membrane available for removal to acceptor particles in the medium (16, 18). We have previously shown that net depletion of cellular cholesterol mass by tyrosylated HDL cor-

![Fig. 4. Reduction in cholesterol esterification by reconstituted HDL containing control or tyrosylated apoAI and apoAII. Fibroblasts were cholesterol-loaded and incubated with rHDL containing the indicated concentration of control or tyrosylated apoAI and apoAII (molar ratio of proteins 1:1, apoAI/apoAII$_{\text{monomer}}$), prepared using a molar ratio of lipid extract phospholipid:total protein of 80:1. ApoAI and apoAII were exposed to tyrosyl radical oxidation individually, (e.g. TyrAI/TyrAII, ○), or combined and oxidized together by tyrosyl radical (TyrAI+TyrAII, ▲), prior to reconstitution with the whole lipid fraction of HDL. HDL (○) and tyrosylated HDL (TyrHDL, ▲) are shown in dashed lines and indicate the reduction of cholesterol esterification by intact HDL particles. Results are expressed as in Fig. 1. Cholesterol esterification by cells incubated with the Tyr(AI+AI) sample was significantly less than all other rHDL samples containing various combinations of control or oxidized apoAI and apoAII ($p < 0.001$ at all concentrations).](image)

![Fig. 5. Cellular cholesterol esterification following exposure to control and tyrosylated LpAI and LpAI/AII. HDL was separated into apoAI-only containing particles (LpAI, A) or particles containing both apoAI and apoAII (LpAI/ AII, B) by immunoaffinity chromatography prior to oxidation by tyrosyl radical. The ability of control particles (C) and particles exposed to tyrosyl radical ($\Delta$) to deplete cholesterol available for esterification are shown. Results expressed as in Fig. 1. New cholesteryl ester formation following exposure of cells to tyrosylated LpAI/AII was significantly lower than LpAI/AII at all concentrations tested ($p < 0.01$).](image)
relates directly with depletion of radiolabeled cholesterol in cells, and movement of this label and cholesterol mass into the medium (18). Following the incubation with LDL and 20 μg/ml rHDL protein, cells exposed to rHDL containing either the apoAI-apoAII monomer or apoAI-apoAII heterodimers showed a 50% decrease in free cholesterol accumulation compared with cells incubated with LDL alone (Fig. 7). This striking ability of the heterodimer-containing rHDLs to prevent cholesterol accumulation from LDL exceeded that of intact tyrosylated HDL, in which the effect of the apoAI-apoAII heterodimers would be diluted by the other apoprotein species. No loss of cell protein or alteration of cell morphology was seen in cells treated with rHDL containing the heterodimers. Reconstituted HDL containing the apoAI dimers or trimers showed modest increases in ability to prevent cholesterol accumulation compared with control HDL, but these did not reach statistical significance, and could not account for the enhanced activity seen in intact tyrosylated HDL.

DISCUSSION

In the current study, we have shown that the enhanced ability of HDL oxidized by tyrosyl radical to deplete cellular cholesterol is a result of modification of its protein components, and that formation of apolipoprotein AI-AII heterodimers is responsible for this effect. HDL and spherical rHDL particles containing cross-linked heterodimers of apoAI and apoAII showed a markedly increased ability to deplete cells of the regulatory pool of cholesterol available for activation of ACAT, as compared with particles with non-cross-linked apoproteins or particles containing control or oxidized apoAI or apoAII alone. Cholesterol-depleted cells also showed a markedly decreased net accumulation of free cholesterol from LDL when coincubated with rHDL containing the apoAI-apoAII cross-linked heterodimers. Enhanced cholesterol efflux by tyrosyl radical-oxidized HDL could not be attributed to modification of its lipid components, as reconstitution using the whole lipid fraction from these particles did not alter the activity of HDL or tyrosylated HDL apoproteins relative to particles reconstituted using the whole lipid fraction of HDL.

Several lines of evidence suggest that the formation of apoAI-apoAII heterodimers is responsible for the enhanced ability of this oxidized form of HDL to deplete cellular cholesterol. First, tyrosyl radical oxidation of free apoAI or apoAII alone failed to increase their capacity in either lipid-free or lipid-bound forms to deplete ACAT-accessible cholesterol from cholesterol-loaded cells. Cooxidation of apoAI and apoAII by tyrosyl radical, however, resulted in a product that in rHDL reproduced or bettered the ability of intact tyrosylated HDL to promote the removal of this regulatory pool of cholesterol. Second, tyrosyl radical oxidation of intact HDL particles containing only apoAI (LpAI) or apoAII failed to enhance their capacity to promote cholesterol efflux. In contrast, oxidation by tyrosyl radical of particles containing both apoAI and apoAII (LpAI/AII) significantly enhanced their capacity to deplete ACAT-accessible cholesterol. Finally, isolation of the major cross-linked and non-cross-linked apoprotein species of tyrosylated HDL allowed us to test their individual abilities to deplete cellular cholesterol directly. Reconstituted HDL containing both the apoAI-apoAII monomer and apoAI-apoAII heterodimers showed a dramatically increased ability to prevent net accumulation of LDL-derived cholesterol by cholesterol-depleted fibroblasts as compared with intact tyrosylated HDL or rHDL containing other tyrosylated HDL apoprotein species. These data, taken together, indicate that cross-linked species containing apoAI and apoAII formed during tyrosyl radical oxidation are capable of enhancing the depletion of ACAT-accessible cholesterol and of cellular cholesterol mass when presented to cells on the surface of spherical HDL particles.

The delipidated whole apoprotein fraction of tyrosylated

**FIG. 6.** Preparative electrophoresis and immunoblot analysis of individual apolipoprotein species of tyrosylated HDL. 7–20% SDS-PAGE using silver stain (A), anti-apoAI immunoblot (B), and anti-apoAII immunoblot (C) of HDL (Ctl), tyrosylated HDL (Tyr), and the individual apolipoprotein species of tyrosylated HDL isolated by preparative electrophoresis and whole gel elution as described under “Experimental Procedures.” A, 5 μg of protein/lane; B and C, 3 μg of protein/lane. Molecular mass standards and apoproteins AI and AII are indicated.

**FIG. 7.** Free cholesterol mass in fibroblasts incubated with LDL and rHDL containing individual tyrosylated HDL apoprotein species. Human fibroblasts were grown from 60% to full confluence in 10% lipoprotein-deficient serum to up-regulate LDL receptors. Cells were then incubated for 24 h in SFM containing LDL (50 μg of protein/ml) and 20 μg of protein/ml of HDL or rHDL containing the indicated tyrosylated apoprotein species isolated as in Fig. 6. HDL and TyrHDL represent cholesterol accumulation from LDL in cells treated with intact HDL and tyrosylated HDL. Polymer represents apoprotein fraction 7 as indicated in Fig. 6. Results are mean ± S.D. of four determinations. *, p < 0.05; **, p < 0.001 compared with cells incubated with HDL.
HDL showed no increased ability to deplete cells of ACAT-accessible cholesterol relative to lipid-free apoproteins of HDL. A modest but significant increase in this activity was seen when cell incubations were performed using discoidal rHDL containing the tyrosylated HDL apoprotein fraction. A striking increase in activity was seen, however, when the protein fraction of tyrosylated HDL or the product of cooxidation of apoAI and apoAII was reconstituted with the whole lipid fraction of HDL to generate spherical rHDL particles. These results suggest that the enhanced activity of tyrosylated HDL apoproteins, and specifically the apoAI-apoAII heterodimers, requires a lipid-bound state and a particular conformation present when the proteins are bound to the surface of a sphere, but not (or to a less extent) when bound to lipid discs. A conformation of apoAI bound to the surface of spherical rHDL (generated by LCAT treatment of discoidal rHDL) distinct from that seen on discoidal rHDL has been reported by Jonas and colleagues (45). Although the crystal structure of a truncated human apoAI has recently been reported (46), little is known about the tertiary structures of the full-length apoproteins in these different lipid-bound states. Interestingly, our results indicate that the initial cross-linking of apoAI and apoAII by tyrosyl radical to form active heterodimers is not dependent on a lipid-bound conformation of the apoproteins, as cooxidation of the lipid-free proteins produced a complex with enhanced activity when re-lipidated in spherical rHDL.

The requirement for lipid-bound proteins in order to reproduce the enhanced activity of intact tyrosylated HDL suggests that this effect is unlikely related to the dissociation of the modified apoproteins off the lipoprotein surface. Tyrosylated HDL enhances the mobilization of cellular cholesterol from the regulatory pool available for esterification by ACAT and inhibition of HMG-CoA reductase (as evidenced by increased new cholesterol synthesis following exposure to tyrosylated HDL), to sites on the cell surface available for removal by acceptor particles (16, 18). The mechanism by which a lipid-bound heterodimer of apoAI and apoAII achieves this effect is unknown. One possible explanation is that cross-linking of apoAI to the more lipophilic LpAII-apoAII serves to tether apoAI to the HDL surface, while still allowing the apoAI interaction with cell surface proteins or domains that initiates a pathway for the delivery of ACAT-accessible cholesterol to efflux-accessible sites. The tethering of apoAI to the particle surface by apoAII may serve to accentuate or prolong the apolipoprotein-cell interaction over and above that seen with lipid-free or lipid-poor apoAI dissociated from the lipoprotein surface.

Previous studies have suggested an impaired ability of HDL containing both apoAI and apoAII to promote cholesterol efflux relative to apoAI-only HDL, whereas others have found no significant difference in efflux by these particles (reviewed in Refs. 11 and 47). Our results do not confirm or refute a particular ability of native LpAI/AII particles to promote cholesterol efflux, but do indicate that oxidation of these particles by tyrosyl radical, if occurring in vivo, could significantly enhance their ability to prevent cholesterol deposition or to remove excess cholesterol deposits from peripheral cells. Particles in the HDL3 density range used in our studies are reported to contain apoAI and apoAII in molar ratios of 1:1 to 2:1 (48), in keeping with our finding of cross-linked oligomers of apoAI as well as heterodimers of apoAI and apoAII induced by tyrosyl radical oxidation. The lack of an increase in size in tyrosylated HDL on nondenaturing gradient gel electrophoresis (16) suggests that the cross-linking of HDL apoproteins is an intraparticle rather than interparticle phenomenon.

An unexpected finding in our studies was the presence, based on molecular weight by non-reducing SDS-PAGE and immunoblot analysis, of a cross-linked species containing apoAI and monomeric apoAII. No monomeric apoAII was seen on non-reducing SDS-PAGE of our control HDL, although some may have been generated during tyrosyl radical oxidation by an unknown mechanism. ApoAII is felt to exist only as a dimer in human plasma; however, monomeric apoAII is apparently present in rapidly isolated fresh human plasma (49). This suggests that oxidation of HDL by tyrosyl radical in vivo could potentially result in formation of apoAI-apoAII{	extsubscript{monomer}} as well as apoAI-(apoAII){\textsubscript{2}} heterodimers.

Unlike LDL, oxidized HDL from an in vivo source has not previously been described, although numerous investigators have reported an increased susceptibility of HDL to oxidation as compared with LDL (12–15). We have found ultraconsistently isolated HDL to be more susceptible to tyrosyl radical oxidation than LDL. We have also found recently that activated human monocytes and neutrophils can oxidize HDL by the myeloperoxidase-dependent generation of tyrosyl radical (50). This phagocyte-modified HDL exhibits a pattern of apoprotein cross-linking and enhanced cholesterol efflux capacity identical to that seen in tyrosylated HDL generated in vitro. These results suggest that if HDL is susceptible to oxidation by myeloperoxidase-generated tyrosyl radical in vivo, this process may increase the ability of HDL to prevent the accumulation of excess cholesterol by artery wall cells.

A pattern of cross-linking of HDL apoproteins like that induced by tyrosyl radical (including apparent apoAI-apoAII heterodimers) is indicated in previous reports using manganese, copper, cigarette smoke, or aldehydes to oxidize HDL particles (51–54). We have found that incubation of HDL with the cross-linking agent tetranitromethane is indicated in previous reports using manganese, copper, cigarette smoke, or aldehydes to oxidize HDL particles (51–54). We have found that incubation of HDL with the cross-linking agent tetranitromethane generates a similar pattern. The nature of the cross-linking induced by tyrosyl radical, however, appears to be unique, in that cholesterol efflux is reported to be impaired in all other studies of oxidized HDL where this has been examined (reviewed in Ref. 17). Brinton et al. (34) found a diminished capacity of tetranitromethane-cross-linked HDL to promote cholesterol efflux, and we have recently confirmed this. The site(s) of cross-linking of apoAI and apoAII by tyrosyl radical, and the ability of lipid-free apoproteins from other forms of oxidized HDL to promote cholesterol efflux when reconstituted with the non-oxidized whole lipid fraction of HDL are currently being investigated in our laboratory.

In summary, we have described a novel oxidation product of HDL, apoAI-apoAII heterodimers, with a powerful ability to enhance the removal of cholesterol from cultured cells. The ability of tyrosylated HDL and apolipoprotein AI-AII heterodimers generated by tyrosyl radical oxidation to enhance cholesterol excretion in vitro makes them excellent tools for the study of intracellular cholesterol transport and the removal of excess cholesterol from cells. Identification of the cellular pathways activated by these agents may provide important insights into the molecular events underlying early steps in reverse cholesterol transport, and the ability of HDL to prevent vascular disease.

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