High density lipoprotein subfractions: isolation, composition, and their duplicitous role in oxidation

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Abstract **The plasma HDLs represent a major class of cholesterol-transporting lipoprotein that can be divided into two distinct subfractions, HDL_2 and HDL_3, by ultracentrifugation. Existing methods for the subfractionation of HDL requires lengthy ultracentrifugations, making them unappealing for large-scale studies. We describe a method that subfractionates HDL from plasma in only 6 h, representing a substantial decrease in total isolation time. The subfractions so isolated were assessed for a variety of lipid and protein components, in addition to their susceptibility to oxidation, both alone and in combination with VLDL and LDL. We report for the first time a prooxidant role for HDL during VLDL oxidation, in which HDL donates preformed hydroperoxides to VLDL in a cholesteryl ester transfer protein (CETP)-dependent process. Examination of the participation of HDL in LDL oxidation has reinforced its classic role as a potent antioxidant. Furthermore, we have also implicated the second major HDL-associated enzyme, LCAT, in these processes, whereby it acts as a potent prooxidant during VLDL oxidation but as an antioxidant during LDL oxidation. Thus, we have identified a potentially duplicitous role for HDL in the pathogenesis of atherosclerosis, attributable to both CETP and LCAT.—**McPherson, P. A. C., I. S. Young, B. McKibben, and J. McEneny. **High density lipoprotein subfractions: isolation, composition, and their duplicitous role in oxidation.** ***J. Lipid Res.*** **2007.** 48: **86–95.**

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High density lipoproteins are a heterogeneous group of particles whose principal physiological role is that of reverse cholesterol transport, but they also exert important antiinflammatory and antithrombotic effects (1). They can be classified according to their chemical and physical characteristics, including hydrated density (1.063–1.210 kg/l), flotation rate (0–9 Svedbergs), diameter (7–12 nm), and electrophoretic mobility [mainly α-migrating, although some nascent HDLs also display preβ mobility, with the latter particles containing only apolipoprotein A-I (apoA-I)]. Analysis of material found in the 1.063–1.210 kg/l density interval by moving-boundary ultracentrifugation reveals a bimodal distribution of particles exhibiting a Schlieren pattern: an effect attributable to a change in the index of refraction of a light beam, caused by differences in particle concentration. This provides evidence for the subdivision of HDL into at least two distinct subfractions: HDL_2 and HDL_3. Further studies of these subfractions reveal that the larger and less dense particle (HDL_2) is formed by the action of the enzyme LCAT upon the smaller, denser particle (HDL_3). LCAT is also involved in the formation of the HDL_3 subfraction, by rendering it largely cholesteryl ester-replete. These and additional observations relating to HDL's physiological roles have encouraged investigation into HDL and its subfractions, especially in relation to their antiatherogenic properties. A variety of established techniques have been adapted to subfractionate HDL, including precipitation (2, 3), electrophoresis (4, 5), affinity chromatography (6), and ultracentrifugation (7–14). The last of these methods, ultracentrifugation, is by far the most routinely used means for subfractionating HDL, with numerous protocols being published. However, many of these methods depend upon long periods of ultracentrifugation to maximize the recovery of HDL and its components, a factor that paradoxically reduces the recovery of apolipoprotein (15). Recently, a rapid method for the subfractionation of HDL was proposed that yields good HDL recovery; however, it is disadvantaged by the relatively small volumes of HDL_2 and HDL_3 isolated (16).

A number of additional antiatherogenic properties associated with HDL's chemical composition have been described, mainly with regard to the prevention of oxidative modification of LDL, a key stage in atherogenesis (17). One such component of HDL displaying antioxidant properties is apoA-I, an amphipathic polypeptide

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Abbreviations: apo[a], apolipoprotein [a]; apoA-I, apolipoprotein A-I; CETP, cholesteryl ester transfer protein; cHDL, crude high density lipoprotein; $t_{1/2max}$, time at half maximum (an equivalent of lag time).

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tightly associated with HDL. In environments with a high oxidation potential, the methionine residues of apoA-I are preferentially oxidized, reducing the availability of superoxide ($O^{\cdot}_2{}^{-}$) for LDL oxidation (18). These native antioxidant properties of HDL are supplemented by at least two HDL-associated proteins: paraoxonase and transferrin. Paraoxonase (aryldialkylphosphatase; EC 3.1.8.1) is a calcium-dependent arylesterase that has been found to associate with the apoA-I component of HDL; it mediates the enzymic hydrolysis of potentially oxidized fatty acids in LDL (19, 20). Transferrin, an iron binding protein, operates in a slightly different manner by reducing the availability of redox reactive iron, thereby reducing the oxidative modification of LDL in the intima (21).

In addition to reverse cholesterol transport and the prevention of LDL oxidative modification, HDL and its subfractions exert a number of pleiotropic effects on the vessel wall, including prevention of monocyte chemotaxis, downregulation of cell adhesion molecules, and inhibition of platelet aggregation. The downregulation of cell adhesion molecules, such as intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1, by HDL has received particular attention (22–24), and it appears that HDL may in some way prevent the transcription of cell adhesion molecule DNA, possibly by inhibiting the activation of sphingosine kinase by tumor necrosis factor-α (25).

We describe a method that can subfractionate HDL into HDL_2 and HDL_3 from plasma in only 6 h, a significantly shorter isolation time than with other reported methods (26). This procedure provided sufficient volumes of each subfraction to enable their characterization in terms of lipid and protein content, in addition to an examination of the in vitro antioxidant potential of HDL_2 and HDL_3 themselves, and also in combination with VLDL or LDL. We describe a prooxidant role for HDL (and its subfractions) when VLDL is oxidized in their presence, a process that may be attributable in part to the action of cholesteryl ester transfer protein (CETP). When LDL is oxidized in the presence of HDL and its subfractions, the classic antioxidant effect of HDL is observed. We have also demonstrated that the second major HDL-associated enzyme, LCAT, exhibits a prooxidant effect during VLDL oxidation but, conversely, an antioxidant effect during LDL oxidation.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and obtained from Sigma Chemical Co. (Poole, Dorset, UK) unless stated otherwise.

Plasma separation

Fasting peripheral venous blood samples were collected from healthy normolipidemic laboratory personnel by standard venipuncture of a prominent vein in the antecubital fossa. Collected blood was transferred into K_3-EDTA tubes (Vacuette) and subjected to centrifugation at 1,100 g_{max} for 10 min in a Beckman J-6B centrifuge. The plasma was recovered, pooled, and stored as 2 ml aliquots at −75°C until required.

Preparation of density solutions

Solutions with the following densities were prepared for the isolation and subfractionation of HDL: 1.006, 1.063, 1.125, and 1.210 kg/l. Initially, a solution of normal saline (d = 1.006 kg/l) was prepared by the addition of 11.42 g of NaCl to exactly 1 liter of water. From this, solutions of the desired density were prepared by the addition of solid KBr, according to masses calculated by the formula of Radding and Steinberg (27).

$$M_{KBr} = \frac{V(d_2 - d_1)}{(1 - \bar{v}_5)d_2}$$

where M_{KBr} = mass of KBr, V = volume to be adjusted, d_1 = density of the first solution at 5°C, d_2 = density of the second solution at 5°C, and $\bar{v}_5$ = partial specific volume of KBr at 5°C. The densities of each solution were verified by measurement on a Digital Density Meter (DMA 35; Paar Scientific). In addition, all solutions used for ultracentrifugation were spiked with EDTA (0.1%, w/v, final concentration) to minimize the autooxidation of lipoproteins during ultracentrifugation.

HDL isolation

To facilitate the subfractionation of HDL, crude high density lipoprotein (cHDL) was isolated by a rapid sedimentation ultracentrifugation method, an adaptation of the method of Chung et al. (26). The background density of 1.2 ml of plasma in a 3 ml ultracentrifuge tube (Polyallomer Bell-top; Beckman) was adjusted to d = 1.063 kg/l by the addition of solid KBr. The density-adjusted plasma was then gently overlaid with an aqueous solution of KBr (d = 1.063 kg/l), and the ultracentrifuge tube was sealed using a Beckman Tube Topper Sealer. Ultracentrifugation was performed in a Beckman Optima MAX-E Table Top Ultracentrifuge using a Beckman fixed-angle rotor (TL100.3) for 120 min at 541,000 g_{max} and 4°C. Upon completion of ultracentrifugation, the supernatant (VLDL and LDL) was removed by tube slicing (Beckman Tube Slicer) at 1.2 cm and aspirated in a volume of 1.8 ml. The remaining infranatant corresponds to cHDL (HDL and plasma proteins), and this was recovered in a volume of 1.2 ml.

Before subfractionation, the purity of the isolated cHDL was confirmed by lipoprotein agarose gel electrophoresis using a commercially available kit (Midigel; Biomidi, Toulouse, France). Electrophoresis was performed according to the manufacturer's instructions.

HDL subfractionation

The subfractionation of cHDL into HDL_2 and HDL_3 was achieved by two sequential rapid flotation ultracentrifugation steps. cHDL was density-adjusted by the addition of solid KBr to the previous infranatant, giving a final density of 1.125 kg/l. This was then overlaid by a solution of identical density and ultracentrifuged for 120 min at 541,000 g_{max} and 4°C. HDL_2 was recovered by tube slicing at 1.8 cm and aspirated as supernatant (volume, 0.8 ml), with the infranatant containing crude HDL_3 (HDL_3 and plasma proteins; volume, 2.2 ml). The density of the $cHDL_3$ isolate was adjusted to 1.210 kg/l, and the isolate was overlaid by a KBr solution of identical density and subjected to ultracentrifugation for 120 min at 541,000 g_{max} and 4°C. Upon completion of ultracentrifugation, HDL_3 was removed as supernatant by tube slicing at 1.8 cm, giving a volume of 0.8 ml. During the initial isolation experiments, the infranatant from this final ultracentrifugation step was collected to ensure complete harvesting of HDL_3.

For comparison, HDL subfractions were isolated according to the method of Havel, Eder, and Bragdon (14), which was adapted

for use with a Beckman TL100.3 rotor and a Beckman Optima MAX-E Table Top Ultracentrifuge. In brief, the ultracentrifugation conditions for the adapted method were as follows: 8 h at 541,000 g_{max} and 12°C for crude HDL isolation; 4 h at 541,000 g_{max} and 12°C for particles in the range 1.1063–1.125 kg/l; and 4 h at 541,000 g_{max} and 12°C for particles in the 1.125–1.210 kg/l range.

VLDL and LDL isolation

VLDL and LDL were isolated from the same plasma pool as the HDL subfractions, according to the methods of McEneny et al. (28) and McDowell, McEneny, and Trimble (29). For VLDL isolation, 1.8 ml of EDTA-plasma was overlaid by a solution of normal saline (d = 1.006 kg/l) and ultracentrifuged for 60 min at 541,000 g_{max} and 4°C. Upon completion of ultracentrifugation, VLDL had floated to the top of the ultracentrifuge tube and was removed by tube slicing at 1.8 cm. LDL was isolated by discontinuous density gradient ultracentrifugation. First, the density of the plasma was adjusted to d = 1.300 kg/l by the addition of 0.4451g of solid KBr to 0.9 ml of plasma. After gentle mixing, this solution was then overlaid with normal saline and ultracentrifuged for 60 min at 541,000 g_{max} and 4°C. LDL was removed by aspiration of the orange band ~10 mm from the top of the tube.

HDL_2 and HDL_3 composition

The composition of the HDL subfractions isolated by this rapid method was assessed by examining the following properties.

Total protein determination. The concentration of total protein for each of the subfractions was determined by a commercially available reagent (Bio-Rad) based upon the Coomassie Blue reaction with protein. This facilitated standardization of the concentration of each lipoprotein before oxidation (as described below).

Cholesterol, phospholipid, and triglyceride. The recovery of total cholesterol, unesterified (free) cholesterol, and phospholipid in the HDL subfractions was determined using commercially available enzymatic assays on a Cobas Fara Autoanalyser. The quantity of cholesteryl ester associated with the HDLs was calculated as the difference of total cholesterol and unesterified cholesterol.

Apolipoprotein determination. The concentration of apoA-I, apoA-II, apoB, and apoC-II in each subfraction was determined by the single radial immunodiffusion method as described by McEneny et al. (28), a modification of the original methods of Mancini, Carbonara, and Heremans (30) and Becker (31). Briefly, a 1% agarose gel was prepared in 50 mM barbital buffer (pH 8.6), followed by the addition of monoclonal antibody [23.8 μl/ml apoA-I (Immunoturb 4700010); 47.6 μl/ml apoA-II (Immunoturb 4700115); 2.5 μl/ml apoB (Immunoturb 4700210); and 7.9 μl/ml apoC-II (Chemicon AB823)]. A 6.3 ml portion of the gel was cast onto glass slides (53 × 70 mm; DiaSys Europe) and allowed to set. Circular wells (diameter, 2 mm) were cut from the gel, and a volume of 4 μl of sample/standard (apoA-I, 0.25–2.50 mg/dl; apoA-II, 0.05–0.75 mg/dl; apoB, 0.25–2.20 mg/dl; and apoC-II, 0.50–7.00 mg/dl) was added. Gels were incubated in a humidified atmosphere at 37°C for 48 h. Once this time had elapsed, gels were then pressed and dried under a stream of air and stained using Coomassie Blue.

During the initial experiments, a small quantity of apoB was detected in the HDL_3 isolate, a density interval in which no apoB-containing lipoproteins should be present. Apolipoprotein [a] (apo[a]), however, slightly overlaps the HDL density interval, so we postulated that the apoB contamination could be attributable to the anti-apoB antibody cross-reacting with apo[a]. To determine whether this was the case, Western blotting was performed on the HDL_2 and HDL_3 isolates, using an antibody specific for apo[a]. Initially, HDL_2 and HDL_3 were delipidated (32) and the protein components separated by SDS-PAGE, according to the method of Laemmli (33). Separated proteins were then electroblotted onto polyvinylidene difluoride membrane, followed by probing with an antibody specific for apo[a] (Autogen Bioclear UK). Detection was achieved by the addition of HRP-conjugated goat anti-mouse IgG_1 (Autogen Bioclear UK) and visualized by the addition of 3,3′-diaminobenzidine tetrahydrochloride reagent (Pierce).

Albumin determination. Albumin is a known antioxidant whose presence could potentially mask the native antioxidant properties of HDL and as such could hinder oxidation studies. The detection of albumin was achieved by the single radial immunodiffusion method, as described by McEneny et al. (28), using 3.0 μl/ml anti-human albumin antibody (Dako A0001). Standards were prepared from human serum albumin in the range 20–200 mg/dl.

Transferrin determination. Transferrin has been found to associate with HDL's apoA-I component and as such could potentially increase its antioxidant capacity. The concentration and recovery of transferrin were determined by a single radial immunodiffusion method, based on the technique of McEneny et al. (28). Anti-human transferrin (Sigma T6265) was added to a 1% agarose gel (as described above) at a concentration of 0.79 μl/ml. Standards were prepared in the range 0.50–50.0 μg/ml for calibration of the assay.

Oxidation potential of HDL and its subfractions

Size-exclusion chromatography of lipoproteins. Trace contaminants such as ascorbate, urate, potassium bromide, and EDTA were removed from ultracentrifugally isolated lipoproteins by size-exclusion chromatography using PD10 columns (Amersham Pharmacia; Sephadex G-25M) and 0.01 M PBS (pH 7.4) to effect elution. In brief, 0.5 ml of lipoprotein was added to the column and allowed to enter, followed by 2 ml of PBS, and the resulting eluents were discarded. An additional 2 ml of PBS was added, with this fraction being collected on ice. The now purified lipoproteins were subjected to total protein determination to facilitate subsequent standardizations, which are required before oxidation.

Optimum copper concentration. As established previously for VLDL and LDL, a relationship exists between lag time and the concentration of Cu^{2+} (28, 29). This is characterized by a decrease in lag time with increasing Cu^{2+} concentration; however, a concentration of Cu^{2+} is reached at which further addition has no effect on lag time. For HDL (total HDL, HDL_2, and HDL_3), we examined Cu^{2+} concentrations between 0.05 and 10.0 μM. We found that HDL and its subfractions demonstrated a similar trend to that found for VLDL and LDL (28, 29): a concentration of copper was reached at which no further enhancement of oxidation occurred. We demonstrated that at Cu^{2+} concentrations >5.0 μM, no further decrease in lag time was observed. Therefore, we used 5.0 μM Cu^{2+} to mediate oxidation for all subsequent oxidation experiments, with total HDL and HDL_3 being standardized to 200 μg/ml total protein and HDL_2 standardized to 40 μg/ml total protein. The production of conjugated dienes at 37°C was followed by recording the change in absorbance at λ = 234 nm in an automated plate reader. The kinetic parameter time at half maximum ($t_{1/2max}$), an equivalent of lag time, was evaluated using SoftMax Pro version 4.8 computer software (Molecular Devices Corp.).

Antioxidant properties of HDL and its subfractions

Combination of HDL subfractions with VLDL and LDL. To determine the antioxidant effect of HDL and its subfractions on the oxidation of VLDL and LDL, increasing concentrations of total HDL, HDL_2, and HDL_3 were coincubated with either VLDL or LDL. The routine procedures were as follows: *1*) coincubations of VLDL (25 μg/ml) with total HDL (5–200 μg/ml), HDL_2 (10–40 μg/ml), or HDL_3 (5–200 μg/ml) were oxidized in the presence of 17.5 μM Cu^{2+} (the Cu^{2+} concentration routinely used to mediate VLDL oxidation); *2*) coincubations of LDL (50 μg/ml) with total HDL (5–200 μg/ml), HDL_2 (10–40 μg/ml), or HDL_3 (5–200 μg/ml) were oxidized in the presence of 2 μM Cu^{2+} (the Cu^{2+} concentration routinely used to mediate LDL oxidation). The production of conjugated dienes at 37°C was followed in an automated plate reader as described above.

Distribution of oxidized products within the lipoprotein classes. VLDL and LDL were coincubated with total HDL, HDL_2, or HDL_3, as described above, but in sufficient volumes to allow for their reisolation by ultracentrifugation. On completion of oxidation, lipoprotein mixtures were overlaid by a solution of normal saline for VLDL reisolation (d = 1.006 kg/l) or an aqueous solution of KBr for LDL reisolation (d = 1.063 kg/l) and separated by single spin ultracentrifugation at 541,000 g_{max} and 4°C for 120 min. VLDL or LDL was recovered by tube slicing at a distance of 1.8 cm, and total HDL, HDL_2, or HDL_3 was removed by aspirating the infranatant in a volume of 0.9 ml. Upon removal, an aliquot of each lipoprotein was subjected to agarose gel electrophoresis to ensure adequate separation, with the remaining samples being retained for lipid phase hydroperoxide assessment by the ferric oxidation of xylenol orange version II assay.

Lipid phase hydroperoxides. The concentration of hydroperoxides was determined by the ferric oxidation of xylenol orange version II method of Wolff (34) and Naurooz-Zadeh, Tajaddini-Sarmadi, and Wolff (35) and as described by McEneny et al. (28).

Roles of CETP and LCAT during VLDL and LDL oxidation. The role of HDL-associated CETP in VLDL oxidation was examined by pretreating HDLs with a specific inhibitor of CETP, 4,4′-dithiodipyridine, at a maximal concentration of 1.0 μM (36). VLDL was then oxidized in the presence of 4,4′-dithiodipyridine-treated HDLs, as described previously. The participation of LCAT in HDL's dual role in lipoprotein oxidation was assessed by generating LCAT-depleted lipoproteins (LDL and HDL) by a modification of the method originally described by Holmquist (37); in our adaptation, we used prepacked columns of Q-Sepharose to generate LCAT-depleted plasma. LCAT-depleted LDL and LCAT-depleted HDL were then isolated from the recovered plasma as described above. In these preliminary experiments, in which we only examined the role of LCAT-depleted total HDL (and not the HDL subfractions), VLDL and LDL were oxidized as described above in the presence of either native HDL or LCAT-depleted HDL.

Statistical analysis

Nonparametrically distributed paired samples were analyzed using the Mann-Whitney *U*-test available in the Statistics Package for Social Sciences (SPSS) for Windows. Results are given as means ± SD unless stated otherwise. $P < 0.05$ was considered statistically significant.

RESULTS

HDL_2 and HDL_3 isolation: purity of isolated crude HDL and its subfractions

Figure 1 displays typical electrophoresis results for HDL subfractions isolated by rapid ultracentrifugation; it clearly demonstrates that crude HDL was devoid of any contamination by VLDL or LDL and exhibits typical α-mobility. The absence of any α-migrating lipoproteins in the VLDL/LDL lane demonstrates the complete recovery of HDL particles during the initial sedimentation ultracentrifugation step.

HDL_2 and HDL_3 composition

Cholesterol, phospholipid, and triglyceride. The percentage composition of HDL_2 and HDL_3 is shown in **Table 1**. For comparison, the compositions of subfractions obtained using the prolonged method of Havel, Eder, and Bragdon (14) are also shown. No statistical difference was observed between the two methods for each subfraction ($P > 0.05$). The percentage recoveries for the individual lipid components (expressed as percentage recovery from unsubfrac-

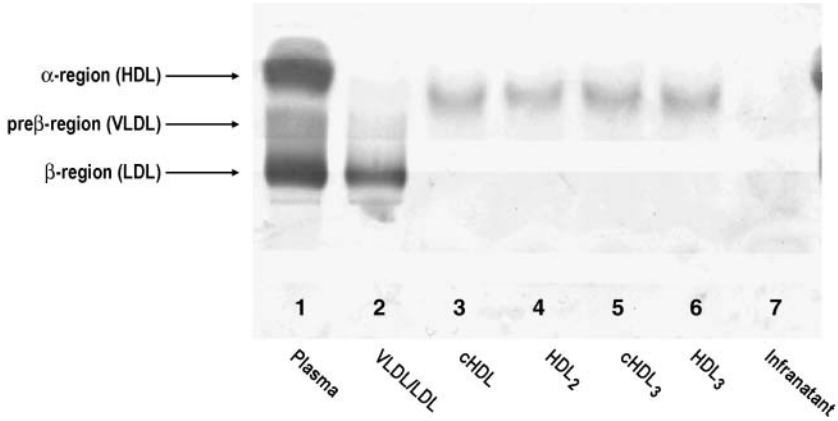

Fig. 1. Purity of crude HDL, HDL_2, and HDL_3 as demonstrated by agarose gel electrophoresis and staining with Sudan Black lipid stain. The absence of any α-migrating lipoproteins in lane 2 indicates the complete recovery of HDL during the initial sedimentation ultracentrifugation. cHDL, crude high density lipoprotein.

TABLE 1. Chemical composition of the two major HDL subfractions, HDL_2 and HDL_3, isolated by rapid ultracentrifugation (column A) and by the prolonged method of Havel, Eder, and Bragdon (column B)

	HDL_2		HDL_3	
Variable	A	B	A	B
Chemical composition (%)				
Total protein	40.2 ± 8.6	40.5 ± 3.5	59.6 ± 3.3	54.1 ± 10.3
Total lipid	59.8 ± 4.9	59.5 ± 2.5	40.4 ± 2.4	45.9 ± 4.1
Lipid composition (%)				
Free cholesterol	9.3 ± 2.6	9.9 ± 1.8	7.9 ± 1.7	7.4 ± 1.1
Cholesteryl ester	35.5 ± 9.5	36.8 ± 3.5	23.0 ± 1.7	25.5 ± 9.6
Phospholipid	52.1 ± 6.6	52.7 ± 4.3	67.3 ± 5.5	66.4 ± 4.6
Triglyceride	3.0 ± 0.9	0.5 ± 0.2	1.7 ± 0.6	0.44 ± 0.9

Results are expressed as percentage of total composition ± SD (n = 3).

tionated HDL isolated by an established technique) are extremely favorable: total cholesterol, 101.74 ± 1.51; cholesteryl ester, 102.00 ± 6.97; phospholipid, 102.89 ± 1.15; and triglyceride, 93.33 ± 9.94.

Apolipoprotein and transferrin. The concentrations of apoA-I and apoA-II **(Table 2)** detected by our single radial immunodiffusion technique are in agreement with those found by Miller et al. (38) using an electroimmunoassay and by Alberts, Cheung, and Wahl (39) using single radial immunodiffusion. The low concentration of apoB detected in the HDL_3 isolate could be attributable to the presence of apo[a], because the major apoB-containing lipoproteins such as LDL have a density much less than 1.210 kg/l. This was confirmed by Western blotting analysis for apo[a]. **Figure 2** demonstrates that the apoB-reactive reacting material in the HDL_3 isolate (as detected by single radial immunodiffusion) was attributable to apo[a]. However, it is worth noting that this contamination accounts for only 0.6% when the results are expressed as a percentage of the total apolipoprotein composition, compared with 1.2% when using the prolonged method of Havel, Eder, and Bragdon (14). The distribution of transferrin between the two subfractions shows transferrin enrichment of the smaller, denser subfraction, with HDL_3 containing more than twice the level of transferrin than HDL_2 (5.9 ± 0.9 and 14.8 ± 1.0 μg/ml, HDL_2 vs. HDL_3; $P < 0.05$), which may explain, in part, why this subfraction is a more proactive antioxidant. Comparison of the protein composition of the HDL subfractions obtained using the two methods revealed only the albumin content as being statistically different, with the more prolonged method of Havel, Eder, and Bragdon (14) yielding an HDL_3 isolate containing more than twice the concentration of albumin compared with our method (3.2 ± 2.2 vs. 9.4 ± 2.7 mg/dl; $P < 0.05$).

TABLE 2. Protein characteristics of HDL_2 and HDL_3 isolated by rapid ultracentrifugation (column A) and by the method of Havel, Eder, and Bragdon (column B)

	HDL_2		HDL_3	
Variable	A	B	A	B
ApoA-I (mg/dl)	24.3 ± 4.1	18.9 ± 6.8	85.6 ± 5.8	79.1 ± 6.2
ApoA-II (mg/dl)	11.2 ± 2.9	8.9 ± 4.8	37.6 ± 7.0	32.7 ± 2.0
ApoB (mg/dl)	0[a]	0.3 ± 0.0	0.8 ± 0.0	1.4 ± 0.1
ApoC-II (mg/dl)	2.9 ± 0.2	1.4 ± 0.2	4.0 ± 0.6	3.2 ± 2.2
Albumin (mg/dl)	0[a]	0[a]	2.4 ± 1.1	9.4 ± 2.7
Transferrin (μg/ml)	5.9 ± 0.9	3.6 ± 0.8	14.8 ± 1.0	12.3 ± 3.6

ApoA-I, apolipoprotein A-I. Results are expressed as means ± SD (n = 3). The coefficients of variation for the single radial immunodiffusion technique used for the measurement of the protein composition of the HDLs, taken over a number of determinations, were as follows: 4.8% (apoA-I), 3.2% (apoA-II), 7.1% (apoB), 7.9% (apoC-II), 8.1% (albumin), and 3.1% (transferrin).

[a] None detected.

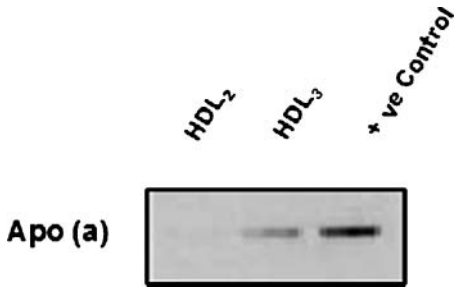

Fig. 2. Western blot for apolipoprotein [a] (apo[a]) in delipidated HDL_2 and HDL_3 isolates. Results show a positive binding reaction between anti-apo[a] and HDL_3, demonstrating a small degree of contamination of HDL_3 by apo[a].

Antioxidant properties of HDL subfractions

Optimum copper concentration. The optimum concentration of Cu^{2+} needed for complete oxidation of 50 μg/ml total HDL, HDL_2, or HDL_3 was found to be 5 μM, as shown in **Fig. 3**.

Combination of HDL subfractions with VLDL and LDL. Owing to VLDL's more resistant nature to oxidation compared with LDL, the peroxidation process was followed for a longer time span when examining VLDL. The oxidation of VLDL in the presence of total HDL, HDL_2, and HDL_3 produced unexpected results and has, for the first time, provided evidence that under these conditions HDL may act as a prooxidant species. Results **(Table 3)** demonstrate that during coincubation of VLDL with total HDL, HDL_2, and HDL_3, increasing the concentration of HDL decreases the $t_{1/2max}$ for VLDL ($P < 0.05$). The converse is true of coincubations of LDL with total HDL, HDL_2, or HDL_3, with $t_{1/2max}$ increasing with increasing concentrations of HDL and HDL displaying its more customary antioxidant effect **(Table 4)**.

Roles of CETP and LCAT during VLDL and LDL oxidation. Oxidation of VLDL in the presence of HDL treated with a CETP inhibitor resulted in a significant increase in the $t_{1/2max}$ of VLDL compared with VLDL + native HDL (75.3 ± 1.5 min vs. 36.2 ± 1.5 min) **(Fig. 4)**. This is consistent with a CETP-mediated transfer of oxidized lipid from HDL to VLDL, in which the donated lipids increase the rate of lipid oxidation and thus accelerate VLDL's own oxidation. To exclude any independent effect of the CETP inhibitor, HDL with and without inhibitor and VLDL with

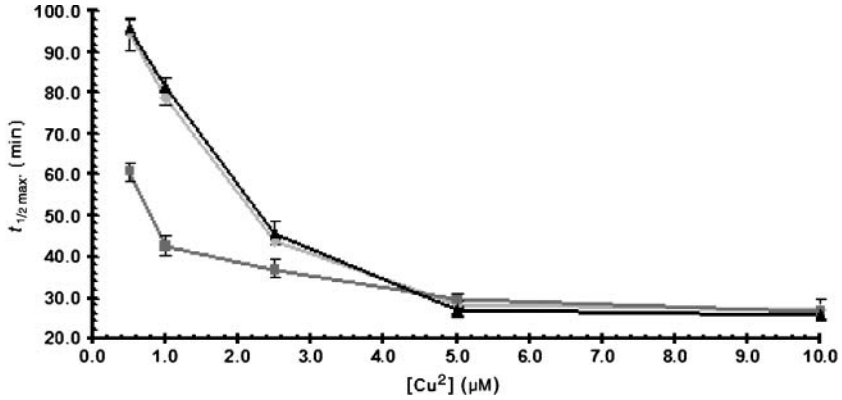

Fig. 3. Optimum copper(II) chloride concentration required for the oxidation of total HDL (triangles), HDL_2 (squares), and HDL_3 (diamonds). A final concentration of 5 μM was chosen because this value corresponds to the last point on the graph before the plateau region. Results are means ± SD (n = 6). $t_{1/2max}$, time at half maximum.

and without inhibitor were also oxidized in each set of experiments (results not shown).

Oxidation of VLDL in the presence of native HDL (nHDL) or LCAT-depleted HDL (dHDL) demonstrated that VLDL was more readily oxidized in the presence of native HDL (VLDL + nHDL, 39 ± 1.7 min vs. VLDL + dHDL, 106 ± 5.9 min; $P < 0.05$, n = 6). These results reveal the involvement of HDL-associated LCAT in the increased oxidation of VLDL. Oxidation of LDL in the presence of native HDL or LCAT-depleted HDL supports the role of LCAT in HDL's sacrificial protection of LDL and is in agreement with the findings of Vohl et al. (40). Oxidation of LDL in the presence of native HDL resulted in a prolonged $t_{1/2max}$ compared with that of LDL oxidized in the presence of LCAT-depleted HDL (LDL + nHDL, 90 ± 3.3 min vs. LDL + dHDL, 54 ± 2.1 min; $P < 0.05$, n = 6). In this instance, HDL's endogenous LCAT may help spare LDL from oxidation until later in the oxidation process.

When LCAT-depleted HDL was treated with the CETP inhibitor and oxidized in the presence of VLDL, a $t_{1/2max}$ was obtained that was significantly longer than that of VLDL cooxidized with native HDL (165 ± 5.1 min vs. 36.2 ± 1.5 min; $P < 0.05$, n = 6). These findings suggest that both CETP and LCAT are major causative agents for the prooxidant effect of HDL in VLDL's oxidation.

Distribution of oxidized products with the lipoprotein classes. During incubation of VLDL with total HDL, HDL_2, or HDL_3, the concentration of hydroperoxides in VLDL increased **(Fig. 5A)**, whereas total HDL displayed a subtler increase in lipid hydroperoxides (Fig. 5B). For the HDL subfractions (Fig. 5B), the levels of hydroperoxides appeared to increase concurrently in VLDL and the HDL subfractions, suggesting that an equilibrium-like state was established between the lipoproteins (i.e., the rate of formation of hydroperoxide in the HDLs was equal to the rate of transfer). In contrast, a control sample of total HDL, HDL_2, or HDL_3 undergoing oxidation in the absence of VLDL (but under otherwise identical conditions) showed

TABLE 3. $t_{1/2max}$ for combinations of VLDL (standardized to 25 μg/ml) with increasing concentrations of total HDL, HDL_2, and HDL_3

Variable	Lipoprotein Combination		
	VLDL + Total HDL	VLDL + HDL_2	VLDL + HDL_3
VLDL	241.4 ± 3.9	252.0 ± 4.7	230.3 ± 4.9
Concentration of HDL total protein (μg/ml)			
1	241.7 ± 5.1	258.3 ± 2.8	238.9 ± 2.6
2	204.0 ± 3.7	216.8 ± 6.4	199.4 ± 2.8
3	173.5 ± 2.8	207.3 ± 3.9	169.5 ± 9.3
4	159.0 ± 5.1	194.4 ± 3.9	144.2 ± 6.1
5	142.7 ± 8.1	150.2 ± 6.9	134.2 ± 2.0
10	119.3 ± 3.6	123.4 ± 5.6	110.2 ± 1.6
20	84.8 ± 4.4	94.0 ± 4.2	78.9 ± 2.7
30	67.2 ± 2.9	69.3 ± 3.6	68.1 ± 2.2
40	59.5 ± 4.6	60.3 ± 0.6	60.4 ± 4.1
50	58.6 ± 7.0	—[a]	57.5 ± 8.4
100	49.9 ± 3.8	—[a]	44.3 ± 4.1
150	40.0 ± 3.5	—[a]	36.2 ± 1.6
200	38.1 ± 4.2	—[a]	33.9 ± 1.0

$t_{1/2max}$, time at half maximum. Results are means ± SD (n = 6).
[a] Combinations of VLDL and HDL_2 were not analyzed at these protein concentrations because the concentration of total protein in HDL_2 rarely exceeds 40 μg/ml.

TABLE 4. $t_{1/2max}$ for combinations of LDL (standardized to 50 μg/ml) with increasing concentrations of total HDL, HDL_2, and HDL_3

Variable	Lipoprotein Combination		
	LDL + Total HDL	LDL + HDL_2	LDL + HDL_3
LDL	66.1 ± 2.2	63.2 ± 2.7	67.2 ± 3.7
Concentration of HDL total protein (μg/ml)			
5	70.4 ± 6.1	62.1 ± 2.8	66.1 ± 3.1
10	73.9 ± 3.1	67.3 ± 2.9	72.8 ± 3.6
20	88.2 ± 0.6	72.8 ± 2.1	85.5 ± 2.6
30	98.0 ± 3.7	81.9 ± 3.1	94.1 ± 2.7
40	108.4 ± 3.1	97.6 ± 4.7	116.1 ± 9.1
50	132.2 ± 4.6	—[a]	124.3 ± 2.0
100	154.8 ± 7.1	—[a]	149.0 ± 6.7
150	168.2 ± 5.1	—[a]	179.0 ± 5.1
200	189.0 ± 4.8	—[a]	182.0 ± 3.8

Results are means ± SD (n = 6).
[a] Combinations of LDL and HDL_2 were not analyzed at these protein concentrations because the concentration of total protein in HDL_2 rarely exceeds 40 μg/ml.

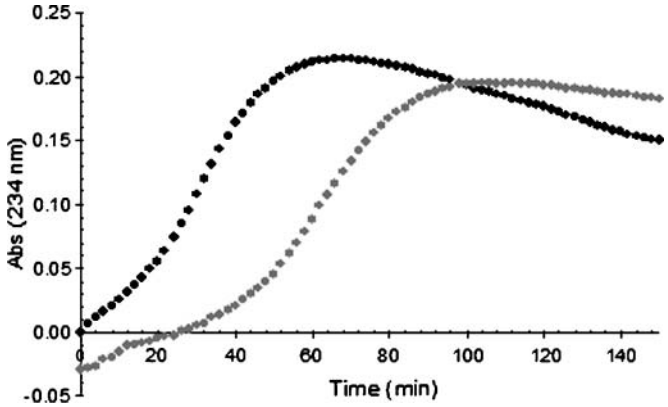

Fig. 4. Comparison of VLDL oxidized in the presence of native HDL (black circles) and VLDL oxidized in the presence of cholesteryl ester transfer protein (CETP)-inactivated HDL (gray circles). This graph shows a significant increase in VLDL $t_{1/2max}$ when oxidized with CETP-inactivated HDL. Abs, absorbance.

markedly higher concentrations of hydroperoxides. Expressed as percentage change (control HDLs vs. HDLs reisolated from coincubations with VLDL), the results were as follows: total HDL, 50.0% versus 71.7%; HDL_2, 21.5% versus 61.6%; HDL_3, 18.3% versus 59.2%. This supports our hypothesis that HDL may act as a source of hydroperoxides that are donated to VLDL during the oxidation process. Coincubation of LDL with total HDL, HDL_2, or HDL_3 demonstrated a less well-marked increase of hydroperoxides within LDL, but with a rapid production of hydroperoxides in HDL (**Fig. 6A, B**). Interestingly, an examination of HDL oxidation in the presence of LDL versus HDL oxidized alone identified a small difference between the two treatments. In the combination of HDL + LDL, the rate of oxidation was marginally faster than in HDL alone (5.1 vs. 4.8 nM/min; $P < 0.05$, n = 3). This supports the current understanding that HDL is preferentially oxidized in the presence of LDL.

DISCUSSION

We describe a rapid flotation ultracentrifugation method that isolates HDL_2 and HDL_3 from plasma in only 6 h, representing a significant reduction in total ultracentrifugation time compared with existing methods. The composition of the HDL subfractions so obtained is in agreement with published sources (41–43), thus making this an appealing method for use in large-scale studies in which quality preparations are required under pressure of time.

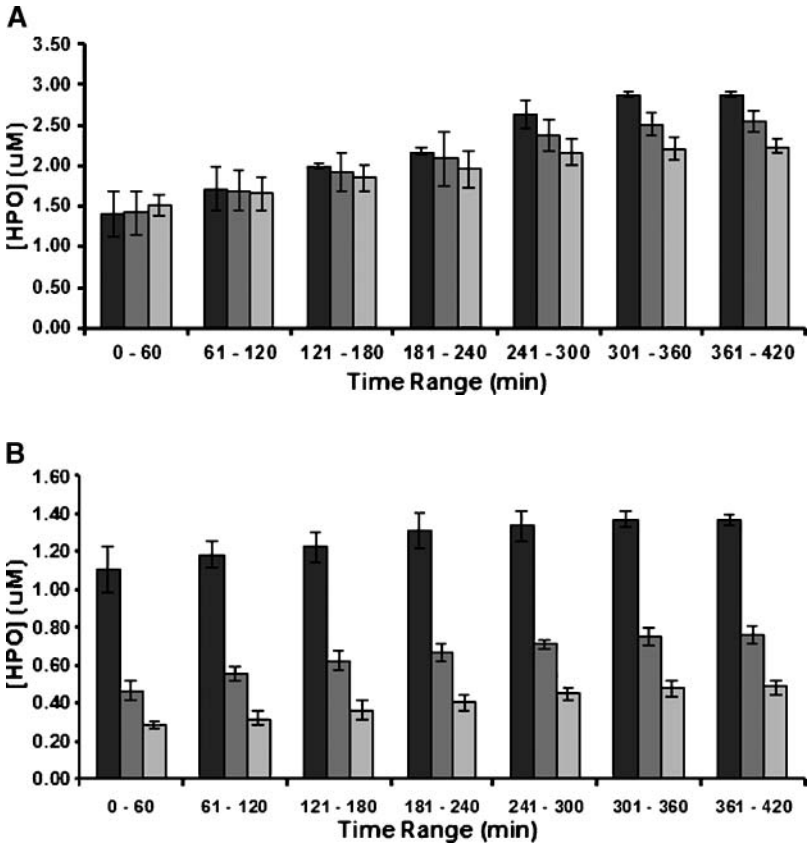

Fig. 5. A: Variation in lipid phase hydroperoxides (HPO) in VLDL reisolated from coincubations with total HDL (dark gray), HDL_2 (medium gray), and HDL_3 (light gray) collected over several time ranges. Results are means ± SD (n = 6). B: Variation in lipid phase hydroperoxides in total HDL (dark gray), HDL_2 (medium gray), and HDL_3 (light gray) reisolated from coincubations with VLDL collected over several time ranges. Results are means ± SD (n = 6).

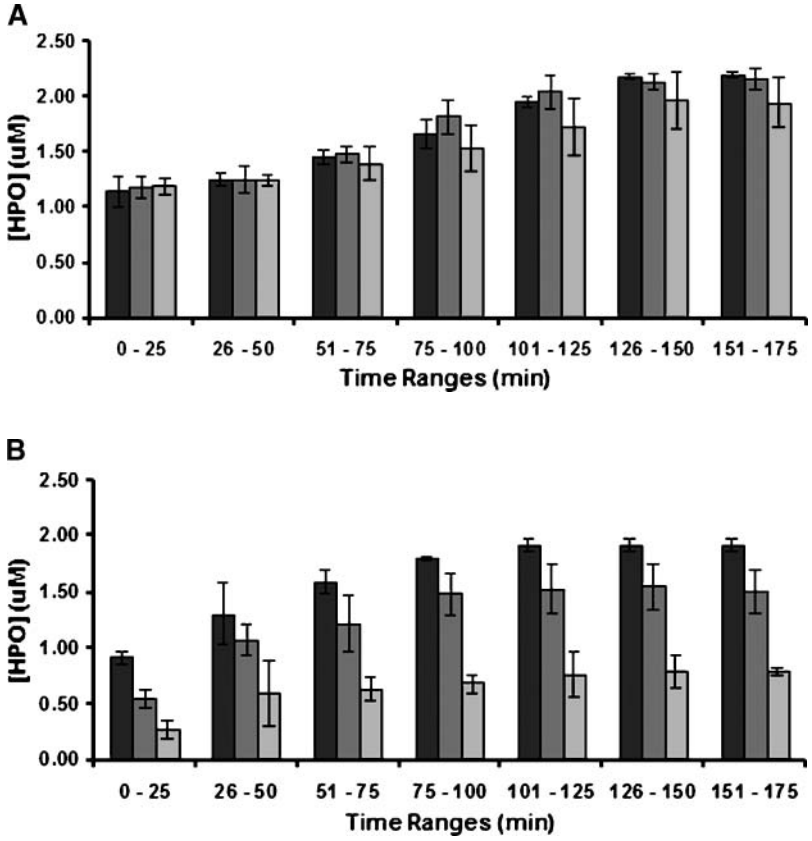

Fig. 6. A: Variation in lipid phase hydroperoxides (HPO) in LDL reisolated from coincubations with total HDL (dark gray), HDL_2 (medium gray), and HDL_3 (light gray) collected over several time ranges. Results are means ± SD (n = 6). B: Variation in lipid phase hydroperoxides in total HDL (dark gray), HDL_2 (medium gray), and HDL_3 (light gray) reisolated from coincubations with LDL collected over several time ranges. Results are means ± SD (n = 6).

Indeed, compared with the compositions originally reported by Havel, Eder, and Bragdon (14) (the method that was adapted for comparison with the protocol reported here, which required 16 h of ultracentrifugation), the method we propose produces HDL subfractions in closer agreement with those likely to be found in vivo. We have also shown transferrin enrichment of the smaller, denser subfraction (HDL_3), which may in part explain the significant antioxidant properties of this species.

The use of copper(II) ions to initiate free radical reactions within lipoprotein particles has been widely cited (44, 45). Application of copper-mediated oxidation to HDL_2 and HDL_3 produced consistently reproducible results, with freshly isolated subfractions displaying almost identical oxidation parameters as frozen subfractions (data not shown). The oxidation rates of the two subfractions followed a predictable pattern, with HDL_2 requiring a slightly longer incubation time to achieve full oxidation and progress into the decomposition phase of oxidation. This is in agreement with previous studies showing that larger lipoprotein particles require longer periods of time to reach full oxidation. The largely linear oxidation profiles of HDL_2 and HDL_3 fit the current understanding that HDL particles are largely devoid of at least those antioxidant molecules capable of inhibiting copper-induced oxidation and thus have a very short lag period.

When the interrelationship between LDL and HDL subfractions was examined, the customary protective (antioxidant) role of HDL was observed, as characterized by an increase in LDL lag time with increasing concentrations of HDL_2 or HDL_3. In this respect, the more proteinaceous HDL_3 subfraction appears to be more effective at preventing the oxidative modification of LDL, possibly by a form of sacrificial protection via selective oxidation of methionine residues on apoA-I, as suggested by Garner et al. (18). We also found that LCAT protected LDL against oxidation, a finding in complete agreement with that previously reported by Vohl et al. (40), who demonstrated that the antioxidant properties of LCAT were attributable to a catalytically active serine residue (serine 181), which serves as a proton donor, neutralizing carbon-centered radicals and halting the peroxidation chain reaction.

This investigation has identified, for the first time, a prooxidant role for HDL in the oxidative modification of VLDL. Ostensibly, copper(II) ions have the capacity to decompose peroxides to peroxyl and alkoxyl radicals via a seeding mechanism. The peroxyl radicals thus formed can participate in hydrogen abstraction from methyl groups on

PUFA side chains, thus perpetuating the free radical chain reaction (46). Our results suggest that HDL acts as a reservoir of preformed hydroperoxides, which are then donated to VLDL in carrier protein-mediated events. Two such proteins have been identified as CETP and LCAT. In our preliminary experiments, we have demonstrated that both CETP and LCAT have prooxidant activity during VLDL oxidation, with VLDL acting as a sink for HDL's preformed lipid hydroperoxides, which are transferred by CETP. The donated hydroperoxides then appear to seed additional oxidations within VLDL, under the influence of copper(II) ions. Bowry, Stanley, and Stocker (47) demonstrated that HDLs are a major carrier of lipid hydroperoxides, and they suggested that HDLs exert their beneficial effects by attenuating the buildup of oxidized lipids in LDL. These findings may go some way to explain why some individuals with hyperalphalipoproteinemia still develop premature atherosclerosis, despite such an antiatherogenic phenotype (48–50).

HDL, particularly the HDL_3 subfraction, has been linked to the downregulation of cell adhesion molecules on vascular endothelial cells, and as such it may represent a significant impediment to the progression of atherosclerosis. The effect of oxidized HDL on this process has not been fully elucidated; however, it seems likely that oxidation would reduce HDL's ability to downregulate cell adhesion molecule expression. The significance of this effect would be greater in individuals with significant hypertriglyceridemia, because this population is known to have a particularly atherogenic lipoprotein profile (high VLDL and low HDL). The excess of triglyceride would serve as an ideal substrate for oxidation reactions, and in light of our recent findings, even low levels of HDL could potentially accelerate the propagation of VLDL oxidation and as such promote LDL oxidation by donation of preformed hydroperoxides. Such a chain of hydroperoxide donations may play an integral step in atherosclerosis, which identifies all lipoprotein particles as being potentially atherogenic.

Conclusion

We have proposed a method that isolates HDL_2 and HDL_3 in only 6 h, a significantly shorter time than previous methodologies. Upon coincubation with VLDL, we have shown for the first time a prooxidant effect of HDL in vitro, which may go some way to explain the complex pathogenesis of atherosclerosis. Application of this method to various patient groups may also help uncover the biochemical abnormalities associated with a variety of clinical presentations and lead to explanations for their more atherogenic profiles.

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