High-Density Lipoprotein Apolipoproteins in Urine: I. Characterization in Normal Subjects and in Patients with Proteinuria

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A high-resolution two-dimensional electrophoretic method for protein, with silver staining, has been used to characterize and identify urinary high-density-lipoprotein apolipoproteins (HDL-Apos) and their isoforms in healthy subjects and in patients with kidney disease. Analytical techniques based on both molecular mass and ultracentrifugal flotation properties were used to isolate urinary lipoprotein particles with characteristics identical to those of HDL in plasma. HDL-Apos identified in urine of normal subjects and patients with glomerular proteinuria were Apos A-I, A-II, and C. Five isoforms of Apos A-I were present. Immunostaining of electrophoreted proteins further confirmed the presence of HDL-Apos in urine. Creatinine clearance rate was decreased in the patients with proteinuria, and ranged from 32.5 to 40 mL/min. Concentrations of cholesterol and triglycerides in serum were greater in the patients’ groups, whereas mean HDL-cholesterol (0.68, SD 0.10 mmol/L) and Apos A-I (0.953, SD 0.095 g/L) were significantly (each P <0.01) lower. Results of this study suggest that measurement of urinary Apos A-I will reflect excretion of HDL in urine.

Additional Keyphrases: apolipoprotein A-I • high-density lipoprotein • kidney disease • two-dimensional electrophoresis • polyacrylamide gel electrophoresis • isoelectric focusing • electrophoresis • ultracentrifugation • cholesterol • sex-related differences

The proteins in urine can be useful indicators of biochemical and physiological changes in an individual. The characterization and identification of individual urinary proteins have shown that certain urinary proteins may be useful markers of certain disease states (1). Qualitative and quantitative changes in urinary proteins are considered clinically significant as indicators of filtration and reabsorption events in the kidney nephron. In healthy persons the renal output of total protein is less than 150 mg/day (2–4), contrasted with gram quantities of protein measured in the urine of patients with kidney disease. Concentrations of certain analytes in plasma also reflect concurrent kidney disease. Researchers well recognize that renal disease, in particular nonselective glomerular proteinuria, is associated with hyperlipoproteinemia (5).

High-density-lipoprotein apolipoproteins (HDL-Apos) in serum, in particular Apos A-I, have been extensively studied in health and in disease (6) because of their association with coronary heart disease.⁴ In contrast, there have been few studies of urinary apolipoproteins. The urinary excretion of apolipoproteins by normal subjects is not well understood. HDL-Apos may be excreted as small HDL particles (7, 8) or possibly filtered as individual Apos that recombine to form HDL-like particles.

Recently, high-resolution 2DE (9, 10), coupled with silver staining (11), has found widespread application in separation and identification of proteins. One of the problems encountered in preparing urinary proteins for 2DE analysis is the removal of other substances such as salts, organic acids, polyamines, pigments, and drug metabolites that are or may be present in substantial quantities. Also, because urinary proteins other than albumin are present in very low concentrations, their assay requires concentration of the sample. Among the procedures used to concentrate and desalt urinary proteins are ultracentrifugation, precipitation, chromatography, dialysis, and lyophilization (12, 13).

In this investigation we sought to study the excretion of urinary HDL-Apo in healthy volunteers and in patients with glomerular proteinuria, so as to better understand the characteristics of urinary HDL and the mechanism of hyperlipoproteinemia seen in renal disease and to help diagnose proteinuria, which is noted especially in disease associated with glomerular dysfunction.

Materials and Methods

Specimens

Random (untimed) urine specimens from healthy adult volunteers (Centers for Disease Control, Atlanta, GA) were pooled individually into vessels kept at 4 °C and containing a solution of (per liter) 1 g of sodium azide, 5 mg of aprotinin, and 1 g of sodium carbonate. Similarly, 24-h urine specimens were collected from four healthy subjects—two men and two women. Twelve more 24-h urine specimens were collected (at 4 °C, without preservative) from three male and three female volunteers and from six adult patients (three men and three women, all more than 30 years of age) with biopsy-proven glomerular disease.

After a 12-h fast, adhered to during the 24-h urine collection, 5-mL blood samples were collected into sterile, evacuated collection tubes containing EDTA. Serum was separated by low-speed centrifugation, removed, and stored at 4 °C before prompt analysis or at −20 °C if analyzed later.

Antisera

For the immunodetection of Apos A-I and C, we used goat antisera to human Apos A-I and C (Clinical Biochemistry Branch, Center for Environmental Health and Injury Control, Centers for Disease Control, Atlanta, GA 30333), rabbit

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⁴ Nonstandard abbreviations: HDL-Apos, high-density-lipoprotein apolipoproteins; 2DE, two-dimensional electrophoresis; Apos, apolipoprotein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered isotonic saline; VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins.

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anti-goat IgG, and goat anti-rabbit antisera conjugated with horseradish peroxidase (EC 1.1.11.7; Cappel, Division of Cooper Diagnostics, Cochrannville, PA) as the primary, secondary, and tertiary antibodies, respectively. Apo A-II was detected with monoclonal antibody to Apo A-II (from L. K. Curtiss, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA) and goat anti-mouse antibodies affinity-purified and conjugated to horseradish peroxidase (Calbiochem–Behring Diagnostics, La Jolla, CA).

Analytical Procedures

Albumin and creatinine in serum and creatinine and total protein in urine were determined with an automated analyzer (14). The rate of creatinine clearance was then calculated according to standard criteria (15). Cholesterol and triglycerides in serum were determined enzymatically (14), and HDL-cholesterol was measured by enzymatic cholesterol assay (DuPont acc Clinical Tests, DuPont Co., Wilmington, DE) after precipitation of LD and VLDL by phosphotungstate precipitation buffer (14). Apo A-I was quantified by double-antibody radioimmunoassay (16).

Urine Sample Preparation

Concentration and desalting. Figure 1 summarizes the procedures we used to concentrate and desalt urinary proteins and to separate urinary lipoproteins. The final concentration factors ranged from 100- to 1000-fold. All procedures were carried out at 4 °C.

After aliquots for creatinine and total protein analyses were removed, insoluble debris was removed by low-speed centrifugation. A 40-mL aliquot (aliquot A) was set aside for further studies. The remaining urine sample was concentrated and diafiltered (desalted) simultaneously by passage through an ultrafiltration system (Amicon, Division of W. R. Grace & Co., Danvers, MA; 400-mL capacity, 50 000- Da-cutoff type PM membrane). After concentrating the sample 10-fold, we removed a 40-mL aliquot (aliquot B) from the retained portion, then concentrated the remaining sample twofold further (aliquot C). The two 40-mL urine aliquots (aliquots A and B) were then concentrated with a Centricon 10™ Microconcentrator (Amicon) with a 10 000- Da-cutoff, type YM membrane as follows: about 2 mL of the urine aliquot was introduced and centrifuged at 6000 × g at 4 °C for 30 to 60 min; more of the urine was repeatedly added and centrifuged with the same Centricon 10 device until the sample volume was about 0.2–0.5 mL. The final concentrate was then treated with urea mix as described later and stored at −50 °C.

Isolation of lipoproteins. Lipoproteins were isolated from urine samples that had been concentrated 20-fold (aliquots C). Lipoprotein fractions (VLDL and LDL, d = 1.006–1.063; HDL, d = 1.125–1.21) of serum and concentrated urine were separated sequentially by preparative ultracentrifugation (17). The isolated lipoproteins were dialyzed at 4 °C against a solution containing, per liter, 8.5 g of NaCl, 0.1 g of EDTA, 0.4 g of sodium azide, and 1.2 g of thimerosal. Sodium azide and thimerosal were omitted from this solution (physiological saline) for the dialysis of apo B-containing lipoproteins.

We used the method of Lowry et al. (18) to determine protein in each lipoprotein fraction, with bovine serum albumin as calibrator (Standard Reference Material No. 926; National Bureau of Standards, Shady Grove, MD).

Two-dimensional electrophoresis. Serum lipoproteins and urine samples obtained from each final concentration step (Figure 1) were analyzed by the "Iso-Dalt" technique (9, 10). The serum lipoprotein fractions were treated with "SDS mix" (diluted fivefold with de-ionized water) and then heated to 95 °C for 5 min. The SDS mix consists of 50 mmol of cyclohexylaminoethanesulfonic acid (Calbiochem–Behring Corp.), 200 mg of SDS (Bio-Rad Labs, Richmond, CA), 10 g of dithioerythritol (Bio-Rad Labs), and 200 mL of glycerol (Fisher Scientific, Fair Lawn, NJ) per liter, adjusted to pH 9.5 with ammonium hydroxide solution (250 mL/L).

The concentrated urinary lipoprotein fractions and concentrated urine (Figure 1) were mixed with urea mix—per liter, 9 mol of urea (Bio-Rad Labs), 5 mL of 2% ampholytes (pH 3–10; Pharmacia, Uppsala, Sweden), and 50 g of dithioerythritol (Bio-Rad Labs)—with 2 mL of urea mix being added to the final concentrate of urinary samples (about 0.5 mL). These treated samples were further concentrated with a Centricon 10 (10 000-Da cutoff) by centrifugation at 6000 × g until the sample volume was reduced to about 0.2 mL.

Proteins were separated in the first dimension in 1.5 × 135 mm gel rods by isoelectric focusing for 14 000 V h with carrier ampholytes of either pH 4–6.5 or pH 3–10 (Pharmacia). At the end of an isoelectric focusing run, the gels were eluted from the tubes into individual vials containing 1 mL of "equilibration buffer"—100 mL of glycerol (Fisher Scientific), 500 mL of Tris buffer (pH 6.8, 24.5 mmol/L), 400 mL of de-ionized water, 20 g of SDS (Sigma Chemical Co., St. Louis, MO), and 0.1 g of bromphenol blue (Serva Feinbiochemica, Heidelberg, F.R.G.)—then, without delay, frozen on solid CO2.

The second-dimension electrophoresis was in a slab gel (160 × 170 × 1.5 mm) with a linear polyacrylamide gradient from 10% to 20%, and was carried out at 0.06 A per gel for about 4 h until the bromphenol blue dye reached the end of the gel. After electrophoresis, the proteins were made
visible by either silver staining (11) or immunostaining as described later.

Electrotransfer and immunological detection of HDL-Apos on nitrocellulose membranes. The general electroblotting method of Towbin et al. (19) as modified by Burnette (20) was used to transfer proteins from 2DE gels to nitrocellulose. We used a "Trans Blot" electrophoretic transfer cell (Bio-Rad Labs) at 60 V and at a constant temperature of 15 °C overnight. The nitrocellulose (0.45-μm pore size) was from Schleicher & Schuell, Keene, NH. The electrotransfer buffer consisted of 25 mmol/L Tris HCl buffer, pH 8.3, containing 43.34 g of glycine and 200 mL of methanol per liter.

After the electrophoretic transfer was completed, we soaked the nitrocellulose in phosphate-buffered isotonic saline (PBS, pH 7.2) containing, per liter, 10 g of nonfat dry milk (Carnation Company, Los Angeles, CA) for 1 h; the milk protein blocks any unbound protein-reactive sites on the nitrocellulose. To wash the nitrocellulose, we then shook it four times, for 5 min each, with PBS containing polyoxyethylene (20) sorbitan monolaurate (Twee 20), 0.5 mL/L, but no milk.

Goat anti-human primary antisera to each individual HDL-Apo (Apo A-I, A-II, and C) in PBS buffer with nonfat milk were added to the nitrocellulose sheet and incubated for 1 h at room temperature, then washed as described above. Rabbit anti-goat secondary antibody was prepared in PBS-Tween 20 containing 10 g of nonfat milk per liter, added to a tray containing the nitrocellulose sheet, and incubated at 37 °C for 30 min, with constant agitation. We then washed the nitrocellulose as described above. A goat anti-rabbit tertiary antibody was introduced, and the contents were incubated at 37 °C for 30 min. The wash procedure was repeated.

Three milligrams of 3,3-diaminobenzidine added to 2 mL of dimethyl sulfoxide (Burdick and Jackson Laboratories Inc., Muskegon, MI) was dissolved in 100 mL of PBS-Tween 20 containing 25 μL of hydrogen peroxide solution (300 mL/L), then poured onto the nitrocellulose to develop a visible precipitate. The reaction was stopped by adding de-ionized water. The antibody concentrations were determined by titration experiments for optimal concentrations.

Results

The creatinine clearance ranged from 65 to 128 mL/min in the control subjects and from 32.5 to 40 mL/min (significantly different, P <0.001) in the patients.

Serum lipids and lipoprotein. The concentrations of cholesterol, HDL-cholesterol, triglycerides, Apo A-I, and albumin in serum and the daily urinary protein loss are shown in Tables 1 and 2. We observed no significant sex-related differences in the volunteers with respect to lipids and albumin in serum or protein concentrations in urine. Apo A-I values were not significantly sex-related in the patients but were significantly different (P <0.01) in the volunteer group (men 107.92, SD 0.21 g/L; women 129.71, SD 0.41 g/L). Serum cholesterol and triglycerides and urinary protein were increased in the patients; serum albumin, Apo A-I, and HDL-cholesterol were decreased (Table 2).

Identification of HDL-Apos in urine. Examples of 2DE protein patterns of serum HDL and of urinary protein separated at d = 1.21 (i.e., HDL-like particle) are shown in Figure 2. These 2DE protein patterns show several similarities and some major differences. The positions of Apo A-I, A-II, and C in the 2DE protein patterns are similar, with Apo A-I exhibiting several isoforms in both serum and urinary HDL. Also, the molecular mass and isoelectric points of serum and urinary Apo A-I isoforms were almost identical. The positions of Apo A-II and Apo C were superimposable (i.e., identical in the electrophoreograms). In addition, the Apo A-I was the predominant protein in the electrophoreograms.

Differences between the protein patterns of HDL from serum and urine in the 2DE electrophoreograms were also apparent. These differences were most evident just below the Apo A-I isoforms. A cascade of proteins that differed in size and charge were revealed in the urinary HDL gels, but these proteins were absent in the serum HDL gels. Visual comparisons of 2DE HDL proteins in urine specimens from men and women revealed no sex-related differences.

The 2DE protein pattern of urine separated at d = 1.063 showed no proteins in the gels (not shown), even in the position corresponding to Apo B, indicating that VLDL and LDL particles were absent from the urine of healthy volunteers. In addition, immunostaining with antisera specific for Apo B detected no proteins in this region, even though the detection level of this technique is in the low nanogram range.

Immunoreactivity of Apo in urine. Further identification of HDL-Apos separated by 2DE was confirmed by immunodetection. Figure 3 illustrates HDL-Apo A-I from urine immunostained with antisera to Apo A-I, showing the main Apo A-I isoforms and a cascade of proteins that differ from the main isoforms both in size and charge. The molecular masses of these cascade proteins appear to range from 500 to 2000 Da smaller than the main Apo A-I
isoforms. Thus, these cascade variants have Apo A-I immunoreactivity but different size characteristics. Other HDL-Apos, A-II and C, were similarly identified (not shown).

Identification of Apos in whole urine. Figure 4 shows typical examples of 2DE patterns of concentrated HDL and other proteins in 24-h urine. The urinary protein patterns from healthy individuals were similar to those from patients with glomerular disease, although qualitative differences were apparent. Quantitatively, Apo A-I was detected by silver-staining in 2DE gels of 24-h urines specimens from healthy volunteers after they had been concentrated by more than 450-fold (Figure 4, left), whereas Apo A-I could be detected in 24-h urines of glomerular disease patients after the urine had been concentrated less than 100-fold (Figure 4, right). Apo A-II and C were difficult to identify in the 24-h urines of both normal subjects and patients with glomerular disease. Normally, these proteins in HDL constitute less than 25% of the total protein content. The 2DE pattern of urinary HDL, separated by sequential ultracentrifugation, however, showed the presence of these proteins (Figure 2, panels A and B), confirming their presence in 24-h urines from both patients and healthy volunteers.

Discussion

Plasma-derived apolipoproteins in urine may establish urinary profile patterns that would be useful for diagnosing glomerular disease. In normal persons the glomerular selectivity excludes high-molecular-mass proteins (>60 000 Da), whereas in glomerular disease the selectivity is altered, allowing more of the high-molecular-mass proteins found in plasma to pass through and appear in urine. The molecular mass of HDL ranges from 175 000 to 360 000 Da (21), and the masses of HDL-Apos are <46 000 Da (22). Thus, HDL-Apos could be excreted unhindered if they were present as single proteins, even though intact HDL would be retained in plasma. Measurable quantities of HDL particles have, however, been reported in urine of normal subjects (6),
suggesting passage of intact HDL through the glomerular apparatus. The 2DE patterns of HDL protein from both the serum and the urine in this study (separated ultracentrifugally at d > 1.21) were similar, suggesting that urinary HDL is derived from plasma. The HDL proteins from serum and urine have similar charge characteristics, size, apolipoprotein distributions, and immunoreactivity. This indicates that the urinary Apo C and A-II were only detected in 2DE gels of highly concentrated urinary HDL is consistent with the normal distribution and lower concentrations of these proteins in native HDL.

This study has further characterized HDL in urine and the qualitative distribution of proteins associated with the plasma HDL-like particle. The study of urinary HDL by 2DE analysis in renal disorders may yield useful information for detecting and differentiating various types of renal disease, primarily distinguishing glomerular from tubular disorders.

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