Preparation of Soluble Apolipoproteins A-I, B, and C-II by a Chromatofocusing Column Method, and Evaluation of Their Concentrations in Serum in Pulmonary Disease

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A chromatofocusing column method for isolating ApoB is described. LDL is first isolated by sequential ultracentrifugation and delipidated with n-butanol/diisopropyl ether. Chromatofocusing of ApoC-DL yielded a large ApoB peak at pl 5.0–5.3. ApoA-I and ApoC-II were prepared analogously, with HDL and VLDL as the source of apoprotein. Antisera were raised in rabbits, and electroimmunoassay techniques were used for determination. ApoB was water-soluble after chromatofocusing. Intra-assay precision (CV) was 4.7% for ApoA-I, 7.8% for ApoB in the "rocket" electrophoresis. Interassay precision (CV) was 6% for ApoA-I and 8% for ApoB. Apolipoprotein concentrations were measured in subjects who had undergone lung resection and patients with obstructive pulmonary disease. After lung resection, the concentration of ApoA-I in serum was significantly decreased (p < 0.001) and that of ApoB significantly increased (p < 0.001) as compared with controls. The ApoA-I/ApoB ratio was significantly lower in the lung-resection group. ApoA-I and ApoB concentrations were unchanged in obstructive pulmonary disease. ApoC-II concentrations in each group were similar to those for control subjects. Of the lipids, values for total cholesterol were above normal after lung resection (p < 0.002), as those for triglycerides (p < 0.02).

Additional Keyphrases: lung resection · chronic obstructive pulmonary disease · electroimmunoassay standardization · cholesterol · triglycerides · "rocket" immunoelectrophoresis

The liver and intestine evidently are the main sites of synthesis of apolipoproteins and formation of lipoprotein particles (1). Normal synthesis of apolipoproteins is essential for the formation of these particles. ApoB appears to be necessary for the transport of triglycerides from liver and intestine, and ApoB-containing lipoproteins can be viewed as the transport medium for plasma triglycerides (2). Triacylglycerols are transported in the blood in large particles, mainly in chylomicrons and very-low-density lipoproteins (VLDL) (3, 4). The triacylglycerols within these particles are hydrolyzed by lipoprotein lipase (LPL; EC 3.1.1.34) in such extrahepatic tissues as adipose tissue, muscle, and lung (5). This enzyme is activated by a peptide, apolipoprotein C-II (ApoC-II) (6). For normal lipoprotein metabolism the reaction catalyzed by lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) is essential. Apolipoprotein A-I (ApoA-I) is its specific cofactor and is the main structural protein in HDL (7). Determination of ApoB or the ratio ApoA-I/B can perhaps be used to evaluate the risk for atherosclerosis (8).

There are few reports concerning different pulmonary diseases and lipid metabolism. The pulmonary capillary bed is the first large endothelial surface with which chylomicrons come in contact after their secretion into the plasma compartment. In addition to respiration and a role in the regulation of steroid metabolism (9–11), synthesis of the surface-active material that lines the alveoli is an essential function of lung tissue (12). In addition to taking part in the synthesis of surfactant, pulmonary LPL may act to remove accumulated triglyceride-rich particles from the lungs in cases of fat embolism (14); LPL activities increase in both serum and lung during experimental fat embolism (15).

We describe here a chromatofocusing method for isolating soluble ApoB from the LDL fraction. In addition, electroimmunoassay standardization is described for it and ApoA-I. Furthermore, we have measured different lipids, ApoA-I, ApoB, ApoC-II, and enzyme activities in subjects with lung resection and in patients with chronic obstructive pulmonary disease to see if these impaired lung functions have any effect on these variables.

Materials and Methods

Subjects

Patients. We studied 15 patients who had had lung resection: four women, ages 49 to 63 years, and 11 men, ages 51 to 69 years. The obstructive-disease group consisted of eight men, ages 43 to 63 years.

Controls. The control group consisted of 35 persons: 21 men, ages 25 to 43 years, and 14 women, ages 25 to 38 years. None had any history of lung disease. They were selected without conscious bias from the staff and clients of the Rehabilitation Research Centre of the Social Insurance Institution, Turku.

Procedures

Preparation of lipoproteins. All lipoprotein fractions were isolated by ultracentrifugal flotation in a Kontron TGA 65 ultracentrifuge. The centrifugations were performed at 8°C for 18 h at 105 000 × g (16). Densities were adjusted with KBr. VLDL were isolated by ultracentrifugation of pooled normolipemic sera at d = 1.006. After centrifugation, VLDL were refloated at d = 1.006, with use of NaCl solution to remove contaminating proteins. This fraction was used for ApoC-II isolation (17). LDL and HDL were isolated at d = 1.063 and 1.210, respectively. These fractions were also washed at appropriate densities.

Lipoprotein fractions were delipidated by a method modified from Cham and Knowles (18). The extraction mixture consisted of n-butanol/diisopropyl ether (1:2 by vol), with extraction at room temperature. After a 1-h extraction, two

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5 Nonstandard abbreviations: Apo, apolipoprotein; LPL, lipoprotein lipase; VLDL, very-low-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; d, relative density (specific gravity).

Received April 25, 1983; accepted June 20, 1983.
additional extractions were carried out with diisopropyl ether. The residual ether was removed at 37 °C, under reduced pressure.

Isolation of apolipoproteins A-I, B, and C-II. ApoA-I, ApoB, and ApoC-II were fractionated by a chromatofocusing method (19). The source of ApoA-I was apoHDL, and A-I was eluted at pH 7.7, as described earlier (20). When delipidated LDL (apoLDL) was chromatofocused, the column was equilibrated with imidazole hydrochloride (35 mmol/L, pH 7.7) containing, per liter, 3 mol of urea and 5 mL of Triton X-100 surfactant. ApoLDL (50 mg) was eluted with pH 4.1 "Polybuffer 74 HCl" (Pharmacia Fine Chemicals, Uppsala, Sweden) diluted sixfold with distilled water containing 3 mol of urea and 5 mL of Triton X-100 per liter. After elution, fractions corresponding to the ApoB peak were dialyzed overnight against a pH 7.4 solution containing, per liter, 10 mmol of Tris, 100 mmol of NaCl, 10 mmol of NaN3, and 100 mg of EDTA, then for 30 h against distilled water. The dialyzed fractions were lyophilized and stored at −80 °C. ApoC-II was fractionated from apoVLDL as described elsewhere (17). Homogeneity of all apoproteins used in this study was checked by polyacrylamide gel electrophoresis in SDS and urea (21), by a modified Laemmli discontinuous buffer system (22), and by double immunodiffusion techniques with use of specific antisera raised against each apoprotein.

Preparation of antisera. Antiserum against ApoC-II was prepared as described elsewhere (17). Anti-ApoA-I and anti-ApoB were prepared in New Zealand White rabbits. The purified proteins, dissolved in sterile isotonic saline and mixed with equal volumes of Freund's complete adjuvant, were injected into the upper back of rabbits. Three booster injections were given at intervals of four weeks, again with the proteins solubilized in saline and mixed with incomplete adjuvant. With this schedule, rabbits produced precipitating antibodies of sufficient titer. Antisera to ApoA-I, ApoB, and ApoC-II were found to be specific, as judged from double immunodiffusion according to Ouchterlony (23).

Electroimmunoassay of apoproteins. ApoC-II rocket electrophoresis was performed as described earlier (17). ApoA-I and ApoB concentrations were determined by quantitative immunoelectrophoresis according to Laurell (24). Antiserum concentrations were 5 µg/mL of gel for ApoA-I and 7.5 µg/mL of gel for ApoB. Agarose, 10 g/L of barbital buffer (10 mmol/L, pH 8.7, ionic strength 0.02) containing, per liter, 70 mmol of glycine and 40 mmol of Tris, was the best combination for quantifying both ApoA-I and ApoB. Wells 4 mm in diameter were filled with 10 µL of serum diluted with buffer containing 10 mL of Triton X-100 per liter, 50-fold for ApoA-I and 30-fold for ApoB determination. Electrophoresis was for 2.5 h in the case of ApoA-I, 3.5 h for ApoB. The slides were then blotted, thoroughly washed in saline, dried, and stained with Blau R (Serva, Heidelberg, F.R.G.). The basic calibration curves were constructed with purified apoproteins dissolved in electroimmunoassay buffer. Pooled normal serum, for which the ApoA-I and B concentrations had previously been established by electroimmunoassay against ApoA-I and B reference material prepared as described above, was used for secondary calibration (1:30, 1:40, 1:60, and 1:80 dilutions for ApoA-I; 1:10, 1:20, 1:30, and 1:40 dilutions for ApoB). Pooled serum was frozen batchwise at −80 °C.

To expose antigenic determinants of ApoA-I and ApoB, we used 10 mL of Triton X-100 per liter in the barbital sample diluent. This slightly increased the measured ApoA-I and ApoB content and improved the shape of the rockets. The curve was linear over the range 0.6–2.4 g of ApoA-I per liter of serum (r, the coefficient of regression, was 0.996).

The curve for ApoB was slightly curvilinear and the calibration curves for the assay were used in the concentration range 0.4–2.5 g of ApoB per liter of serum (r = 0.981, for the whole range, including curvilinearity). The nonlinear region was omitted. The intra-assay CVs calculated from measurements on 10 samples of pooled serum were 4.7% for ApoA-I and 7.8% for ApoB. Corresponding interassay values were 6% for ApoA-I, 8% for ApoB.

Lipid analysis. Venous blood for serum analysis was sampled the morning after an overnight fast. Concentrations of total cholesterol, free cholesterol, and triglycerides in the serum was measured by enzymatic assays (Boehringer Mannheim GmbH, Mannheim, F.R.G.). HDL-cholesterol was measured enzymatically after VLDL and LDL were precipitated with dextran sulfate/MgCl2 (25).

Results

Isolation of Apoprotein B

We found the modified chromatofocusing column technique to be suitable for fractionating ApoB as well as ApoA-I and ApoC-II (17, 20). ApoB eluted in the pl range 5.0–5.3 (Figure 1), and this large peak had two shoulders (II-1 and II-2). Two other peaks eluted, just at the beginning of the run (pl 7.6) and during salt elution (peaks I and III). These protein fractions did not react with the antisera to fraction II. The proteins represented by the shoulders II-1 and II-2 gave a slight reaction with this antisera.

Electroimmunoassay of ApoA-I and ApoB

Figures 2 and 3 show standard curves for the ApoA-I and ApoB electroimmunoassay. When the standard curves were constructed with pooled serum containing known amounts of ApoA-I and ApoB, the reactions with antisera were similar and the curves were parallel.

The temperature at which the secondary standard is stored is critical. ApoB values significantly declined, from 820 to 650 mg of ApoB per liter, within two months when the standard was preserved at −20 °C. The decline began after one month of storage, until which time ApoA-I and ApoC-II values did not change. This effect was not found when storage was at −80 °C.

ApoA-I, ApoB, and ApoC-II in Controls and Patient Groups

Apoprotein concentrations are presented in Figure 4. ApoA-I concentration was significantly lower in lung-resection patients, the concentrations being about 68 and 72% of the values for the obstructive-disease and control groups,

![Fig. 1. Chromatofocusing of delipidated apoLDL in a bead-formed polybuffer (PBE 94; Pharmacia, Uppsala, Sweden) excchanger gel column](image-url)

Column bed height 32 cm, diameter 1 cm. Sample volume: 5 mL, containing 50 mg of apoLDL. Elution rate: 25 mL/h. 5-mL fractions were collected, with continuous measurement of absorbance at 280 nm and of pH. Salt elution begun at point indicated by arrow and S (NaCl, 1 mol/L).
ApoA-I rockets

Fig. 2. Relation between rocket height and ApoA-I content of purified ApoA-I (X) and secondary standard (C).
The regression equation refers to the curve made with purified ApoA-I (X). Insert: typical patterns for different concentrations of ApoA-I standards (the first four rockets at the left side for secondary standard, and the other four rockets for purified ApoA-I).

Lipid Analysis

Figure 5 depicts the total cholesterol, free cholesterol, HDL-cholesterol, and triglyceride concentrations in the experimental groups. As compared with the control group (Figure 5), the lung-resection group had higher cholesterol ($p < 0.002$) and triglyceride ($p < 0.02$) concentrations. There were no differences in these lipid values between chronic obstructive patients and controls. HDL-cholesterol showed a tendency to be higher in the obstructive-disease group than in the lung-resection group. HDL-cholesterol values did not differ between the lung-resection group and controls.

Discussion

Many different techniques have been applied to obtain purified apolipoproteins from lipoprotein fractions, the most common probably being gel filtration and ion-exchange chromatography (26, 27). However, these methods are quite laborious and time consuming. As earlier reported (19, 20), the chromatofocusing technique is useful for apoVLDL and apoHDL fractionation. When delipidated apoLDL was chromatofocused with detergent in the elution buffer, the fraction containing ApoB maintained its water-solubility after lyophilization and also was soluble in electroimmunoassay buffer (pH 8.7). In addition, with different apoLDL preparations the principal soluble ApoB peak repeatedly appeared in the same pH range. The insolubility of this protein in water after isolation is certainly the main reason for the slow progress in elucidating its primary structure or its use for method standardization (28, 29). In the solution, protein precipitation was noticed if the pH was <8.0. This pH-dependence of ApoB precipitation varies with the detergent used. A DEAE–Sepharose column method (30) also yields apolDL that is water soluble.

Immunochromic quantitation procedures demand that, in the assay system being used, the antigen in the test samples must behave identically with the standard samples (31). In this study, standardization of our pool serum against different ApoA-I and ApoB preparations gave reproducible results, and the rockets formed by ApoA-I and serum and by ApoB and serum had the same shape. This agrees well with earlier results for ApoA-I (32, 33). Of all the apoproteins, standardization of ApoB has been one of the most difficult. Several methods have used earlier to isolate LDL preparations for quantification of ApoB (34, 35). However, during storage at either 4°C or −20°C solutions of LDL reportedly (36) became opalescent, precipitate formed, and there was loss of ApoB immunoreactivity. Using plasma samples, Havekes (36) found ApoB immunoreactivity to be constant until −80°C for at least 20 days; this accords well with our observations. We found that pooled serum containing known amounts of ApoB as the assay standard could be stored at −80°C with no change in immunoreactivity for six months.

The linear concentration ranges used for ApoA-I and ApoB assays in this study agree well with recently published values; the range reported for ApoA-I was 0.75–3.90 g/L (32) and for ApoB 0.50–2.40 g/L (35) and 0.55–2.10 g/L (37).

For the patient group with lung resection we found significantly diminished ApoA-I and significantly greater ApoB concentrations as compared with controls. Earlier, workers found significant decreases of ApoA-I and HDL-cholesterol concentrations during peripheral vascular disease (38). In the lung-resection group HDL-cholesterol was unchanged. There appear to be at least two ApoA-containing particle populations in the HDL fraction (39). Possibly ApoA-I-containing particles decrease relatively more than HDL-cholesterol after lung-resection. Furthermore, because the lung-resection group had high triglyceride concentrations, the fractional catabolism of ApoA-I might be enhanced (40). Reportedly, the best differentiation between
normal individuals and atherosclerotic patients can be obtained by use of the ApoB and ApoA-I/B ratio (8, 29). Thus the lung-resection group may have an increased risk for atherosclerotic vascular changes.

A recent report claims that patients with chronic expiratory airflow obstruction and increased work of breathing also have higher HDL-cholesterol concentrations (41), but our study does not confirm this.

The LPL activator ApoC-II concentrations did not differ in the two groups of patients as compared with controls, although the triglyceride concentration was increased in the resection group. In addition LPL activity was the same in each group. Carlson and Ballantyne (42) observed that the ApoC-II/C-III ratio of d < 1.006 lipoproteins was lower in patients with hypertriglyceridemia than in normotriglyceridemic subjects. Perhaps, after lung resection, synthesis of LPL activator protein is less than that of LPL inhibitor protein (ApoC-III) (5), and this may affect pulmonary LPL activity and facilitate slightly higher triglyceride concentrations.

**References**


