papers and notes on methodology

Isolation and characterization of polymorphic forms of porcine apoC-II by chromatofocusing

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Abstract Chromatofocusing, which separates proteins on the basis of their different isoelectric points, was used to isolate isoforms of apoC-II from porcine very low density lipoproteins. This method was found to be time-saving and the yield of protein recovery was high. With chromatofocusing, three polypeptides were obtained which were characterized by amino acid analysis, double immunodiffusion, and by their ability to activate bovine milk lipoprotein lipase. The three polypeptides had the same amino acid composition, gave a reaction of identity against a monospecific antiserum to porcine apoC-II, but had different isoelectric points between pH 4.8 and 4.4. They all enhanced the activity of lipoprotein lipase, but to a lesser degree than native porcine serum. There was no indication of the existence of apolipoproteins that correspond to human apoC-III polypeptides.—**Knipping, G., E. Steyrer, R. Zechner, and A. Holasek.** Isolation and characterization of polymorphic forms of porcine apoC-II by chromatofocusing. *J. Lipid Res.* 1984. **25:** 86–91.

Supplementary key words lipoprotein lipase activity

The apolipoprotein C-polypeptides of human serum play an important role in the regulation of the catabolism of serum lipoproteins, especially of very low density lipoproteins (VLDL) and chylomicrons. Human apolipoprotein C-polypeptides are designated as apoC-I, apoC-II, and apoC-III. It has been suggested that apoC-I is an activator protein of lecithin:cholesterol acyltransferase (1). ApoC-II is required to achieve maximal activation of lipoprotein lipase (LPL) in vivo and in vitro (2–6). ApoC-III exhibits charge polymorphism, and therefore appears in three different polymorphic forms owing to the differing sialic acid content (7). In vitro, apoC-III can inhibit LPL (8); in vivo it is supposed to be involved in the regulation of LPL activity, but the exact mechanism is not yet known.

In previous studies, an apolipoprotein analogous to human apoC-II was isolated from pig serum (9–11), which, when purified, partially lost its ability to activate LPL. This apolipoprotein represents the most prominent band of the fast migrating polypeptides from delipidated lipoproteins in polyacrylamide gel electrophoresis (PAGE). The present investigation was designed to characterize additional faint bands in the C-peptide region. For isolation of these apolipoproteins, a relatively new method was used, namely chromatofocusing. This method separates proteins according to their different isoelectric points (12).

MATERIALS AND METHODS

Isolation of lipoproteins and apolipoproteins

Porcine serum was prepared from pooled blood of pigs of both sexes by low speed centrifugation. After addition of 1 mg/ml EDTA, VLDL was isolated by ultracentrifugation at plasma density (110,000 *g*, 18 hr, 15°C) in a Beckman 60 Ti rotor. After dialysis against distilled water, the pH was adjusted to 8.2 with ammonium carbaminate, and the VLDL was lyophilized and delipidated with diethylether and ethanol–diethylether 3:1. For comparison with human apolipoproteins, VLDL from one male human donor was isolated and delipidated in the same manner.

For chromatofocusing, a column (40 × 1 cm) was packed with polybuffer exchanger 94 (Pharmacia Fine

Abbreviations: VLDL, very low density lipoproteins; IEF, isoelectrofocusing; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; LPL, lipoprotein lipase.

Chemicals) and overlayered with 1 cm of Sephadex G 25. The column was equilibrated with 25 mM histidine-HCl buffer, pH 6.2, containing 6 M urea. Urea solutions were purified on amberlite CG-120 I (Serva, Heidelberg, G.F.R.) immediately before use. Porcine apoVLDL was extracted twice with 0.01 M Tris-HCl buffer, pH 7.4, containing 6 M urea. About 20 mg of soluble VLDL apolipoproteins (5–10 ml) was applied to the column, followed by 5 ml of 25 mM histidine-HCl buffer. The pH gradient in the column was then developed with 25 ml of polybuffer 74 (Pharmacia Fine Chemicals), diluted to 200 ml, containing 6 M urea, pH 4.0, at 4°C. The flow rate was about 25 ml/hr. The eluant was continuously monitored with a LKB Uvicord II at 280 nm. The fractions were checked by double immunodiffusion in 1% agarose against antibodies to porcine apoVLDL, albumin, and porcine apoC-II (9). They were further analyzed by 10% PAGE (13), 12.5% SDS-PAGE (14), and by analytical isoelectrofocusing (IEF) using a pH gradient of 4 to 6 (15). The isoelectric points of the isolated apolipoproteins were determined by cutting an unfixed and unstained IEF-gel into slices, each of which was transferred into 1 ml of distilled water. After 24 hr, the pH of each segment was measured.

Hydrophobic interaction chromatography was used to remove the polybuffer. For this purpose, a column (5 × 1 cm) was packed with phenyl-Sepharose (Pharmacia Fine Chemicals) and equilibrated with 4 M NaCl. Each of the protein fractions obtained by chromatofocusing was brought to 4 M with NaCl, and applied to the column. The column was then washed with 4 M NaCl until the base line of the recorder was again reached; 20 ml of 10% ethyleneglycol in 0.9% NaCl was then added to the column. The elution of the C-polypeptides was performed with distilled water, adjusted to pH 10 with ammonium carbaminate. The fractions thus obtained were used either without further work-up or were lyophilized after dialysis against distilled water, using a Spectrapor dialysis membrane (Fisher Scientific Co.).

For the determination of the amino acid composition, 50 μg of the isolated apolipoproteins was hydrolyzed in 6 N HCl under vacuum at 110°C for 24 hr. Cysteine was determined by performic acid oxidation (16). The analyses were run on a Biotronic LC 7000 amino acid analyzer.

Analytical methods

After PAGE, the protein bands were stained for carbohydrates with periodic acid according to the procedure of Zacharius et al. (17). For sialic acid digestion, sialidase from *Clostridium perfringens* (EC. 3.2.1.18, Sigma Chemicals, Munich) was used. Four μg of the enzyme was incubated with 100 μg of human or porcine VLDL apolipoproteins in 80 mM ammonium acetate buffer, pH 5.8, at 37°C for 3 hr. Aliquots were removed for PAGE. The protein content was estimated according to Lowry et al. (18), using bovine serum albumin as a standard.

Radial immunodiffusion

For quantification of porcine apoC-II, 6 ml of 1% agarose (Behring Werke, G.F.R.) containing 100 mg of Dextran T 70, 30 mM sodium barbital (pH 8.6) and 0.01% (w/v) NaN_3, were heated, cooled to 56°C, mixed with 0.5 ml of anti-porcine apoC-II (9), and poured on plastic discs. After solidification, 12 wells were punched and 5 μl of either standard or sample solutions was applied. Porcine apoC-II (9) served as the standard, and was diluted to give five different concentrations ranging from 2 to 12 mg/100 ml. To determine their apoC-II content, porcine serum and native VLDL samples were delipidated with equal amounts of 4.2 M tetramethylurea. Standards and samples were allowed to diffuse over a 48–72-hr period. The immunoprecipitations, after being intensified by addition of 0.25% phosphotungstate solution for 15 min, were then measured in 0.1-mm units using a measuring projector (Model VDC 02, Behring Werke, Marburg, G.F.R.). The interassay coefficient of variation was within 6%.

Lipoprotein lipase activity assay

For the determination of the activation properties of the isolated apolipoproteins, we used bovine milk lipoprotein lipase. The enzyme was isolated from fresh milk by heparin-Sepharose affinity chromatography as described by Bengtsson and Olivecrona (19). The assay procedure was based on the method of Schotz et al. (20) with slight modifications. The triolein emulsions contained 1 μCi of tri[1-^{14}C]oleoylglycerol, 1.33 ml of 10% Intralipid®, 6 ml of 20% albumin solution, pH 8.6, 0.9 ml of 1% Triton X-100, and 3.4 ml of 0.4 M Tris-HCl, pH 8.6. The mixture was sonicated for 4 min at 4°C and used immediately after sonification. Activator peptides in different concentrations were added to 0.6 ml of assay mixture and incubated in the presence of 20 μl LPL (10 U/ml) for 20 min at 37°C. The released free fatty acids were determined by the liquid–liquid partitioning system described by Belfrage and Vaughan (21).

RESULTS

Preliminary experiments with a lower or a higher starting pH resulted in a poor separation of the fast migrating apolipoproteins from each other or from the minor components of apoVLDL. Therefore, the pH gradient of 6.2 to 4 was used throughout the study. The elution profile of one typical chromatofocusing experiment is shown in **Fig. 1.** The minor components of porcine apoVLDL,

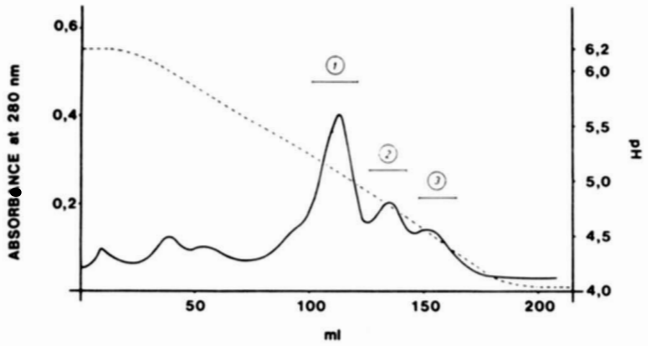

Fig. 1. Chromatofocusing of 20 mg of porcine soluble apoVLDL. The column (40 × 1 cm) was packed with polybuffer exchanger 94 and equilibrated with 25 mM histidine-HCl buffer, containing 6 M urea, pH 6.2. The pH gradient was developed with 25 ml polybuffer 74, diluted to 200 ml, containing 6 M urea, pH 4.0, at 4°C.

e.g., apoE and apoA-I, eluted between pH 6.2 and 5. They were not further analyzed. Three peaks followed between pH 5 and 4, which are designated 1, 2, and 3 in Fig. 1. Peak 1 represents the previously recognized apoC-II; peaks 2 and 3 represent those apolipoproteins that migrate into a position similar to that of human apoC-III_1 and apoC-III_2 in 10% PAGE. The shoulder of peak 1 varied with the amount of material applied to the column and represented dimers of apoC-II, as could be seen from their electrophoretic mobility and immunochemical behavior. **Fig. 2** shows the migration pattern

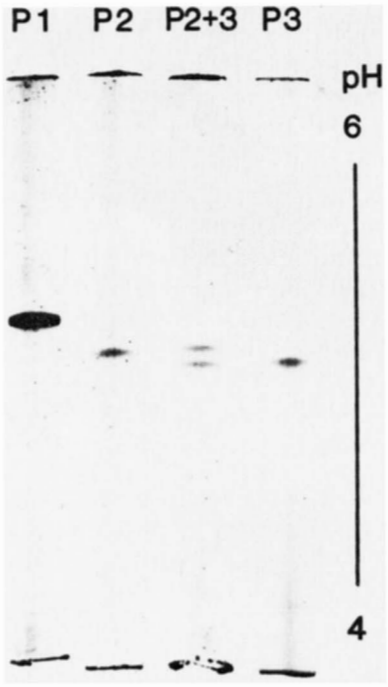

Fig. 2. Migration behavior of the isolated apolipoproteins from chromatofocusing in 7.5% IEF-gels, pH 4–6. P1 corresponds to peak 1; P2 corresponds to peak 2; P2 + 3 is a mixture of apolipoproteins corresponding to peaks 2 and 3; P3 corresponds to peak 3.

of the apolipoproteins corresponding to peaks 1, 2, and 3 in IEF-gels of pH 4 to 6. The isoelectric points were found to be 4.82 ± 0.05, 4.6 ± 0.04, and 4.49 ± 0.04 (n = 4), respectively.

To determine the protein content and the amino acid composition, the polybuffer had to be removed from the system. Precipitation of the apolipoproteins with saturated ammonium sulfate or 20% trichloroacetic acid led to a loss of at least 50% of the material originally applied to the column. Therefore, we used hydrophobic interaction chromatography on phenyl-Sepharose, which removed polybuffer and urea and concentrated the protein solutions at the same time. The yield of protein recovered after chromatofocusing and phenyl-Sepharose chromatography was between 75 and 80%.

The amino acid composition of the isolated polypeptides is displayed in **Table 1.** We could not find any significant difference in the amino acid composition of porcine apoC-II (peak 1) and the two faster migrating apolipoproteins of peaks 2 and 3. All of them lack histidine and cysteine. Upon testing these apolipoproteins by double immunodiffusion against a monospecific antiserum of apoC-II, a reaction of identity was obtained (**Fig. 3**). Therefore, it was possible to measure the content of apoC-II with its isoforms in porcine serum by radial immunodiffusion. We found an amount of 9–13 mg/100 ml of apoC-II, testing 20 different pig sera. This is about two to three times as much as in human serum, where values of 3–6 mg/100 ml are reported (22, 23).

Sialic acid digestion of porcine apoVLDL did not alter the electrophoretic behavior of apoC-II and its polymorphic forms in 10% PAGE, whereas human apoVLDL treated with neuraminidase in the same way exhibited a

TABLE 1. Amino acid composition of apolipoproteins corresponding to peaks 1, 2, and 3 from chromatofocusing experiments

Amino Acid	Peak 1	Peak 2	Peak 3
Asp	111.4 ± 6.8[a]	110.7 ± 5.3	107.9 ± 5.6
Thr	106.9 ± 8.5	104.2 ± 7.6	101.7 ± 9.0
Ser	137.6 ± 10.5	138.6 ± 5.9	134.8 ± 8.2
Glu	134.3 ± 6.0	133.5 ± 5.1	132.7 ± 4.8
Pro	42.6 ± 4.2	42.3 ± 2.6	46.7 ± 3.6
Gly	43.7 ± 2.0	41.0 ± 3.0	44.9 ± 3.2
Ala	72.1 ± 3.5	73.1 ± 2.1	70.3 ± 4.0
1/2 Cys	0	0	0
Val	55.7 ± 4.3	57.2 ± 3.5	58.5 ± 3.1
Met	13.3 ± 6.8	14.4 ± 7.0	13.5 ± 6.0
Ile	18.3 ± 2.7	19.0 ± 2.0	19.4 ± 3.0
Leu	60.4 ± 5.7	62.1 ± 3.1	65.2 ± 4.4
Tyr	35.0 ± 3.6	36.4 ± 2.5	37.1 ± 3.4
Phe	42.7 ± 3.8	43.3 ± 4.0	43.6 ± 4.1
His	0	0	0
Lys	100.1 ± 4.2	96.8 ± 5.2	94.8 ± 5.3
Arg	26.0 ± 2.4	27.4 ± 1.2	28.9 ± 2.2

[a] The values represent average numbers of four different preparations, expressed as mol per 1000 moles.

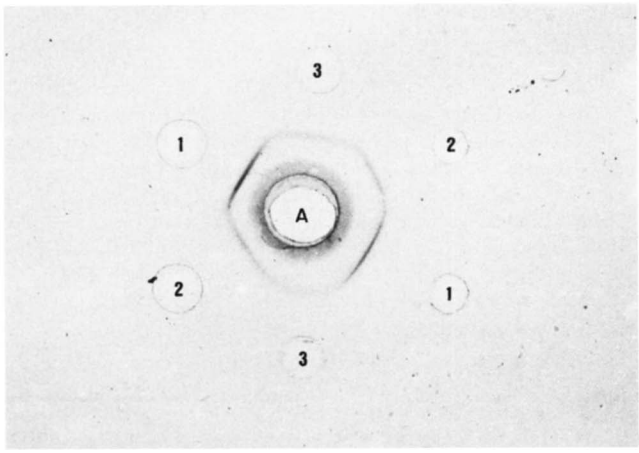

Fig. 3. Double immunodiffusion of isolated apolipoproteins from chromatofocusing. 1 corresponds to peak 1; 2 corresponds to peak 2; 3 corresponds to peak 3. A represents anti-porcine apoC-II.

significant broadening of the apoC-III$_0$ band. Carbohydrate staining demonstrated that none of these porcine polypeptides contains carbohydrates.

In order to investigate the extent to which porcine serum, native VLDL, delipidated VLDL, and the isolated apolipoproteins of peaks 1, 2, and 3 activate LPL, an LPL assay was performed. The concentration of activator proteins added to this assay was determined by radial immunodiffusion. As illustrated in **Fig. 4,** 200–250 μl of porcine serum, corresponding to 20–25 μg of apoC-II, was required to achieve maximal activation. Native fresh VLDL enhanced the activity of LPL to the same extent as porcine serum, but lost its ability for activation within a few days. In contrast, delipidated VLDL and the isolated apolipoproteins of peaks 1, 2, and 3 never produced maximal activation of LPL in our assay system. Unlike porcine serum and native VLDL, addition of apoVLDL resulted in a lower enhancement of LPL activity, achieving 85% of the maximal value. The polypeptides of peaks 1 and 3 activated LPL equally, with a maximal stimulation of about 25% of that of porcine serum. For the apolipoprotein of peak 2, the maximal stimulation was approximately 45–50% of that with porcine serum. It is noteworthy that after storage at −20°C, the isolated apolipoproteins totally lost their activating ability within 1 month, whereas porcine serum did not lose this property even after repeated freezing and thawing.

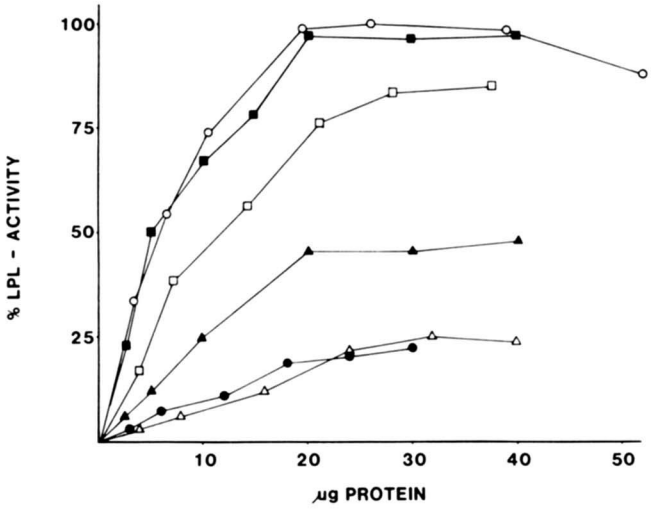

Fig. 4. Activation of LPL by: porcine serum (○); native VLDL (■); delipidated VLDL (□), and the polypeptides corresponding to peak 1 (△), to peak 2 (▲), and to peak 3 (●) from chromatofocusing.

DISCUSSION

Several different methods are used for the isolation of apolipoproteins (24–28). Some of them are time-consuming, or the yield of recovered protein is very low. Separation of proteins by chromatofocusing in a narrow pH range only takes about 8 hr. The necessary removal of the polybuffer is a disadvantage. This problem can easily be overcome, however, using phenyl-Sepharose chromatography. This requires another 1 or 2 hours (depending on the fraction volume) until a desalted, concentrated sample is obtained. The yield of recovered protein after chromatofocusing and phenyl-Sepharose chromatography was about 75–80%.

With chromatofocusing we were able to isolate three polypeptides, which seem to represent different forms of the same apolipoprotein, namely apoC-II. This was verified on the basis of amino acid compositions, their reaction against an antibody to porcine apoC-II, and their activation of LPL. The reason for their differing migration in PAGE could not be established. The main polymorphic form (peak 1 in Fig. 1) accounts for about 65–70% of apoC-II, while that of peak 2 represents about 20–25%, and that of peak 3 less than 10%. The results of eight different VLDL preparations and chromatofocusing runs demonstrated that there is no indication for the existence of proteins corresponding to human apoC-III polypeptides, with respect to their carbohydrate content, their immunochemical behavior, and the amino acid analyses.

Recently, Havel, Kotite, and Kane (25) found that a second form of apoC-II in human VLDL, which also contained no carbohydrate residues, showed no significant difference in the amino acid composition to the previously described apoC-II, yet migrated faster in PAGE or IEF. In contrast to our findings on porcine activator proteins, this second form of human apoC-II activated LPL to the same extent as the previously recognized one. In former studies on pig apolipoproteins, Fidge (10) found no enhancement of LPL activity by isolated porcine apoC-II.

Jackson et al. (11), however, demonstrated that porcine apoC-II, isolated by DEAE-chromatography, showed only one-thousandth the activity of the crude peak of small molecular weight proteins derived from Sephadex G 150 chromatography. The reason that the porcine apolipoproteins retained more ability to activate LPL in our study may be the less time-consuming isolation procedure. Our results show that only native VLDL can activate LPL to the same extent as porcine serum, while delipidated VLDL and the isolated polypeptides lost their activating ability to a considerable extent.

It has been suggested that for maximal activation of LPL, apoC-II has to interact with the phospholipids of VLDL and chylomicrons as well as with the enzyme (29). It seems reasonably obvious that the structure of porcine apoC-II becomes changed during delipidation and storage, and that the hydrophilic sites of the polypeptide therefore cannot fully interact with the enzyme. Since human apoC-II maintains its ability for activation, the structural features of human and porcine apoC-II appear to be different. In a recent study by Astrup and Bengtsson (28), three activator proteins were isolated from bovine plasma which may also represent isoforms, since their amino acid composition is only slightly different, but distinct from that of human and porcine apoC-II. Unfortunately, nothing is reported about their activating ability in the native or delipidated state. We conclude that porcine and human apoC-II show structural differences, which may have consequences on the activation of LPL. Additionally, the absence of apolipoproteins corresponding to human apoC-III indicates that LPL activation in the pig may be regulated by an alternative mechanism.

We are indebted to Miss A. Birchbauer for excellent technical assistance and to Mrs. G. Hödl for writing the manuscript. This work was supported in part by the Österreichischer Fonds zur Förderung der wissenschaftlichen Forschung under project number P-5158, and by the Österreichische Nationalbank.

Manuscript received 18 August 1983.

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