papers on methodology

Rapid determination of apolipoprotein E phenotypes from whole plasma by automated isoelectric focusing using PhastSystem™ and immunofixation

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Abstract The influence of the genetic apolipoprotein (apo) E isoforms on human plasma lipoproteins is well established. There is, however, still a need for a phenotyping procedure applicable in laboratories not specialized in lipid research. To this end, we developed a rapid, automated electrophoresis method for apoE phenotyping. Either self-made or commercially available precasted gels can be used. Fresh or frozen samples corresponding to 0.1 μl of plasma are applied automatically after lipid extraction in a urea-containing buffer onto the gel and isoelectric focusing is carried out for 45 min. Thereafter, apoE bands are precipitated by specific polyclonal antibodies and visualized by automated silver staining. The method is reliable, easily and quickly performed, and not restricted to specialized laboratories.—**Hackler, R., J. R. Schäfer, S. Motzny, S. Brand, T. O. Kleine, H. Kaffarnik, and A. Steinmetz.** Rapid determination of apolipoprotein E phenotypes from whole plasma by automated isoelectric focusing using PhastSystem™ and immunofixation. *J. Lipid Res.* 1994. **35:** 153–158.

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Human apolipoprotein E is a protein constituent of plasma chylomicrons, very low density lipoproteins, intermediate density lipoproteins, and high density lipoproteins. The 299 amino acid-containing glycoprotein obviously plays a regulatory role in lipoprotein metabolism by interacting with the apoB/E (LDL) receptor and also with the low density lipoprotein receptor-related protein (1–3).

Three common apoE isoforms occurring in the population (apoE2, E3, and E4) are coded for by three alleles (ε2, ε3, and ε4) at the apoE gene locus (4, 5). The charge difference of these isoforms was shown to be due to cysteine for arginine substitutions at positions 112 and 158 of the amino acid sequence (6, 7). Although significant differences in the apoE allele frequencies were reported among populations, apoE3 has always been the predominant isoform and the effect of the apoE type on cholesterol levels did not differ significantly among the populations (reviewed in refs. 8–10). Compared to ε3, the ε2 allele lowers total plasma cholesterol, apoB, and LDL cholesterol levels, and increases apoE levels, whereas the opposite is true for ε4 (11; for a review, see refs. 8 and 12). Although there exist conflicting results about the influence of the apoE isoforms on the development of atherosclerosis (for a review, see ref. 12), phenotyping is increasingly performed for risk assessment. In addition, the diagnosis of type III hyperlipidemia requires apoE phenotyping and rare mutants of apoE may lead to dyslipoproteinemias (for a review, see ref. 12).

Numerous methods have been published to determine apoE phenotypes, initially from isolated VLDL, later on from whole blood plasma (reviewed in ref. 12), including the development of site-specific monoclonal antibodies (13). The procedures available today are still laborious in that they require immunoblotting or the preparation of special gels by the user. Here we report on a simple procedure also possible to be performed with commercially available gels. In addition, the method avoids immunoblotting by introducing specific immunofixation. This method is no longer restricted to specialized lipoprotein laboratories.

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; HDL, high density lipoproteins; IEF, isoelectric focusing; Vh, volt-hours; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate-disodium; HAc, acetic acid; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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SUBJECTS, MATERIALS AND METHODS

Subjects

Plasma from patients of the lipid outpatient clinic in the department of Endocrinology and Metabolism was used. Blood was drawn from subjects, after overnight fasting, into tubes containing EDTA (1 mg per ml of blood). Plasma was then immediately obtained by low speed centrifugation (3500 *g*, 20 min at 4°C). In a few cases blood samples were drawn in parallel into tubes without EDTA and allowed to clot at room temperature for 1 h. Serum was then obtained as described for plasma.

Materials

Ethanol, ether, ammonium peroxydisulfate, urea, sodium acetate, sodium chloride, calcium chloride, acetic acid, Tris, TCA, glutardialdehyde, silver nitrate, sodium carbonate, sodium thiosulfate pentahydrate, and formaldehyde of analytical grade were obtained from Merck (Darmstadt, Germany). Acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'tetramethylenediamine (TEMED) were provided by Serva (Heidelberg, Germany). DTT was provided by Sigma Chemie (Deisenhofen, Germany). Decyl sodium sulfate was from Kodak Eastman Company (Rochester, NY). Pharmalytes pH 5–6 and 5–8 were purchased from Pharmacia/LKB (Freiburg, Germany).

Antibodies to human apoE were produced in rabbits as described (14). In addition, anti-human apoE antibodies were provided by Behringwerke (Marburg, Germany) (rabbit antibodies) or by Immuno GmbH (Heidelberg, Germany) (sheep antibodies). Further rabbit antibodies were purchased from Daiichi (Tokyo, Japan), goat antibodies from Greiner Biochemica (Flacht, Germany), from Immuno-Chimie Internationale (Paris, France), or from Midland Bioproducts Corp. (Scarborough, ME).

Methods

Apolipoprotein E phenotyping by established procedures. The apoE phenotypes of the patients investigated in the study presented here had been determined by established procedures, either by immunoblotting after isoelectric focusing from plasma or after isoelectric focusing in immobilized pH gradients of apoVLDL (12, 14).

ApoE quantification. To evaluate the sensitivity of the IEF-immunofixation procedure, total plasma apoE levels were determined by electroimmunodiffusion as previously outlined (15).

Sample preparation. Ten microliters of plasma or serum were injected into 2.5 ml of a mixture of ethanol–ether 3:1 (v/v) in capped Eppendorf reaction tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) at −20°C. Samples were stored in the solvent at −20°C (for a minimum time of 2 h) until IEF was carried out. The tubes were then centrifuged at 0°C for 15 min at 2000 *g*. The solvent was removed by aspiration and the pellet was suspended for 1 h in 2 ml cold ether (−20°C). Centrifugation was repeated, the solvent again aspirated, and the wet pellet was dissolved in 100 μl of 100 mmol/l Tris-HCl buffer, pH 8.0, containing 3 mol/l urea, 0.1% decyl sodium sulfate, and 0.2 mmol/l DTT (sample solution). The ether delipidation did not lead to apparent artificial bands. Alternatively, chloroform–methanol solvent could be used for lipid extraction. Ten microliters plasma was vortexed for 10 min at room temperature with 1.5 ml of chloroform–methanol 2:1 (v/v) in capped Eppendorf reaction tubes. Then 0.5 ml methanol was added and vortexing was continued for an additional 5 min. After centrifugation at 2000 *g* for 15 min, the solvent was removed and the pellet was processed as above.

One microliter of this sample solution was then applied to each lane of the IEF gel, when 8 samples per gel were analyzed. Alternatively, 0.3 μl of the sample solution was applied to each lane of the IEF gel, when 12 samples per gel were analyzed.

Production of IEF gels. In addition to the use of commercially available gels (CleanGels™) we produced IEF gels as follows. Polyacrylamide gels (T = 5%, C = 3%; 43 × 50 × 0.45 mm) adhering to a plastic support film (GelBond™ PAG film, Biozym-Diagnostik, Hameln, Germany) were prepared in a capillary casting mold similar to that described by Esen (16). To prepare two gels, 0.72 g solid urea, 1.0 ml acrylamide stock solution (19.4% w/v acrylamide; 0.6% w/v bisacrylamide), 220 μl Pharmalyte 5–6, 30 μl Pharmalyte 5–8, 6 μl TEMED were mixed and made up to 3.72 ml with double-distilled water. After degassing with a vacuum pump, 0.28 ml of a 1.2% (w/v) of freshly prepared ammonium peroxydisulfate solution was added and the solution was transferred into the mold. When polymerization was complete (after approximately 30 min) gels were ready for use.

Preparation of ready-made IEF gels. Ready-made dehydrated gels (CleanGels™) were used as provided by the manufacturer (Pharmacia/LKB, Freiburg, Germany). These gels were rehydrated for 2.5 h at room temperature with 800 μl of a solution containing 3 mol/l urea, 10 μl Pharmalyte 5–8 and 40 μl Pharmalyte pH 5–6, in a reswelling device (GelPool™, Pharmacia/LKB, Freiburg, Germany).

Isoelectric focusing. Various conditions for sample treatment and IEF (reswelling solution, pH gradient, sample application area, focusing time) were tested and the final conditions are outlined. Time required is given in brackets.

Gels were placed on the cooling plate of the Phast-System™ (2 min) and prefocused for 75 Vh at 2000 V (7 min). One μl or 0.3 μl of the sample solution, respectively, per lane was pipetted directly onto the applicator (Sample Applicator 8/1 or 12/0.3, Pharmacia/LKB, Freiburg, Germany) (3 min). Applicators were inserted into the sample applicator arm (closest position to

TABLE 1. Protocol the IEF conditions for separation of apoE isoforms using the PhastSystem™

Sample applied down at			1.2	0 Vh	
Sample applied up at			1.3	100 Vh	
Extra alarm to sound at			1.1	73 Vh	
Sep. 1.1	2000 V	2.5 mA	3.5 W	15°C	75 Vh
Sep. 1.2	200 V	2.5 mA	3.5 W	15°C	15 Vh
Sep. 1.3	1000 V	2.5 mA	3.5 W	15°C	100 Vh
Sep. 1.4	2000 V	2.5 mA	3.5 W	15°C	510 Vh

Samples were applied after 75 Vh of prefocusing and IEF was carried on for a total of 700 Vh.

cathode) and 8 or 12 samples per gel were applied automatically onto the gel. IEF was continued for a total of 700 Vh (ca. 33 min). The focusing protocol is outlined in detail in **Table 1.**

Immunofixation procedure (17, 18). After IEF, gels were removed from the cooling plate and covered with 175 μl of diluted antibody solution (usually 1:3 to 1:6), depending on the titre of the antibody. An even distribution of the antibody solution was achieved by covering the gel with plastic foil cut to the size of the gel. The foil was removed after incubation of the gel with antibody for 40 min at room temperature in a moist chamber. Unprecipitated proteins were removed by washing the gel in a solution of 150 mmol/l sodium chloride overnight under vigorous agitation. Fifteen to 20 h of washing was required to thoroughly remove unprecipitated proteins. This was conveniently done overnight. The immunoprecipitated apoE bands were then visualized by silver staining. All polyclonal antibodies listed in the Material section were suitable for immunofixation of apoE after isoelectric focusing.

Silver staining. Silver staining of the gels was carried out in the PhastSystem Development Unit™ with reagents prepared as proposed by the manufacturer (19). The details of the staining procedure are given in **Table 2.** Stained gels were dried in air and kept for documentation. They could fit into slide frames and could be demonstrated directly.

RESULTS

Phenotyping analysis, reproducibility, reliability

Analysis of the isoforms was performed by visual comparison of the bands in relation to an internal standard of known apoE phenotype (apoE 2/3) run in one lane of each gel. These internal standards were kept as aliquots in capped tubes at −20°C (10 μl of plasma each precipitated in 2.5 ml of ethanol-ether 3:1 (v/v)). One tube was processed with sample tubes each time a gel was run. The standard aliquots were stable at least up to 6 months at −20°C. The IEF procedure gave reproducible patterns as checked by these internal standards in all runs (n = 46). **Fig. 1** shows two self-made gels where 8 samples (Fig. 1a) and 12 samples (Fig. 1b) were applied for analysis.

The conditions for the IEF system chosen allow definite identification of apoE phenotypes. Potential pitfalls caused by cofocusing of sialylated and nonsialylated isoforms in heterozygous individuals are avoided in the present system. This applies specifically to the discrimination between apoE 2/3 and apoE 3/3 individuals with a high degree of sialylated isoforms. As can be seen in **Fig. 2,** the apoE2 of the apoE 2/3 individual (lane D, arrow head) can be clearly discriminated from the sialylated apoE isoforms of an apoE 3/4 phenotype (lane C, arrow). Non-

TABLE 2. Details of the silver staining procedure automatically carried out by the PhastSystem Development Unit™

Step	Solution	In-Port	Out-Port	Time	Temperature
				min	°C
1	20% TCA[a]	1	0	5	20
2	10% Ethanol, 5% HAc[b]	3	0	2	50
3	10% Ethanol, 5% HAc[b]	3	0	4	50
4	5% Glutardialdehyde[a]	4	0	6	50
5	10% Ethanol, 5% HAc[b]	3	0	3	50
6	10% Ethanol, 5% HAc[b]	3	0	5	50
7	Double distilled water	5	0	2	50
8	Double distilled water	5	0	2	50
9	0.4% Silver nitrate[a]	6	0	10	40
10	Double distilled water	5	0	0.5	30
11	Double distilled water	5	0	0.5	30
12	Developer[c]	7	0	0.5	30
13	Developer[c]	7	0	4.5	30
14	Background reducer[d]	8	0	2.0	30
15	Double distilled water	5	0	5	50

[a] Weight/volume.
[b] Volume/volume.
[c] Two hundred ml 2.5% (w/v) sodium carbonate with 100 μl formaldehyde (37%).
[d] Tris (3.7%, w/v) pH 5.5, 2.5% sodium thiosulfate pentahydrate (w/v).

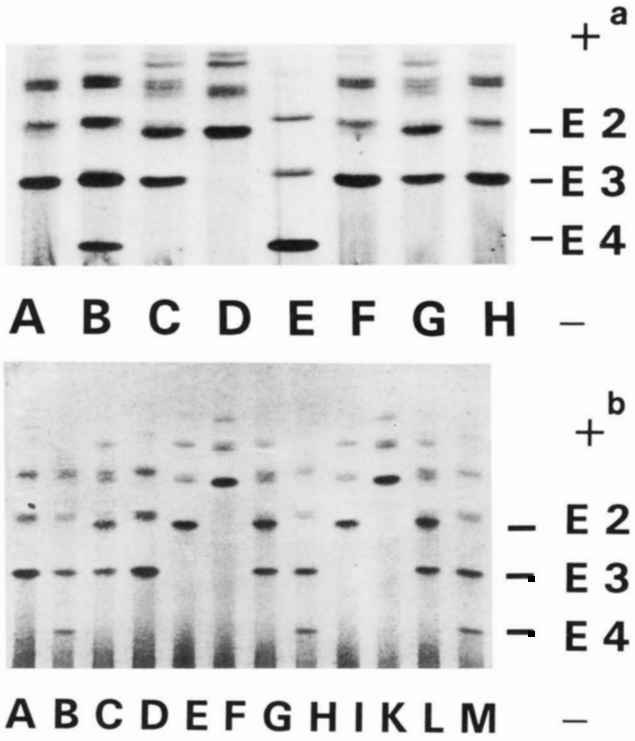

Fig. 1. Phenotyping of apoE by using the PhastSystem™ and self-made IEF gels. Plasma was delipidated and 8 samples (a) or 12 samples (b) per gel were applied close to the cathode. After IEF apoE bands were immunoprecipitated and stained with silver. Phenotypes are: Gel a: apoE 3/3 (lane A), apoE 3/4 (lane B), apoE 2/3 (lane C), apoE 2/2 (lane D), apoE 4/4 (lane E), apoE 3/3 (lane F), apoE 2/3 (lane G), and apoE 3/3 (lane H). Gel b: apoE 3/3 (lane A), apoE 3/4 (lane B), apoE 2/3 (lane C), apoE 3/3 (lane D), apoE 2/2 (lane E), apoE 1/1 (lane F), apoE 2/3 (lane G), and apoE 3/4 (lane H), apoE 2/2 (lane I), apoE 1/1 (lane K), apoE 2/3 (lane L), and apoE 3/4 (lane M). Plasma from apoE 2/2 and apoE 1/1 was diluted 10-fold further in sample buffer.

sialylated apoE2 focuses with a slightly more basic pI than the sialylated apoE3 forms (see also Fig. 1a, lanes F, G, H). Also, the sialylated isoforms of the heterozygous patterns are visibly separated (see asterisk in Fig. 2, lane C).

Samples from 82 patients with different apoE phenotypes were blinded and analyzed by the new procedure (2 apoE 2/2, 12 apoE 2/3, 45 apoE 3/3, 19 apoE 3/4, 2 apoE 2/4, 1 apoE 4/4, and 1 apoE 1/1). All agreed with the phenotype of these patients that had been determined by IEF in large gels as previously described (12, 14). In no case was there a discrepancy between the phenotyping procedures.

Comparison of self-made gels with commercially available gels

For comparison, samples were run on self-made and commercially available gels in parallel. The bands of the apoE isoforms were not straight over the entire CleanGel™. The lanes localized at the rim of the gel appeared somewhat crooked. The phenotyping of the samples was, however, not influenced by this difference as the sialylated isoforms could be used to define focusing positions (see arrow and arrow heads in Fig. 2).

Sensitivity of the procedure

Levels of apoE vary widely among apoE phenotypes, being lowest at apoE4/4 and highest in apoE2/2 subjects (20), especially upon expression of type III hyperlipidemia. Optimal results were obtained by applying 5 ng of apoE per microliter of sample solution. Despite the high variability of apoE levels, the dilution of the sample as given above allowed phenotyping in all subjects, including those with high lipid levels (highest levels tested: triglycerides 2100 mg/dl, cholesterol 730 mg/dl). Only in grossly hyperlipemic patients with type III hyperlipidemia was the apoE2 band broad and heavily overloaded. Then, during a second run, these rare samples were subjected to a further 10-fold dilution in sample buffer. No determination of apoE levels was necessary prior to IEF as the given dilution of samples allows phenotyping in all cases.

DISCUSSION

We report here a new procedure for apoE phenotyping that can use commercially available devices, and can be automated during IEF and subsequent staining procedures. Although the use of self-made gels gives straighter bands and avoids divergence of the outer lanes (see Fig. 1a and b), phenotyping is possible with both types of gels. The reasons for the difference are not entirely obvious but might have to do with the drying or rehydration procedure of the commercial gels, as the self-made ones are used as poured.

In contrast to a recently described method that also uses the PhastSystem™ to perform IEF (21), our procedure does not require the generation of immobilized pH gradient gels, which is technically more difficult and time-

Fig. 2. Phenotyping of apoE by using the PhastSystem™ and a commercially available CleanGel™. Plasma was delipidated and applied to the rehydrated gel. Samples were applied close to the cathode. After IEF apoE bands were immunoprecipitated and stained with silver. Phenotypes read: apoE 3/3 (lane A), apoE 3/4 (lanes B and C), apoE 2/3 (lanes D, E, F), apoE 3/4 (lane G), and apoE 3/3 (lane H). Arrow heads indicate the more basic position of apoE 2 as compared to the sialylated apoE forms (arrow). Asterisk indicates sialylated form of apoE 4.

consuming than the production of IEF gels with carrier ampholytes. Instead, we generally used available dried acrylamide gels that only needed to be rehydrated. This can be performed easily and reproducibly.

In addition, the procedure described here does not require immunoblotting which cannot yet be automated. We could show, on the other hand, that immunofixation is possible even in the presence of up to 8 mol/l of urea (18). Immunofixation clearly has advantages over blotting as the gel itself on the support film can be processed and developed to a final test result. It does not require the transfer to another support with potential loss of protein and the appearance of less sharp bands. The immediate addition of the precipitating antibody avoids diffusion of the apoE bands within the acrylamide gel. Luley, Baumstark, and Wieland (22) also used immunofixation for the detection of apoE isoforms in approximately 3 mol/l urea. They generated agarose gels to screen many samples per run (23). In this respect, the short focusing time of our procedure (45 min) allows several runs a day of two gels in parallel (16 to 24 samples each time) and thus a fair number of phenotypes can be determined in a short time.

Although fresh plasma as well as serum could be used to determine phenotypes, we usually use plasma to avoid proteolysis by thrombin, known to produce 22- and 10-kDa fragments of the apoE molecule (24). We tested plasma that was kept frozen up to 6 months and found it still suitable. We do not, however, generally freeze plasma to be phenotyped. Instead, we inject 10 μl of plasma into ethanol–ether and keep the capped tube at −20°C until enough samples have accumulated for analysis. We have successfully analyzed samples kept this way up to 6 months. For storage reasons we prefer the ethanol–ether delipidation over the chloroform–methanol procedure although the latter gives comparable focusing results.

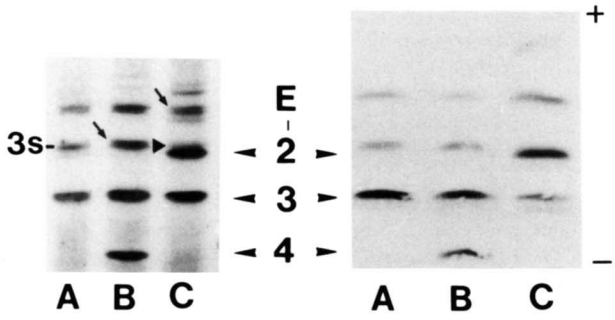

Fig. 3. High resolution of the PhastSystem™ isoelectric focusing combined with immunofixation (left) with IEF and Western blotting in large gels (right) (12, 25). Three different apoE phenotypes are shown for comparison: apoE 3/3 (A); apoE 3/4 (B), and apoE 2/3 (C). Despite the smaller size of the PhastSystem™ gel (43 × 50 mm, left side) the resolution is even better than in the conventional gel (160 × 180 mm), shown for comparison on the right side of the figure. In the PhastSystem™, sialylated apoE3 isoforms (3s) clearly focus in a more acidic position than apoE2, allowing the differentiation between apoE2 and sialylated apoE3. The high resolution of the PhastSystem™ gel is further demonstrated by the doubling of the sialylated forms into two bands of similar staining in heterozygous subjects (arrows). This phenomenon could not be resolved by the conventional blotting procedure. Also the sialylated apoE3 isoform is visible above the apoE2 band (arrow head).

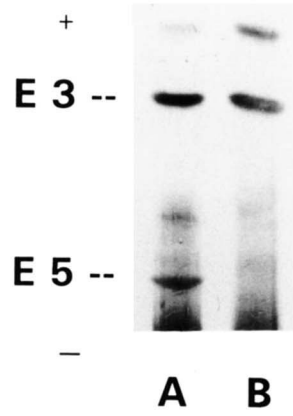

Fig. 4. Detection of a rare basic apoE isoform by PhastSystem™ isoelectric focusing and immunofixation. Plasma of an apoE 3/5 subject (28) (lane A) is shown in comparison to an apoE 3/3 sample (lane B). The pH range of the gradient chosen allows detection of more basic isoforms than apoE4 but also more acidic apoE proteins than apoE2 (see apoE1 in Fig. 1b, lanes F and K).

The method described here does not require neuraminidase treatment of plasma prior to IEF. Treatment with neuraminidase did not add to the security of the determination of the phenotype (data not shown). On the contrary, the presence of sialylated forms helps to define focusing positions to avoid artifacts. In the pH gradient depicted here, the sialylated forms of apoE do not cofocus with nonsialylated isoforms and thus allow clear identification without cleaving sialic acid residues. **Fig. 3** compares the resolution of the new procedure with a conventional immunoblot after IEF in a large gel (160 × 180 mm) (25). The small dimensions of the IEF gels used in PhastSystem™ (43 × 50 mm) thus do not lead to a loss in resolution power. The resolution obtained by IEF on PhastSystem™ even exceeds that of the conventional procedure as can be seen by the doubling of sialylated forms in heterozygous patterns (arrows in Fig. 3). This may be caused by the higher field strength that can be generated in smaller gels as shown by Laas and Olsson (26).

Finally, the pH range of the gels chosen allowed detection of genetic variants of apoE focusing outside of the usual apoE isoform pH range. Fig. 1b shows an example of an additional, more acidic isoform detectable by the new method: apoE1 (27). The pH gradient in the gel was chosen so that variant apoE isoforms more basic than apoE4 could be detected. **Fig. 4** shows an example of an apoE3/5 subject (28) with both isoforms visible. In further experiments, an induced shift of the pH gradient towards an electrode did not result in a loss of a given isoform focusing within the shifted pH range. It was, instead, squeezed towards the electrode and still detectable. Thus, even more basic isoforms than apoE5 would not be missed by this procedure.

In summary, the procedure described here not only simplifies apoE phenotyping, it also allows an automated, reliable, easy, and rapid analysis of apoE patterns and is thus no longer restricted to specialized laboratories.

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