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Isolation of a high-density-lipoprotein conversion factor from human plasma

A possible role of apolipoprotein A-IV as its activator

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1. A high-density-lipoprotein (HDL) conversion factor was partially purified from human plasma by precipitation with $(NH_4)_2SO_4$, ultracentrifugation, cation-exchange chromatography, anion-exchange chromatography and chromatography on a column of hydroxyapatite. 2. This factor modulates the particle size of HDL by converting a homogeneous population into new populations of particles, some of which are smaller and others larger than those in the original population. 3. The isolated HDL conversion factor appeared as one major band and at least three minor bands on SDS/polyacrylamide-gel electrophoresis; attempts to purify this factor further resulted in loss of conversion activity. 4. Preparations of the HDL conversion factor were stable after heating to 58 °C for 1 h, and were shown not to possess proteolytic activity. 5. The conversion factor was distinct from the known apolipoproteins, none of which had HDL conversion activity. 6. Addition of apolipoprotein A-IV had a dose-dependent potentiating effect on the process promoted by the HDL conversion factor.

INTRODUCTION

Epidemiological studies have demonstrated that the risk of developing coronary heart disease is inversely correlated with the concentration of high-density lipoproteins (HDL) (Miller & Miller, 1975; Miller, 1981). The HDL fraction in human plasma is heterogeneous, comprising several discrete subpopulations of particles of distinct density, size and composition (Kostner, 1981; Blanche *et al.*, 1981). There are two major subfractions, HDL_2 containing larger and less dense particles, and HDL_3 containing smaller and more dense particles. The use of gradient-gel electrophoresis to separate particles of different sizes has revealed further heterogeneity, identifying at least two subpopulations of HDL_2 and three subpopulations of HDL_3 (Blanche *et al.*, 1981; Nichols *et al.*, 1983). Studies using immunoaffinity chromatography have also identified heterogeneity in terms of apolipoprotein composition (Cheung & Albers, 1984; McVicar *et al.*, 1984).

Heterogeneity of HDL has some important implications. For example, smaller HDL particles are preferred to larger particles as substrates for plasma cholesterol esterification, the reaction catalysed by lecithin–cholesterol acyltransferase (LCAT) (Barter *et al.*, 1985). This reaction represents a key step in reverse cholesterol transport. Furthermore, the apparent capacity of HDL to protect against coronary heart disease relates to an increase in the concentration of HDL_2 rather than HDL_3 (Shepherd *et al.*, 1980; Anderson *et al.*, 1978). The regulation of HDL particle size is therefore a process of considerable importance.

A number of plasma factors have been shown *in vitro* to influence the size and density of human HDL. In addition to LCAT (Daerr & Greten, 1982), these include the cholesteryl ester transfer protein (Hopkins *et al.*, 1985), lipoprotein lipase (Patsch *et al.*, 1978) and hepatic lipase (Shirai *et al.*, 1981). More recently, another factor has been identified, a putative HDL conversion factor (Gambert *et al.*, 1982; Rye & Barter, 1984, 1986) which promotes conversion of human HDL_3 into new populations, some comprising particles that are smaller than the original HDL_3 and others comprising larger particles in the size range of HDL_2 (Rye & Barter, 1986). The HDL conversion factor is present in human plasma (Rye & Barter, 1986), and has been postulated to have an important physiological function by promoting a continuing supply of small HDL particles to act as substrates in the LCAT reaction. The present study reports on the isolation and characterization of this conversion factor and provides evidence that activity of the conversion factor is markedly potentiated by apolipoprotein A-IV (apoA-IV).

EXPERIMENTAL

Assay for HDL conversion activity

The presence or absence of HDL-conversion-factor activity was determined as described by Rye & Barter (1986). The assay depends on the capacity of the

Abbreviations used: apo, apolipoprotein; FPLC, fast protein liquid chromatography; HDL, high-density lipoproteins; LCAT, lecithin–cholesterol acyltransferase (EC 2.3.1.43).

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conversion factor to convert a homogeneous population of HDL_3 into new populations of particles, some of which are smaller and others larger than the parent particles. HDL_3 ($1.13 < d < 1.21$) were isolated from human plasma as described by Rye & Barter (1986). To ensure homogeneity of the HDL_3, samples were isolated only from the plasma of subjects who had been shown by previous gradient-gel electrophoresis of the total HDL to possess particles of radius 4.3 nm (HDL_{3a}) as the only identifiable subpopulation of HDL_3. Incubations were performed at 37 °C in a shaking water bath. An arbitrary incubation time of 24 h was employed since it was recognized that the conversion process is progressive and may require more than 24 h for completion (Rye & Barter, 1986). Control samples were maintained at 4 °C throughout. After incubation, lipoproteins were isolated by ultracentrifugation as described by Rye & Barter (1986) and then subjected to gradient-gel electrophoresis (Blanche *et al.*, 1981). The gels were stained with Coomassie Brilliant Blue G and scanned with a 2202 Ultroscan Laser Densitometer (LKB) attached to an integrator (Hewlett–Packard 3390A).

Purification of the HDL conversion factor

Plasma samples from several human subjects were combined, and 1800 ml was treated with $(NH_4)_2SO_4$ to precipitate proteins between 35% and 55% saturation; these were recovered and subjected to ultracentrifugation (Rye & Barter, 1986). The fraction of density 1.21–1.25 g/ml was isolated and further separated by cation-exchange chromatography as described by Rye & Barter (1986).

After cation-exchange chromatography, the fractions shown to possess HDL conversion activity were combined and exhaustively dialysed against 0.02 M-Tris/HCl (pH 7.4). This solution (approx. 10 mg of protein in 40 ml) was applied to a column of Mono Q HR 5/5 (Pharmacia Fine Chemicals) attached to a Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The column was pre-equilibrated with 0.02 M-Tris/HCl (pH 7.4) and eluted with a linear gradient of NaCl (0–0.5 M) in the buffer.

After Mono Q chromatography, the fractions containing conversion activity were combined (1–2 mg of protein) and dialysed against 0.001 M-sodium phosphate buffer (pH 7.0) and subjected to chromatography on a column of hydroxyapatite (Bio-Gel HT; 0.9 cm × 5 cm) equilibrated with 0.001 M-sodium phosphate buffer (pH 7.0). HDL conversion activity was eluted with a linear gradient of sodium phosphate (0.03–0.06 M).

Isolation of apoliproteins

Apolipoproteins A-I and A-II (apoA-I and apoA-II) were isolated from human HDL (d 1.085–1.21) (Havel *et al.*, 1955). The apolipoprotein obtained after delipidation of HDL was dissolved in a solution containing 6 M-urea and 0.05 M-Tris/HCl buffer (pH 8.0), dialysed against the same buffer for 24–48 h at room temperature and then loaded on to a Sephadex G-150 column (5 cm × 150 cm), equilibrated with the same buffer (Scanu *et al.*, 1969). Fractions containing apoA-I or apoA-II were combined and rechromatographed on the same column. Apolipoprotein E (apoE) and the C apolipoproteins (apoC) were isolated from human very-low-density lipoprotein ($d < 1.019$) after delipidation and chromatography on Sephacryl S-300 (2.6 cm × 100 cm) equilibrated with 0.05 M-Tris/HCl/5 M-guanidine hydrochloride (pH 8.2). The fractions containing mainly apoE were combined and rechromatographed on Sephadex G-150 (2.6 cm × 100 cm) equilibrated with 0.05 M-Tris/HCl (pH 8.0) containing 8 M-urea.

Human apoA-IV was isolated (Ohta *et al.*, 1984) from lymph obtained from a subject with a chylous pleural effusion. To recover the triacylglycerol-rich lipoproteins, the lymph was centrifuged in a Beckman 35 rotor for 2 h at 30000 rev./min. After washing and delipidation, the chylomicron apolipoprotein was dissolved in 0.05 M-Tris/HCl (pH 7.4) containing 4 M-guanidine hydrochloride, loaded on a Sephacryl S-200 column (2.6 cm × 110 cm) and eluted with the same buffer system. The fraction enriched with apoA-IV was rechromatographed on the same column to produce apparently pure apoA-IV, as judged by SDS/polyacrylamide-gel electrophoresis.

β2-Glycoprotein-1, an apolipoprotein associated with chylomicrons, was isolated by preparative SDS/polyacrylamide-gel electrophoresis as described by Fidge & McCullagh (1981).

Other methods

SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), immunoblotting (Burnette, 1981) and assays for protein (Lowry *et al.*, 1951) were performed as described in the references.

Experiments were performed to determine whether the preparations of partially purified conversion factor also possessed proteolytic activity, which could produce artefactual changes in HDL size as a result of apolipoprotein degradation. Human HDL_3 was isolated and radioiodinated with ^{125}I as described by Fidge *et al.* (1980). Samples of the labelled HDL_3 were incubated at 4 °C or 37 °C for 24 h with partially purified conversion factor or with trypsin at various protein ratios (trypsin/HDL_3 ranging from 1:10 to 1:50, w/w). After incubation, samples were loaded on to Sephadex G-100 columns (1.3 cm × 55 cm) equilibrated and eluted with 0.15 M-NaCl/0.02 M-Tris/HCl (pH 7.5), and 1.0 ml fractions were collected for radioassay. Other samples were subjected to SDS/polyacrylamide-gel electrophoresis, after which the gels were stained, sliced and counted for radioactivity. Further samples were delipidated with ethanol/ethyl ether (3:1, v/v) and then fractionated by gel filtration or SDS/polyacrylamide-gel electrophoresis.

RESULTS

Partial purification of the HDL conversion factor from human plasma

The initial steps in the purification of the conversion factor [precipitation with $(NH_4)_2SO_4$, ultracentrifugation and cation-exchange chromatography] have been described (Rye & Barter, 1986). Fractions containing HDL conversion activity were recovered after cation-exchange chromatography (Fig. 1*a*), combined and subjected to anion-exchange chromatography (Fig. 1*b*). The active fractions recovered from this step were combined and applied to a column of hydroxyapatite, from which the HDL conversion factor activity was eluted at about 0.045 M-sodium phosphate (Fig. 1*c*).

The active fractions recovered after hydroxyapatite chromatography promoted conversion of a single population of HDL_3 (particle radius 4.3 nm) into new

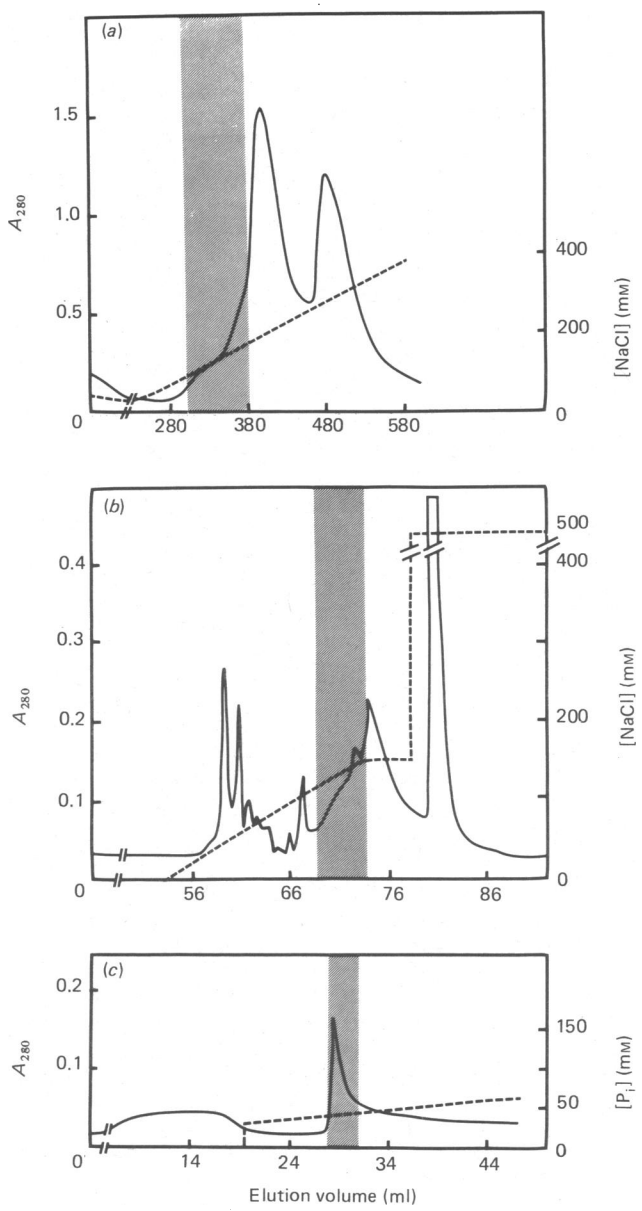

Fig. 1. Isolation of the HDL conversion factor

(*a*) Cation-exchange chromatography. Human plasma (1800 ml) was subjected to precipitation with $(NH_4)_2SO_4$ and ultracentrifugation to recover the fraction of density 1.21–1.25 g/ml. This preparation, containing 225 mg of protein, was applied to a 1.6 cm × 32 cm column of CM-cellulose equilibrated with 0.05 M-sodium acetate buffer (pH 4.5) and eluted with a NaCl gradient (0–0.4 M; ----) at a flow rate of 30 ml/h. The A_{280} was monitored (——). Conversion activity was confined to the fractions indicated by the shaded area. (*b*) Anion-exchange chromatography. The active fractions recovered after cation-exchange chromatography were combined and dialysed (see the text), and a preparation containing 10 mg of protein was applied to a 0.5 cm × 5 cm Mono Q column (pre-equilibrated with 0.02 M-Tris/HCl, pH 7.4) attached to a FPLC system. Bound proteins were eluted with a NaCl gradient (0–0.5 M; ----) at a flow rate of 1.0 ml/min, and the A_{280} was monitored (——); 1.0 ml fractions were collected. The shaded area indicates the fractions which contained HDL conversion activity. (*c*) Hydroxyapatite chromatography. The active fractions recovered after anion-exchange chromatography were combined and dialysed (see the text) and a preparation containing 1–2 mg of protein was applied to a pre-equilibrated column of hydroxyapatite (Bio-Gel HT; 0.9 cm × 5 cm) attached to a FPLC system. Bound proteins were eluted with a phosphate gradient (0.03–0.06 M; ----) at a flow rate of 0.4 ml/min, and the A_{280} was monitored (——); 1.0 ml fractions were collected. The shaded area indicates the fractions which contained HDL conversion activity.

populations of particles with radii ranging from 3.7 to 4.7 nm (Fig. 2).

SDS/polyacrylamide-gel electrophoresis of this preparation of conversion factor revealed one major and at least three minor bands (Fig. 3). Attempts at further purification of this preparation have resulted in a loss of activity. Consequently, the characteristics described in this paper involve studies using this partially pure preparation.

The HDL-conversion-factor activity recovered after hydroxyapatite chromatography was stable when stored at 4 °C, retaining full activity for up to 12 weeks. These fractions were also heat-stable, tolerating incubation at 58 °C for 1 h without loss of activity.

Characterization of the HDL conversion factor

Preparations of HDL conversion factor did not possess proteolytic activity, in that, after incubation of ^{125}I-labelled HDL_3 with either the conversion factor or buffer alone (at 4 °C or 37 °C), all radiolabel was recovered with intact HDL_3 (Fig. 4). Furthermore, after gel-permeation chromatography of the delipidated HDL_3, there was no evidence of label in proteolytic fragments of the apolipoproteins (results not shown). However, after comparable incubations in the presence of trypsin, only 28% of the label remained with intact HDL_3 or with apoA-I or apoA-II. When the conversion factor was incubated with labelled HDL_3, subsequent electrophoresis followed by slicing of the gels and radioactivity counting indicated that more than 95% of the radioactivity was recovered in apoA-I, apoA-II and apoC, with no evidence of loss owing to production of proteolytic fragments.

To address the issue of identity of the conversion factor, several known proteins were tested for HDL conversion activity. At protein concentrations comparable with that present in the conversion-factor preparations, no conversion activity could be detected with β2-glycoprotein, apoA-I, apoA-II, apoA-IV, apoE or a mixture of the C-apolipoproteins. Furthermore, none of these proteins was visible as an identifiable band on SDS/polyacrylamide gels of preparations containing the conversion factor.

Activation of HDL conversion factor by apoA-IV

Addition of apoA-IV to incubations containing the conversion factor and HDL_3 resulted in a marked enhancement of the conversion process. In a typical experiment (Fig. 5), HDL_3 (60 μg of protein) was incubated in the presence of various additions in a final incubation volume of 200 μl. In the absence of conversion factor, addition of up to 30 μg of apoA-IV did not promote any change in HDL particle size. In the presence of HDL conversion factor (37 μg of protein), however, addition of as little as 3 μg of apoA-IV markedly enhanced the conversion process, with a much greater proportion of the original HDL particles (4.3 nm radius)

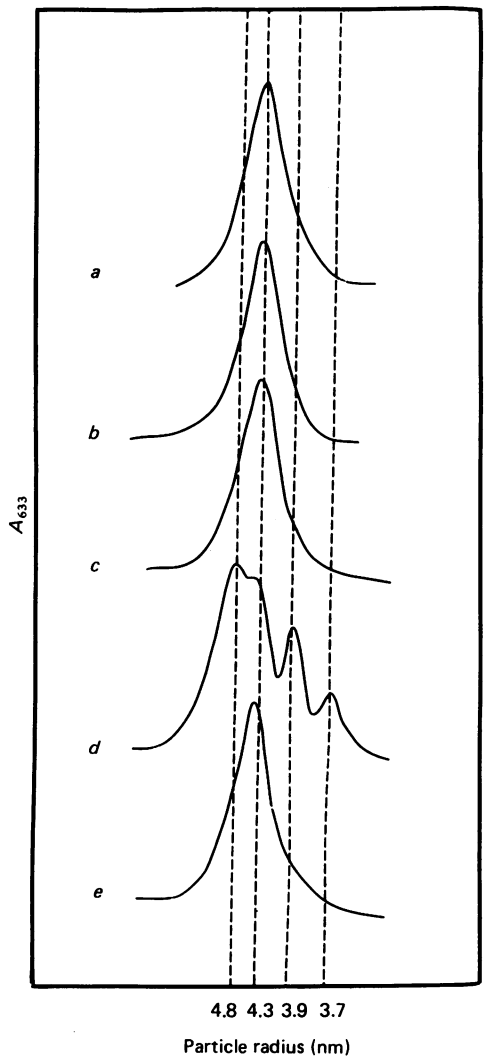

Fig. 2. Particle size distribution of HDL as assessed by gradient-gel electrophoresis

Samples of human HDL_3 (50 μg of protein) were mixed with phosphate-buffered saline, pH 7.4 (profiles *a* and *b*), or with a solution containing 2 mg of human serum albumin (profile *c*) or with 52 μg of a preparation of the conversion factor (profiles *d* and *e*). The total incubation volume in each case was 0.2 ml. Mixtures were incubated at 4 °C (profiles *a* and *e*) or 37 °C (profiles *b*–*d*) for 24 h. After incubation, the lipoproteins were recovered at 1.25 g/ml by ultracentrifugation and subjected to gradient-gel electrophoresis. Laser-densitometric scans of the gels are shown.

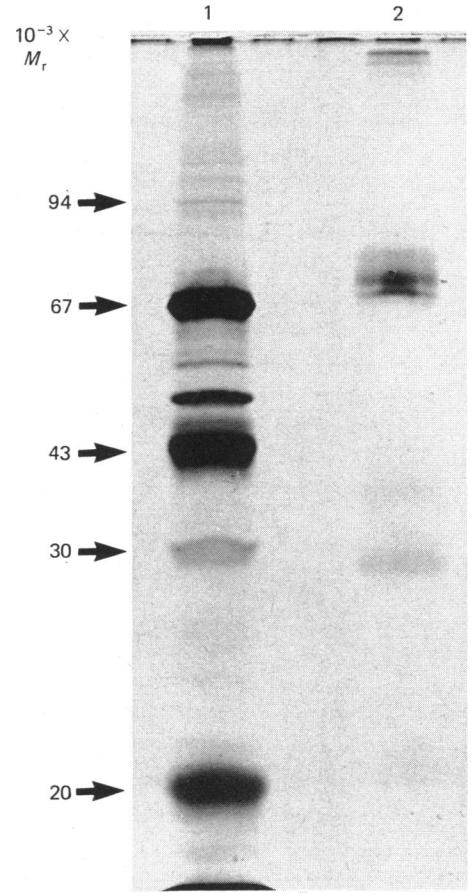

Fig. 3. SDS/polyacrylamide-gel electrophoresis of a preparation containing HDL conversion activity

The active sample (5 μg of protein) recovered after hydroxyapatite chromatography (Fig. 1*c*) was loaded on a 12.5%-polyacrylamide gel (lane 2). After electrophoresis, the gel was developed by silver staining. Lane 1 shows low-M_r standards; bands representing phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) and trypsin inhibitor (20100) are identified.

now being converted into new populations of particles with radii of 3.7 nm and 4.7 nm. The addition of 6 μg of apoA-IV to an incubation mixture containing the conversion factor had an even greater effect. The original population of HDL_3 of radius 4.3 nm was now almost completely converted into two discrete subpopulations, one consisting of particles of radius 3.7 nm and the other containing particles of radius 5.3 nm (Fig. 5). In other experiments (results not shown), the potentiating effect of apoA-IV was apparent with an addition of as little as 1.5 μg of the apolipoprotein.

Despite the obvious potentiating effect of apoA-IV on the conversion process, it was established that conversion activity did not depend entirely on the presence of this apolipoprotein. Immunoblotting capable of detecting 0.1 μg of apoA-IV was unable to detect the apolipoprotein in a preparation of the conversion factor containing 10.7 μg of protein.

In experiments similar to those performed with apoA-IV, other apolipoproteins were shown not to be activators of the conversion process. The addition of up to 30 μg of apoA-I, apoA-II, apoC or apoE had no demonstrable effect on the capacity of the conversion factor to modify the particle size of HDL_3 (results not shown).

DISCUSSION

The conversion process under examination in these studies is one that converts a single population of HDL_3 into new populations of particles, some of which are smaller and others larger than those in the original population (Rye & Barter, 1986). This capacity to

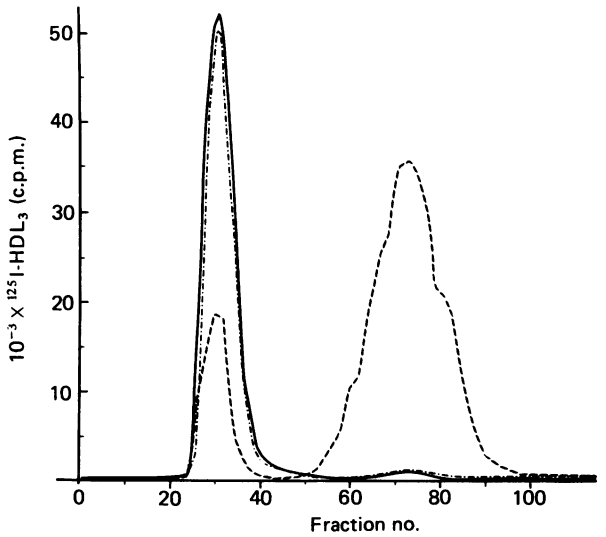

Fig. 4. Gel-permeation chromatography of ^{125}I-labelled HDL after incubation with conversion factor or trypsin

A preparation of active HDL conversion factor was incubated with ^{125}I-labelled HDL_3 at the same concentration and under conditions identical with those used in the assays described in the legend to Fig. 2. After incubation at 4 °C for 24 h, the sample (——) was loaded on a Sephadex G-150 column (1.3 cm × 55 cm) and eluted with 0.15 M-NaCl/0.02 M-Tris/HCl, pH 7.4. Another sample (–·–·) was incubated at 37 °C for 24 h and applied to the column, and a third sample (– – – –) was chromatographed after incubation of radioiodinated HDL_3 with trypsin (5:1, w/w) at 37 °C for 24 h.

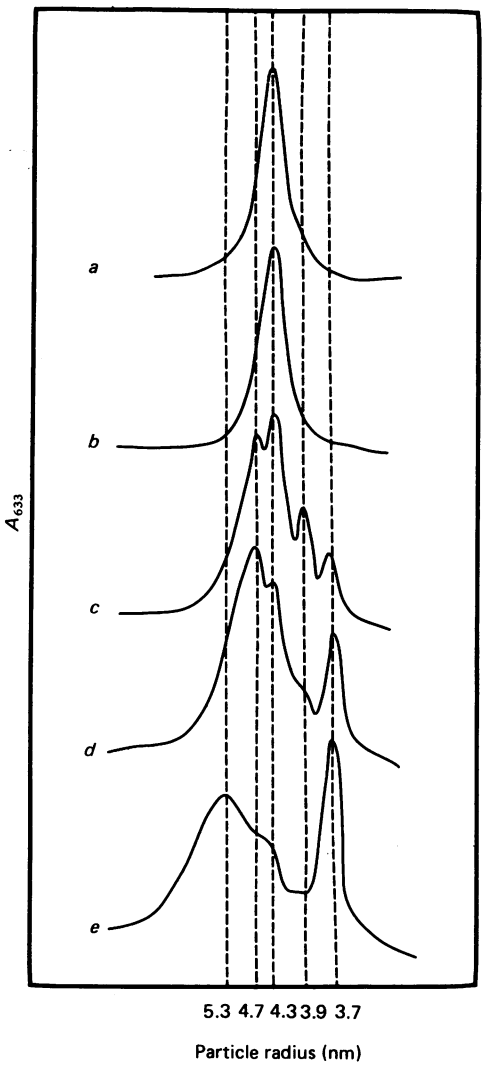

Fig. 5. Effect of apoA-IV on the activity of the HDL conversion factor

The profiles represent the particle size distribution of HDL as assessed by gradient-gel electrophoresis. All incubation mixtures contained HDL_3 at a protein concentration of 0.3 mg/ml in a final volume of 0.2 ml. To *a* and *c* was added HDL conversion factor (final protein concn. 185 μg/ml); *b* contained apoA-IV (final concn. 150 μg/ml) but no conversion factor; *d* contained conversion factor (185 μg/ml) and apoA-IV (15 μg/ml); *e* contained conversion factor (185 μg/ml) and apoA-IV (30 μg/ml). Mixtures were incubated for 24 h at 4 °C (profile *a*) or 37 °C (profiles *b*, *c*, *d* and *e*). After incubation, the samples were processed as described in the legend to Fig. 2.

produce simultaneously both smaller and larger HDL particles is what differentiates activity of the conversion factor from that of several other plasma factors known to influence the size and density of HDL. The factor is known to exist in human plasma (Rye & Barter, 1986), but has previously been isolated only as a component of a crude mixture.

Preparations of the HDL conversion factor isolated in the present studies were still not homogeneous, although there was only one major protein band and three minor bands visible on SDS/polyacrylamide-gel electrophoresis. It had been reported previously (Rye & Barter, 1986) that preparations of conversion factor were deficient in LCAT activity and were apparently distinct from lipid transfer protein. In the present studies the conversion factor was shown not to promote proteolysis of HDL. Furthermore, the demonstrable stability of the conversion factor during either prolonged storage at 4 °C or after heating to 58 °C for 1 h contrasted with the reported lability of LCAT (Glomset, 1968), the plasma phospholipid transfer protein (Albers *et al.*, 1984) and both lipoprotein lipase (Twu *et al.*, 1976) and hepatic lipase (Schoonderwoerd *et al.*, 1981). It was also shown that HDL conversion was not promoted by isolated apoA-I, apoA-II, apoA-IV, apoC or apoE.

An important new finding in these studies was the observation that apoA-IV markedly potentiated the activity of the conversion factor. Isolated apoA-IV possessed no intrinsic conversion activity. But, in contrast with other apolipoproteins, addition of apoA-IV to incubations containing the conversion factor resulted in a major enhancement of the formation of both smaller and larger HDL particles. Furthermore, this potentiating effect of apoA-IV was apparent at concentrations in the range 7.5–30 μg/ml, which are within the reported concentration range of this apolipoprotein in human plasma (Bisgaier *et al.*, 1985; Lefevre & Roheim, 1984; Fidge & Nestel, 1981).

The distribution of apoA-IV in plasma differs markedly from that of other apolipoproteins, with most of the apoA-IV not being associated with any of the major classes of lipoproteins; rather, it exists as a kinetically

distinct pool within the lipoprotein-free fraction of plasma (Ohta *et al.*, 1985; Ghiselli *et al.*, 1986). Under certain conditions, however, such as when LCAT is active in plasma *in vitro*, there is a redistribution of apoA-IV from the lipoprotein-free fraction into HDL (Delamatre *et al.*, 1983). It has also been reported that apoA-IV acts as a cofactor for LCAT, although in this function it is only about one-quarter as efficient as apoA-I (Chen & Albers, 1985). Other studies have indicated that apoA-IV may play a direct role in the efflux of cholesterol from cells (Stein *et al.*, 1986; Mitchell *et al.*, 1987). Nevertheless, the precise physiological function of this relatively abundant apolipoprotein remains to be defined.

The present studies indicate that apoA-IV is a powerful activator or potentiator of a plasma factor which converts HDL_3 into new populations of particles, some larger and some smaller than the original particles. The newly formed larger particles equate in size with naturally occurring HDL_2. The smaller conversion products, by contrast, have no equivalent in plasma freshly collected from normal human subjects, although they do compare in size with the small spherical HDL which accumulate in patients with a deficiency of LCAT (Chen *et al.*, 1984). It is possible that these small particles are very reactive with LCAT and that interaction with the enzyme *in vivo* promotes their immediate reconversion into larger particles.

A process promoting the formation of very small HDL may be an important component of the pathway of reverse cholesterol transport. Not only does it result in a continuing supply of HDL particles that are highly reactive with LCAT, but it also generates particles small enough to migrate with relative ease into the interstitial space, where they can interact directly with tissues that lie outside the vascular space.

Clearly, much more work is needed to define the identity of the conversion factor and to elucidate the physiological significance of the conversion process. The present study provides an additional challenge to determine the precise role of apoA-IV in this potentially very important process. Studies seeking to define the mechanism of this potentiation by apoA-IV and issues such as whether or not the apoprotein is an obligatory cofactor for the conversion process will have to await the availability of the conversion factor in pure form.

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