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

A possible role of apolipoprotein A-IV as its activator

P. J. BARTER,*† O. V. RAJARAM,* L. B. F. CHANG,* K. A. RYE,* P. GAMBERT,† L. LAGROST,† C. EHNHOLM*§ and N. H. FIDGE*

*Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria 3181, Australia, and †Laboratoire de Biochimie Medicale, Faculté de Medicine et CNRS UA 273, Dijon 21034, France

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, HDL₄ containing larger and less dense particles, and HDL₃ 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 HDL₄ and three subpopulations of HDL₃ (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 HDL₃ rather than HDL₄ (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 cholesterol 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 HDL₃ into new populations, some comprising particles that are smaller than the original HDL₃ and others comprising larger particles in the size range of HDL₄ (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
conversion factor to convert a homogeneous population of HDL₃ into new populations of particles, some of which are smaller and others larger than the parent particles. HDL₃ (1.13 < d < 1.21) were isolated from human plasma as described by Rye & Barter (1986). To ensure homogeneity of the HDL₃ 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 (HDL₃a) as the only identifiable subpopulation of HDL₃. 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 Ultrosan 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 (NH₄)₂SO₄ 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 apolipoproteins

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 Sepacryl 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 artifactual changes in HDL size as a result of apolipoprotein degradation. Human HDL₃ was isolated and radioiodinated with ¹²⁵I as described by Fidge et al. (1980). Samples of the labelled HDL₃ were incubated at 4 °C or 37 °C for 24 h with partially purified conversion factor or with trypsin at various protein ratios (trypsin/HDL₃ 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 (NH₄)₂SO₄, ultracentrifugation and cation-exchange chromatography] have been described (Rye & Barter, 1986). Fractions containing HDL conversion activity were recovered after cation-exchange chromatography (Fig. 1a), combined and subjected to anion-exchange chromatography (Fig. 1b). 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. 1c).

The active fractions recovered after hydroxyapatite chromatography promoted conversion of a single population of HDL₃ (particle radius 4.3 nm) into new...
Activation of high-density-lipoprotein conversion factor

1. The preparation of conversion factor was equilibrated protein, The HDL conversion factor was 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 125I-labelled HDL₃ with either the conversion factor or buffer alone (at 4 °C or 37 °C), all radiolabel was recovered with intact HDL₃ (Fig. 4). Furthermore, after gel-permeation chromatography of the delipidated HDL₃, there was no evidence of labeling 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 HDL₃ or with apoA-I or apoA-II. When the conversion factor was incubated with labelled HDL₃, 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 HDL₃ resulted in a marked enhancement of the conversion process. In a typical experiment (Fig. 5), HDL₃ (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)
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 HDL₃ 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.

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

Samples of human HDL₃ (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.

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 HDL₃ (results not shown).

DISCUSSION

The conversion process under examination in these studies is one that converts a single population of HDL₃ 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
A preparation of active HDL conversion factor was incubated with 125I-labelled HDL₃ 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 HDL₃ with trypsin (5:1, w/w) at 37°C for 24 h.

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

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 \textit{in vitro}, there is a redistribution of apoA-IV from the lipoprotein-free fraction into HDL (Delamare 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 HDL₂ 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 HDL₃. 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 \textit{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.

This work was supported by grants from the National Health & Medical Research Council of Australia and from the National Heart Foundation of Australia. We also acknowledge the expert technical assistance of Sheelà Unnithan and Hubert Edelsbacher.

REFERENCES


Ghiselli, G., Krishnan, S., Beigel, Y. & Gotto, A. M., Jr. (1986) J. Lipid Res. 27, 813–827
Glosset, J. A. (1968) J. Lipid Res. 9, 155–167

Received 31 December 1987/29 March 1988; accepted 4 May 1988

P. J. Barter and others

1988