Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 122-128

BINDING OF ApoA-IV-PHOSPHOLIPID COMPLEXES TO PLASMA MEMBRANES OF RAT LIVER

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Received June 17, 1986

SUMMARY: Rat apoA-IV complexes with dimyristoyl phosphatidylcholine (apoA-IV-DMPC) have been prepared and their ability to bind to purified rat liver plasma membranes investigated. Binding equilibrium at 37°C was reached in 30 minutes. Saturation binding experiments and subsequent analysis of the results with Scatchard plots gave results consistent with the presence of a single saturable binding site. DMPC or POPC unilamellar vesicles could not compete with ApoA-IV-DMPC for binding; apoA-I-DMPC competed only partially. ApoE-poor HDL effectively competed with apoA-IV-DMPC. The fact that binding could be greatly reduced (> 70%) by preincubating the membrane with pronase (18 μg/ml), supports the conclusion that a membrane protein is involved in binding. Based on these results, we speculate that the rapid catabolism of apoA-IV in plasma may be mediated by a specific uptake mechanism in the liver. The implications of these results support the hypothesis that apoA-IV is involved in reverse cholesterol transport.

Apolipoprotein A-IV is one of the most actively synthesized apolipoproteins in human and in rat (1). The liver and the intestine are the major sites of synthesis of this apolipoprotein in the rat (2,3). In man, however, the hepatic output of apoA-IV may be insignificant (3). In plasma, apoA-IV is associated with the high density lipoproteins (HDL), but in humans the largest fraction is in the lipoprotein-free fraction (LFF) (4-6). Current evidence on the metabolism of this apolipoprotein indicates that the relative distribution in plasma is a function of the nutritional status (7) and possibly of plasma lecithin-cholesterol acyl-transferase of which apoA-IV is an activator (8,9). ApoA-IV is also one of the most actively catabolized apolipoproteins (6,10) and as such has the potential to be an important specific carrier of lipids to the tissues involved in catabolism. In this report we provide

ABBREVIATIONS. apoA-IV: apolipoprotein A-IV; HDL: high density lipoproteins; LFF: lipoprotein-free fraction of plasma; DMPC: dimyristoyl phosphatidylcholine; POPC: 1-palmitoyl-2-oleoyl-phosphatidylcholine.

0006-291X/86 $1.50

evidence that a specific and saturable binding site for an apoA-IV-phospholipid complex is present on highly purified rat liver plasma membranes.

METHODS

Male Sprague-Dawley rats (175-250 gr) were utilized throughout the study. ApoA-IV and apoA-I were isolated from rat plasma HDL (d = 1.063 - 1.210 g/ml) by a combination of heparin-agarose affinity chromatography and molecular sieve chromatography. Delipidated HDL was dissolved in 5 M Urea, 5 mM Tris-HCl pH 7.4 buffer containing 0.1% β-mercaptoethanol and applied to a heparin-agarose column. The first eluted peak contained apoA-IV, C peptides, and a trace amount of apoA-I. A fraction enriched in apoA-I was eluted by raising Tris-HCl concentrations to 50 mM. ApoA-IV and apoA-I were further purified by chromatography through a Sephadex S-200 (1.5 x 200) column equilibrated with 5 M Urea, 10 mM Tris-HCl pH 7.4. Purity and identity of the proteins were determined by SDS-PAGE, two-dimensional gel electrophoresis and by immunodiffusion techniques utilizing antibodies against rat apoA-I, apoE, and apoA-IV (6). With this procedure the usual yields of apoA-IV and apoA-I were, respectively, 1.5-2.5 mg and 7-10 mg per 100 ml of rat plasma.

ApoA-IV-DMPC and apoA-I-DMPC complexes were prepared by the cholate method of Jonas (11). For this purpose 2.5 mg of DMPC were suspended in 0.5 ml of 100 mM NaCl 50 mM sodium phosphate pH 7.4 and the solution clarified by adding 20% sodium cholate, in the same buffer, drop by drop. One mg of lyophilized apoA-IV or apoA-I was dissolved in this solution and sodium cholate eliminated by extensive dialysis. ApoA-IV or apoA-I and the phospholipids eluted together as a single peak from a BioGel A5m column (1.5 x 50 cm) at the elution volume of HDL consistent with the formation of a protein-phospholipid complex of discrete composition with a calculated MW of 160,000. DMPC and POPC unilamellar vesicles were prepared by sonication (11).

ApoE-poor rat HDL were prepared as described by Hay *et al.* (12) from the d = 1.070-1.170 g/ml plasma fraction. When analyzed by SDS-PAGE the preparation contained apoA-I and apoA-IV as major apolipoproteins with a mass ratio of 3.3 as derived from dye elution and colorimetric reading of the apoA-I and apoA-IV bands (13). Trace amounts of apoC's were present. ApoE accounted for less than 6% of the total HDL apoprotein mass.

Purified plasma membranes of rat liver were prepared as described by Pohl *et al.* (14) with a modification of the method of Neville as previously described (15). Briefly, 7-8 gr of liver were homogenized at 4°C in 25 ml of 1 mM $NaHCO_3$ with 8 strokes of a loose pestle. Eighty ml of bicarbonate buffer were added and the membrane suspension filtered through two layers of cheesecloth. The filtered material was centrifuged at 1,500 g for 10 min in a Sorvall centrifuge and the supernatant discarded. The pellet was resuspended in 1 mM $NaHCO_3$ and brought to 55 ml with 34 ml of 69% sucrose. The material was transferred to polypropylene ultracentrifuge tubes and overlaid with 42.3% sucrose. Partially purified membranes were collected at the top of the tubes after a spin at 90,000 g for 2 hrs in a Beckman SW28 rotor. The membranes were further purified by a final centrifugation at 25,000 g for 1 hr in 1 mM $NaHCO_3$ in a SW28 rotor. All the procedures were carried out at 4°C or in ice. When not used immediately, membranes were frozen at -70°C and resuspended in appropriate buffer just before use. Membrane pronase treatment was carried out by resuspending 100 μg of the membrane pellet as protein in 0.5 ml of 100 mM NaCl, 2 mM $CaCl_2$ and 50 mM Tris-HCl pH 7.4, adding a different amount of pronase and incubating at 37°C for 15 min. The reaction was stopped by adding 20 ml of buffer containing 4% BSA and reisolating the membranes by ultracentrifugation (25,000 g for 1 hr) as described. Membrane protein content was determined by the Bradford method, with BSA utilized as standard.

ApoA-IV-DMPC and apoA-I-DMPC complexes and apoE-poor HDL were radioiodinated by the method of McFarlane as modified by Schaefer (16). Typical specific activity of the preparation was 5,000 cpm/ng. The radioiodinated preparations had the same chromatographic behavior as the unlabelled material. Binding to the liver membranes was carried out in a 100 mM NaCl, 1 mM $CaCl_2$, 2% BSA, 50 mM Tris-HCl pH 7.4 buffer. The final volume of the incubation cocktail was 300 µl. Specific binding was calculated as the difference between the total binding and that observed in the presence of an excess of cold ligand. The binding reaction was stopped by the addition of 2 ml of the incubation buffer and immediate separation of the free from the bound radioiodinated ligand by filtration over a GF/A Whatman membrane, previously soaked in the incubation buffer and held in a Millipore filter holder.

RESULTS

In the first series of experiments, the time and temperature dependence of apoA-IV DMPC binding to rat liver membranes was determined. Equilibrium at 37°C was reached after 30 min. At 4°C, however, only minimal non-specific binding could be detected. Successive saturation binding experiments were carried out at 37°C for 45 min. Increasing concentrations of labelled apoA-IV-DMPC were utilized (0.13-8.4 µg/ml) and non-specific binding was determined by adding to the incubation medium cold apoA-IV-DMPC (75 µg/ml). The results are illustrated in Figure 1. Half-maximal binding saturation could be reached at a ligand concentration of 0.6 µg/ml per mg of membrane protein. A Scatchard plot of the results (Figure 1) was consistent with the presence of a

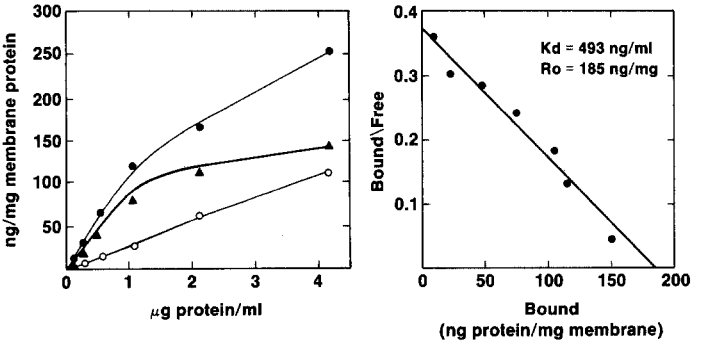
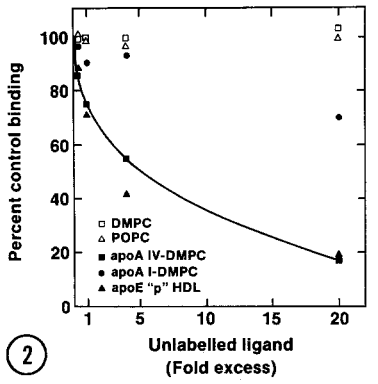


Figure 1: Saturation binding curve for apoA-IV-DMPC (on the left) and Scatchard plot of the results (on the right). Purified rat liver membranes (50 µg) were incubated with increasing concentrations of apoA-IV-DMPC in 100 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 7.4 buffer containing 2% BSA at 37°C for 45 min. At the end of the incubation period, the content of the vials was poured over Whatman GF/A filters and the membranes washed 5 times with 2 ml of the incubation buffer. Total binding (closed circles) was determined by reading the remaining radioactivity associated to the membranes after washing. Aspecific binding (open circles) was determined by adding to the incubation cocktails 22.5 µg of unlabelled apoA-IV-DMPC. The aspecific binding values were subtracted from the total binding values to obtain the specific binding curve (triangles). Each point is the mean of two independent determinations.



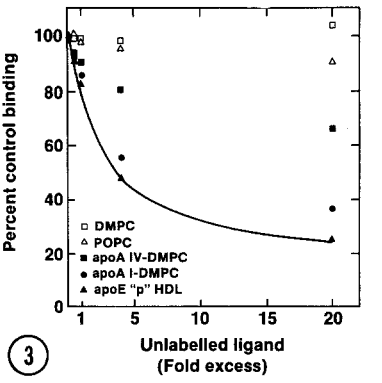


Figure 2: Displacement of radioiodinated apoA-IV-DMPC by increasing concentrations of unlabelled apoA-IV-DMPC, apoA-I-DMPC, apoE-poor HDL, and DMPC and POPC vesicles. A constant concentration of radioiodinated apoA-IV-DMPC (0.26 μg/ml) was used in these experiments ApoA-I-DMPC was added at the same protein concentration as apoA-IV-DMPC. ApoE-poor ("p") HDL had a protein concentration fourfold higher than that of apoA-IV-DMPC, assuming its apoA-IV content was 25% of the total protein mass. DMPC and POPC unilamellar vesicles had the same phospholipid concentration as apoA-IV-DMPC. Each point is the mean of two independent determinations.

Figure 3: Displacement of radioiodinated apoE-poor HDL by increasing concentration of unlabelled apoA-IV-DMPC, apoA-I-DMPC, apoE-poor HDL, and DMPC and POPC vesicles. A constant concentration of radioiodinated apoA-E-poor ("p") HDL (0.2 μg/ml) was used. ApoA-IV-DMPC, apoA-I-DMPC, and apoE-poor HDL preparations were added at the same protein concentration. DMPC and POPC vesicles had the same phospholipid concentration as apoA-E-poor HDL. Each point is the mean of two independent determinations.

single saturable binding site. The calculated dissociation constant was 493 ng/ml (3.1×10^{-9} M); Ro (the saturation concentration per mg of membranes) was 185 ng/mg (1.16 pmoles/mg membrane). Binding of apoA-IV-DMPC was insensitive to the presence of EDTA up to 5 mM. Incubation in calcium-depleted medium did not affect the binding either (data not shown). Thus calcium ion is not required for binding interaction of apoA-IV-DMPC with its binding site on liver membranes. Competition experiments (Figure 2) revealed that apoA-IV-DMPC as well as plasma apoE-poor HDL were able to effectively displace labelled apoA-IV-DMPC from its binding site. ApoA-I-DMPC complexes of the same composition as apoA-IV-DMPC displayed only partial competition. DMPC and POPC unilamellar vesicles were ineffective at all concentrations tested.

In a separate set of experiments, the ability of apoA-IV-DMPC and apoA-I-DMPC to compete for binding with radioiodinated apoE-poor HDL was investigated. Results are illustrated in Figure 3. In this case, apoA-I-DMPC dis-

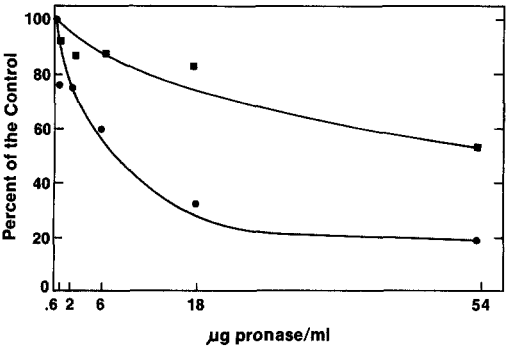


Figure 4: Effect of pronase treatment on the specific binding of radioiodinated apoA-IV-DMPC (●) and apoA-I-DMPC (■) to rat liver membranes. Pronase treatment was carried out as described in the text. After reaction with pronase and reisolation, the membranes were incubated with 0.26 µg/ml of radioiodinated apoA-IV-DMPC or apoA-I-DMPC and the specific binding determined as described in Figure 1. Results are given as percent of the specific binding to membranes not treated with pronase. Each point is the mean of two independent determinations.

played better competition properties than apoA-IV-DMPC. Since our apoE-poor HDL preparation may contain lipoprotein subfractions preferentially enriched in apoA-I and others in apoA-IV (7) both contributing to total binding, we speculate that the relative ability of apoA-I-DMPC and apoA-IV-DMPC to compete in this experiment is directly proportional to the abundance of HDL subspecies present in the radiolabeled apoE-poor HDL that bound. Effective displacement of apoE-poor HDL from the surface of cultured rat hepatocytes by apoA-I-DMPC has also been observed by others (17). Together these data support the view that apoA-IV is the molecular determinant responsible for binding of at least a subclass of HDL particles to the liver plasma membranes.

The effect of pronase treatment of the membranes on apoA-IV-DMPC and apoA-I-DMPC binding was tested. Treatment of the membrane with as little as 6 µg/ml of pronase decreased apoA-IV-DMPC binding efficiency significantly, suggesting that a membrane protein is involved in binding (Figure 4). On the contrary, pronase treatment affected apoA-I-DMPC binding only marginally even at the highest pronase concentration tested (54 µg/ml), which is in agreement with previous reports (18).

DISCUSSION

The synthetic rate of apoA-IV in rat and in human matches that of apoA-I although its plasma concentration is approximately one third and one eighth,

respectively, that of apoA-I, implying a very rapid turnover rate (6,10). The concentration of apoA-IV increases in plasma after a meal, specifically in the lipoprotein-free fraction (7). Current evidence suggests that the increase in LFF-apoA-IV levels is produced by the transfer of lymph LFF-apoA-IV to the plasma compartment (2). Lymph chylomicron apoA-IV in the rat is the major source of apoA-IV in plasma HDL (10). Compositional studies (7) suggest that HDL particles containing predominantly apoA-IV may exist in plasma. Complexes of apoA-IV with phospholipid and cholesterol have been found in rat plasma LFF. A similar complex has been isolated by Otha et al. (19) from human plasma LFF. Turnover studies in rat and humans have shown that apoA-IV either in HDL or LFF has a very fast rate of catabolism and that the liver is a major site of uptake of radioiodinated apoA-IV tracers (20). The data presented in this study are consistent with the view that rat liver membranes possess a saturable binding site which displays a specificity toward apoA-IV-DMPC. We thus speculate that the rapid catabolism of apoA-IV in plasma is mediated by a highly selective and efficient mechanism of uptake by the liver.

A series of observations has led to the view that apoA-IV is implicated in reverse cholesterol transport (1,21). Sloop et al. (21) have found that the peripheral lymph of dog contains apoA-IV associated with discoidal HDL particles which do not contain apoE and only in part apoA-I. Discoidal lipoproteins and apolipoprotein-phospholipid particles are an important functional entity of the reverse cholesterol transport hypothesis (22). In fact, they may be the preferred plasma recipient for the efflux of cholesterol from tissues. The efflux is further promoted by LCAT (23), which catalyzes the esterification of free cholesterol leaving the tissues and apoA-IV is an activator of this enzyme (9). Eventually, these particles mature to the size and shape of plasma HDL. The ultimate fate of these cholesteryl ester-enriched lipoproteins would be the liver since this is the only organ capable of substantial cholesterol catabolism and excretion. The mechanism of uptake is expected to be highly efficient so as to prevent redistribution of the lipid components to other lipoproteins of slower catabolism. Others (24) have

reported that in rat the catabolism of the cholesteryl esters carried in HDL is several fold faster than that of apoA-I and is directed toward the liver. The mechanism whereby HDL deliver cholesteryl ester to the liver is not known. It could occur during a transient binding of HDL to the liver, mediated by apoA-I (17). In addition to this, HDL cholesteryl ester uptake may be mediated by apoA-IV through interaction with a specific binding site. Research aimed at providing further insights into this important aspect of lipoprotein metabolism is underway.

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