Apolipoprotein A-V-heparin Interactions

IMPLICATIONS FOR PLASMA LIPOPROTEIN METABOLISM*

Received for publication, February 10, 2005, and in revised form, May 6, 2005
Published, JBC Papers in Press, May 6, 2005, DOI 10.1074/jbc.M501589200

Aivar Lookene‡, Jennifer A. Beckstead‡, Solveig Nilsson‡, Gunilla Olivecrona‡, and Robert O. Ryan†

From the ‡Department of Medical Biosciences/Physiological Chemistry, Umeå University, SE-901 87 Umeå, Sweden, the §Department of Gene Technology, Tallinn Technical University, Tallinn 12618, Estonia, and the ¶Lipid Biology in Health and Disease Research Group, Children’s Hospital Oakland Research Institute, Oakland, California 94609

Transgenic and gene disruption experiments in mice have revealed that apolipoprotein (apo) A-V is a potent regulator of plasma triglyceride (TG) levels. To investigate the molecular basis of apoA-V function, the ability of isolated recombinant apoA-V to modulate lipoprotein lipase (LPL) activity was examined in vitro. With three distinct lipid substrate particles, including very low-density lipoprotein (VLDL), a TG/phospholipid emulsion, or dimyristoylphosphatidylcholine liposomes, apoA-V had little effect on LPL activity. In the absence or presence apolipoprotein C-II, apoA-V marginally inhibited LPL activity. On the other hand, apoA-V-dimyr- istoylphosphatidylcholine disc particles bound to hepa- rin-Sepharose and were specifically eluted upon application of a linear gradient of NaCl. The interaction of apoA-V with sulfated glycosaminoglycans was further studied by surface plasmon resonance spectroscopy. ApoA-V showed strong binding to heparin-coated chips, and binding was competed by free heparin. ApoA-V enrichment enhanced binding of apoC-II-deficient chylo- microns and VLDL to heparin-coated chips. When LPL was first bound to the heparin-coated chip, apoA-V-enriched chylomicrons showed binding. Finally, human pre- and post-heparin plasma samples were subjected to immunoblot analysis with anti-apoA-V IgG. No differences in the amount of apoA-V present were detected. Taken together, the results show that apoA-V lipid complexes bind heparin and, when present on TG-rich lipoprotein particles, may promote their association with cell surface heparan sulfate proteoglycans. Through such interactions, apoA-V may indirectly affect LPL ac- tivity, possibly explaining its inverse correlation with plasma TG levels.

Using comparative genomics, Pennacchio et al. (1) discovered a new member of the exchangeable apolipoprotein family, termed apolipoprotein (apo) A-V. The gene identified predicted the presence of an open reading frame encoding a 366-amino- acid protein possessing 71% homology with its murine counterpart. To evaluate the function of apoA-V Pennacchio et al. (1) generated transgenic mice that overexpress human apoA-V as well as murine apoA-V gene-disrupted mice. Profound effects were observed in both knock-out and transgenic mice. ApoA-V transgenic mice displayed a 3-fold lower plasma triglyceride (TG) level compared with control littermates. Interestingly, the observed changes in TG levels are directly op- posite those reported for apoC-III knock-out and transgenic mice (2, 3). Whereas apoA-V knock-out mice displayed a 4-fold increase in plasma TG, apoC-III knock-out animals showed a 30% decrease.

To investigate the relationship between apoA-V and plasma TG levels, Pennacchio et al. (1) examined the correlation be- tween DNA sequence polymorphisms in the apoA-V gene and plasma lipid levels in humans. These authors identified four single nucleotide polymorphisms across and surrounding the human APOAV locus. Analysis of APOAV SNPs in a sample set of 501 random unrelated normolipidemic individuals who had been previously phenotyped for various lipid parameters before and after consumption of high and low fat diets, revealed significant associations between plasma TG levels, VLDL mass, and three of the four SNPs. These SNPs were associated with higher TG levels independent of diet. In further studies, Talmud et al. (4) investigated the effect of variation within the APOC3/A4/A5 gene cluster as a determinant of plasma TG levels, obtaining evidence that variation in the APOC3 locus is associated with differences in TGs in healthy men, independent of changes in the APOC3 locus.

Vu-Dac et al. (5) used human hepatocytes and HepG2 cells to test whether fibrates (TG-lowering drugs) modulate APOAV gene expression, thereby influencing plasma TG levels. These investigators found a peroxisome proliferator-activated receptor (PPARα) response element in the APOAV promoter. PPARα agonists strongly up-regulated apoA-V mRNA indicating that the APOAV gene is a positive and direct target of PPARα activators. In another report Prieur et al. (6) reported that the APOAV promoter possesses farnesoid X-activated receptor elements. This is particularly interest- ing because the farnesoid X-activated receptor is an impor- tant regulator of TG levels. Overall, it appears that two nuclear receptors that regulate TG metabolism, PPARα and farnesoid X-activated receptor, modulate expression of the APOAV gene.

Although knowledge of apoA-V gene expression is becoming better understood, key questions remain about the mechanism whereby the apoA-V protein influences plasma TG levels. In the present study we evaluated the hypothesis that apoA-V modulates lipoprotein lipase (LPL) activity and demonstrated that apoA-V is a heparin-binding protein. The potential effects of this interaction on metabolism of TG-rich lipoproteins by LPL and lipoprotein receptors are discussed.
EXPERIMENTAL PROCEDURES

Apolipoproteins and Lipoproteins—Recombinant human apoA-V was prepared as described by Beckstead et al. (7). Normal human lipoprotein fractions were obtained by sequential centrifugation of human EDTA plasma. ApoC-II-deficient chylomicrons were a generous gift from Dr. Bill Skerrett and Dr. Michael Hulley (University of Tennessee, Memphis, TN and Eppendorf, Hamburg, Germany). ApoC-II and apoC-III were isolated from human plasma as described previously (8). The apolipoproteins were dissolved in 5 mM urea, 10 mM Tris-Cl, pH 8.5, and protein concentrations in the stock solutions were determined by the BCA kit (Pierce). Other Reagents and Analyses—Heparin was obtained from Novo Nordisk. Biotinylated heparin was prepared as described by Beckstead et al. (7). Normal human lipoprotein fractions were obtained by sequential centrifugation of human EDTA plasma. ApoC-II-deficient chylomicrons were a generous gift from Prof. Ulrike Beisiegel and Dr. Jörg Heeren, (University Hospital of the University of Munich, Germany). Heparin was obtained from Novo Nordisk. Biotinylated heparin was prepared as described by Beckstead et al. (7). Normal human lipoprotein fractions were obtained by sequential centrifugation of human EDTA plasma. ApoC-II-deficient chylomicrons were a generous gift from Dr. Bill Skerrett and Dr. Michael Hulley (University of Tennessee, Memphis, TN and Eppendorf, Hamburg, Germany). ApoC-II and apoC-III were isolated from human plasma as described previously (8). The apolipoproteins were dissolved in 5 mM urea, 10 mM Tris-Cl, pH 8.5, and protein concentrations in the stock solutions were determined by the BCA kit (Pierce).

Surface Plasmon Resonance—Surface plasmon resonance (SPR) binding studies were performed on a BIACore 2000 (Biacore, Uppsala, Sweden) using CM5 sensor chips (12, 13). Streptavidin was covalently attached to the carboxymethylated surface of the sensor chip activated by N-hydroxysuccinimide and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide. Then biotinylated heparin was bound to the immobilized streptavidin. Binding experiments were carried out at 25 °C in 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl and 50 mM guanidine hydrochloride. A stock solution of apoA-V (1.6 mg/ml) was prepared in 6 mM guanidine hydrochloride, and dilutions into the running buffer were made just before injection. A solution of 10 mM HEPES, pH 7.4, 1 M NaCl was used for regeneration of the surface of the sensorchips. Sensograms were analyzed with the aid of the BIAevaluation software Version 3 (Biacore, Uppsala, Sweden). To obtain curves of specific binding, background sensograms characterizing nonspecific interactions were subtracted from experimental sensograms.

RESULTS

Effect of ApoA-V and ApoC-III on LPL Activity—To determine whether the inverse correlation between apoA-V and plasma TG levels arises from direct stimulation of lipolysis, LPL activity was measured in vitro using three unique substrate lipoparticles. When either VLDL or DMPC liposomes were employed as the substrate, no activation of LPL was observed by apoA-V. ApoC-III in high concentrations caused some inhibition of LPL activity on VLDL (Fig. 1). With the TG emulsion as the substrate there was a slight apparent stimulation of LPL activity at the lowest concentrations of apoA-V, but inhibition was then seen at higher concentrations (>3 μM). The inhibition was not as strong as that with apoC-III, which was compared in the same experiment. In the absence of apoC-III inhibition of baseline LPL activity was observed with apoA-V. ApoC-III was less inhibitory than apoA-V in incubations without apoC-II. Thus, using different substrate lipoparticles, where full activation depends on the presence of apoC-II, little or no effect of apoA-V was observed. On the basis of these data we concluded that apoA-V does not serve as a direct activator of LPL.

Heparin-Sepharose Affinity Chromatography—An examination of the amino acid sequence of mature human apoA-V revealed a stretch of 42 residues that lacks amino acids with negatively charged side chains (residues 186–227). On the other hand, this segment contains 5 Arg and 3 Lys (plus 3 His) residues. Given the strong positive charge density in this region of the protein, we postulated that apoA-V may interact with heparan sulfate proteoglycans. To examine this, apoA-V-DMPC lipid particles were subjected to heparin-Sepharose affinity chromatography (Fig. 2). The results obtained indicated that apoA-V-DMPC lipid particles bind heparin-Sepharose and eluted upon application of a NaCl gradient. ApoA-V-DMPC complexes eluted at 0.36 M NaCl, whereas apoE3-NT, a known heparin-binding protein used as a positive control, eluted at 0.41 M NaCl (not shown).

SPR Spectroscopy—To further explore the interaction of apoA-V with sulfated glycosaminoglycans, SPR binding experiments were performed. Fig. 3 shows a sensogram of apoA-V binding to a heparin-coated chip. A steady, time-dependent increase in binding to the heparin-coated surface was observed that plateaued after ~2600 s, apparently reaching a stable binding state. Whereas dissociation of bound material was slow, it was rapidly released upon addition of free heparin. The interaction was characterized by an association rate constant \( k_a = 4.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) and a dissociation rate constant \( k_d = 0.009 \text{ s}^{-1} \).

To examine the effect of apoA-V on lipoprotein interactions with the heparin-coated chip, apoC-II-deficient chylomicrons were used (Fig. 4A). In the absence of exogenous apoA-V a small amount of binding was noted. By contrast, prior incubation of the heparin chip with apoA-V caused a significant enhancement in chylomicron binding. Similar results were obtained with VLDL (Fig. 4B), where an even greater enhancement in binding was observed in the presence of apoA-V. On the other hand, apoA-V had little effect on the
To extend these binding studies, mixing experiments were performed wherein LPL was first exposed to the heparin-coated chip (Fig. 5). As expected, LPL bound to the heparin surface. Subsequent exposure to apoC-II-deficient chylomicrons induced an enhancement in binding. Moreover, when apoA-V-enriched chylomicrons were introduced, a further increase in chylomicron binding to the LPL-heparin-coated chip was observed.

To determine whether a pool of heparan sulfate proteoglycan-bound apoA-V may exist in vivo, blood samples were obtained from subjects prior to and after injection with 100 units of heparin/kg. ApoA-V levels in the pre- and post-heparin treatment plasma samples were examined by immunoblot analysis. The results, shown in Fig. 6, revealed little or no differences in plasma apoA-V concentration as a function of heparin injection.

**DISCUSSION**

The discovery of apoA-V and its correlation with plasma TG levels through transgenic and gene disruption experiments raises important questions about the mechanism whereby apoA-V exerts its effects. The elegant studies of Pennacchio et al. (1) illustrate the power of comparative sequence analysis in identifying functionally important regions of the genome. The unexpected discovery of a new interaction of HDL with heparin-coated SPR chips (Fig. 4C).

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vascular disease. Although there is strong correlative evidence regarding apoA-V, the molecular mechanism underlying its effects has yet to be elucidated. Understanding details of how apoA-V affects plasma TG levels is a key step toward application of knowledge of this intriguing apolipoprotein to the diagnosis and/or treatment of hypertriglyceridemia. Three major hypotheses currently exist to explain the mechanism of apoA-V action, including 1) a direct modulatory effect on lipolytic enzyme activity, 2) an intracellular effect on hepatic lipoprotein biogenesis and/or secretion, and 3) an indirect effect within the plasma compartment to facilitate TG-rich lipoprotein metabolism or clearance from the plasma compartment.

Mechanism 1, Direct Modulation of LPL Activity—Among hypotheses currently under consideration to explain apoA-V effects on plasma TG levels, a prominent one is a direct activation by apoA-V of LPL activity, thereby facilitating hydrolysis and removal of TG from VLDL and chylomicrons. Such a mechanism would be consistent with apoA-V transgenic and gene disruption experiments in mice. The low abundance of apoA-V in plasma (~1 μg/ml) suggests it is not merely a structural protein. Schaap et al. (14) used adenoviral transfection to increase the levels of apoA-V in mice. These authors found an apoA-V dose-dependent reduction in postprandial hypertriglyceridemia following intragastric fat load and concluded that apoA-V stimulates LPL-dependent clearance of TG-rich lipoproteins. Complementary in vitro experiments with recombinant apoA-V revealed a >2-fold stimulation of LPL activity. In other studies, Fruchart-Najib et al. (15) reported that apoA-V shifts from HDL to VLDL following a fat load in mice expressing human apoA-V. These authors also report that enrichment of human VLDL with recombinant apoA-V induces a 50% increase in LPL activity, leading to the proposal that apoA-V manifests its biological effects on plasma TG levels by activating LPL. In the present studies, however, we found no evidence that apoA-V serves as a direct modulator of LPL activity in vitro. By contrast, apoC-II had a major stimulatory effect on LPL activity. Importantly, in support of our findings, Merkel et al. (16) recently reported that apoA-V did not affect LPL hydrolysis rates in the absence of proteoglycans. Thus, although a clear explanation for the different results is not available at present, it is conceivable that, under certain incubation conditions, apoA-V may alter the substrate emulsion, thereby increasing TG accessibility and LPL activity. Further experiments will be required to determine the basis for the discrepancy between results.

Mechanism 2, Intracellular Mode of Action—Based on the unique interfacial properties of apoA-V (17), an intracellular mode of action has been postulated. In studies with transfected COS-1 cells, control proteins including human serum albumin and apoB-6.6 (17), were efficiently secreted during a 3-h radiolabeling period. By contrast, the majority of apoA-V was retained in the cell lysate, with only small amounts recovered in the medium. Immunofluorescence microscopy studies revealed that albumin and apoB-6.6 displayed diffuse endoplasmic re-
ticulum and prominent heminuclear Golgi staining, whereas apoA-V displayed only perinuclear and diffuse cytoplasmic staining, consistent with localization primarily to the endoplasmic reticulum (17). Thus, it is conceivable that apoA-V does not traffic efficiently from the endoplasmic reticulum to the Golgi, resulting in low secretion efficiency. Additional evidence for an intracellular mode of action has been presented by Schaap et al. (14). These authors observed that adenovirus-mediated apoA-V expression in mice decreased the VLDL-TG production rate in a dose-dependent manner. Interestingly, they found no effect on VLDL particle number, suggesting apoA-V impairs lipida
tion of apoB. When considered in light of the unique interfacial properties of apoA-V (17) the concept that it interferes with nascent lipoprotein secretion efficiency remains viable. This concept is also in keeping with the findings of van der Vliet (18), wherein liver expression of apoA-V was stimulated by partial hepatectomy. In this case, it is conceivable that apoA-
V-mediated inhibition of TG secretion preserves this pool of lipid for cellular needs associated with regeneration.

Mechanism 3, Indirect Effect of ApoA-V on Plasma Lipoprotein Metabolism—Possible mechanisms include serving as a ligand for cell surface lipoprotein receptors or facilitation of alternate cell surface interactions. The presence of a stretch of 42 amino acids possessing strong positive charge character in the apoA-V amino acid sequence led us to examine the heparin binding ability of apoA-V. In contrast to the findings of Frucht-Najib et al. (15), when subjected to heparin-Sepharose affinity chromatography, the binding of apoA-V-DMPC complexes was observed. These studies were followed by SPR characterization of the heparin binding ability of lipoproteins in the presence of apoA-V. The observation that enrichment of a heparin sensor chip with apoA-V induced a significant enhance-
ment in binding of TG-rich lipoproteins suggests that apoA-V can facilitate TG-rich lipoprotein interaction with heparan sulfate proteoglycans in vivo. If so, it may be anticipated that this would increase interaction between LPL and TG-rich lipoprotein substrates, thereby stimulating TG lipolysis with a con-
comitant reduction of plasma TG levels. Support for this concept was obtained in SPR experiments wherein the heparin-coated chip was preloaded with LPL. Subsequent interaction studies with chylomicrons revealed that prior enrichment with apoA-V results in an increased binding. These studies, which provide direct evidence that ternary interactions between LPL, TG-rich lipoprotein, and heparin are enhanced in the presence of apoA-V, are congruent with the recent findings of Merkel et al. (16) who showed that proteoglycan-bound LPL activity is modulated by apoA-V. Attempts to demonstrate the release of cell surface heparan sulfate proteoglycan-associated apoA-V upon injection of heparin into human volunteers, however, failed to show an affect. It is unclear whether sufficient heparin was infused to release an increment of apoA-V that would be detectable in plasma after 10 min. Interestingly, heparin-in
duced release of apoA-V from the SPR chips was relatively slow compared with the behavior of LPL (12). It is conceivable that apoA-V-containing lipoprotein interactions with heparan sulfate proteoglycans in vivo are short lived, culminating in li-
poprotein particle internalization, as proposed by Mahley and co-workers for apoE-containing lipoproteins (19, 20). A “secre-
tion-capture” hypothesis has been enunciated to explain the role of heparan sulfate proteoglycans in facilitation of apoE-de-
pendent lipoprotein clearance. If such a mechanism exists for apoA-V, pre- and post-heparin plasma samples may not reveal differences in apoA-V levels. At present, it is not known whether apoA-V serves as a ligand for cell surface lipoprotein receptors. Although no precise consensus receptor recognition sequence has been identified for members of the low density lipoprotein receptor family, apoE and apoB binding occurs via 

The evidence presented in this work indicates apoA-V can influence lipoprotein interaction with heparan sulfate proteoglycans. In so doing, it may attract TG-rich lipoproteins to an environment that can either facilitate apoC-II-activated lipo-
lysis by LPL or lipoprotein particle uptake. In either case, the result would be consistent with evidence from apoA-V trans-
genic and gene-disrupted mice, providing a molecular explana-
tion for the TG-lowering effects of this protein.

Acknowledgments—We thank Prof. Ulrike Beisigel and Dr. Joerg Heeren for providing apoCII-deficient chylomicon and Dr. Michael N. Oda for the anti-apoA-V IgG.

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J. Biol. Chem. 2005, 280:25383-25387.
doi: 10.1074/jbc.M501589200 originally published online May 6, 2005

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