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Apolipoprotein A-V Interaction with Members of the Low Density Lipoprotein Receptor Gene Family[†]

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ABSTRACT: Apolipoprotein A-V is a potent modulator of plasma triacylglycerol levels. To investigate the molecular basis for this phenomenon we explored the ability of apolipoprotein A-V, in most experiments complexed to disks of dimyristoylphosphatidylcholine, to interact with two members of the low density lipoprotein receptor family, the low density lipoprotein receptor-related protein and the mosaic type-1 receptor, SorLA. Experiments using surface plasmon resonance showed specific binding of both free and lipid-bound apolipoprotein A-V to both receptors. The binding was calcium dependent and was inhibited by the receptor associated protein, a known ligand for members of the low density lipoprotein receptor family. Preincubation with heparin decreased the receptor binding of apolipoprotein A-V, indicating that overlap exists between the recognition sites for these receptors and for heparin. A double mutant, apolipoprotein A-V (Arg210Glu/Lys211Gln), showed decreased binding to heparin and decreased ability to bind the low density lipoprotein receptor-related protein. Association of apolipoprotein A-V with the low density lipoprotein receptor-related protein or SorLA resulted in enhanced binding of human chylomicrons to receptor-covered sensor chips. Our results indicate that apolipoprotein A-V may influence plasma lipid homeostasis by enhancing receptor-mediated endocytosis of triacylglycerol-rich lipoproteins.

Elevated plasma triacylglycerol (TG¹) is an independent risk factor for coronary heart disease (1) and is associated with insulin resistance and type 2 diabetes (2). A recently identified genetic factor affecting plasma TG levels is *apolipoprotein (APO) A5*. Pennacchio et al. showed that *APOA5*, which is located in the *APOA1/C3/A4* gene cluster, encodes a previously unknown 366 amino acid protein, including a 23 amino acid signal peptide (3). Transgenic mice overexpressing human apolipoprotein (apo) A-V had about 30% lower plasma TG levels compared to control mice, while *APOA5* gene disrupted mice displayed a 4-fold increase in plasma TG. Several population studies based on single nucleotide polymorphism analysis in humans have confirmed

an association between apoA-V and plasma TG levels, low density lipoprotein particle size, increased levels of remnant-like particles in plasma as well as a higher risk ratio for cardiovascular disease and familial combined hyperlipidemia (3–12).

ApoA-V is present in human serum at very low concentrations compared to other apolipoproteins and is normally found in association with chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL) (13). Ishihara et al. reported plasma levels of 179.2 ± 74.8 μ g apoA-V/L in healthy humans and found an inverse relationship between plasma apoA-V concentrations and TG levels (14). In other studies such correlations were weak (13). An explanation for these variable results may be differences in the ratio between apoA-V and apoC-III, another known determinant of plasma TG levels (13). There are several reports suggesting that *APOA5* and *APOC3* affect TG levels in an opposite manner (15, 16).

Although the mechanism by which apoA-V affects plasma TG levels is not fully understood, several theories have been proposed including (1) an intracellular effect on hepatic VLDL assembly and secretion and (2) an effect on plasma removal either by (a) direct modulation of the activity of lipoprotein lipase (LPL) or by (b) an indirect effect on lipoprotein metabolism. Recent studies by Lookene et al. (17) and Merkel et al. (18) support the concept that apoA-V indirectly modulates plasma lipoprotein metabolism by facilitating the action of LPL. ApoA-V has high affinity for

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¹ Abbreviations: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenedinitrilo tetraacetic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; RAP, receptor associated protein; SPR, surface plasmon resonance; TG, triacyl glycerol; VLDL, very low density lipoprotein; WT, wild type.

heparin and enhances binding of lipoproteins to heparin, both in the absence and in the presence of LPL. Overexpression of human apoA-V in transgenic mice affected plasma TG levels to a lesser extent when LPL was reduced. ApoA-V did not markedly stimulate hydrolysis by LPL in free solution in the absence or presence of heparin, but when LPL was bound to heparin-covered surfaces, apoA-V significantly increased LPL-mediated hydrolysis of TG.

Still another possibility is that apoA-V affects lipoprotein internalization through interaction with cell surface receptors (17, 19). Similar to previously known apolipoprotein ligands for low density lipoprotein receptor (LDLR) family members, apoA-V contains a segment enriched in positively charged amino acid residues. In the present study we evaluated interactions between apoA-V and two structurally distinct LDLR family members, the LDLR-related protein (LRP) and the mosaic type-1 receptor, SorLA (also known as LR11). The endocytic receptor LRP is abundant in hepatocytes and participates in hepatic clearance of TG-rich particles *in vivo* (20) whereas SorLA is expressed in vascular smooth muscle cells (21). The receptors of the LDL receptor family are dependent on Ca^{2+} for correct folding of their ligand binding sites (22). The extracellular part of LRP contains four clusters of LDL receptor class A (LDL-A) repeats of which clusters II and IV bind a large number of different ligands, including apoE and LPL (23). SorLA has a single cluster composed of 11 LDL-A repeats with ligand binding properties similar to those of LRP clusters II and IV. In fact, the two receptors bind some ligands almost identically (e.g., components of the plasminogen activation system (24)). We hypothesized that LRP and/or SorLA might also bind apoA-V and, thereby, facilitate cellular internalization of apoA-V associated lipoproteins. We show that apoA-V binds to both types of receptors, that a double mutant of apoA-V in which two Lys residues were exchanged for Ala led to reduced binding both to heparin and to LRP, and that prebinding of apoA-V to the receptors dramatically increased the ability of the receptors to interact with human chylomicrons, demonstrating a possible bridging function of apoA-V.

EXPERIMENTAL PROCEDURES

Recombinant ApoA-V. Recombinant wild type (WT) human apoA-V was produced in *Escherichia coli* and isolated as described by Beckstead et al. (25). The expressed protein contained 343 amino acids plus a vector encoded N-terminal His tag extension. The variant R210E/K211Q-apoA-V was generated with mutagenic oligonucleotide primers according to the Quick-Change method (Stratagene). Stock solutions of the lyophilized proteins were prepared in 5 M urea, 10 mM TRIS pH 8.5 for experiments with lipid-free apoA-V, or in 50 mM sodium citrate, pH 3.0 for experiments with complexes with dimyristoylphosphatidyl choline (DMPC). Protein concentrations were determined by the BCA kit (Pierce, USA). Earlier studies have shown that, under the conditions used, the complexes formed are lipid disks (25).

Apolipoprotein A-V-DMPC Disks. ApoA-V-DMPC disks were prepared by dissolving 10 mg of DMPC in chloroform:methanol (3:1 v/v). Solvent was evaporated under a stream of N_2 , and the sample was lyophilized for >2 h to remove residual solvent. The DMPC was subsequently dispersed in 1 mL of 50 mM sodium citrate, pH 3.0, and vesicles were

generated by extrusion through a 0.22 μm filter. Four milligrams of apoA-V was added to the DMPC vesicle preparation, and the sample was gently vortexed until it was homogeneous. It was then incubated for 3 h at 24 °C. Centrifugation for 10 min at 15000 rpm (Eppendorf Centrifuge 5417) resulted in a clear supernatant, which was filtered through a Millex GS 0.22 μm membrane (Millipore). The concentration of lipid was determined by the Phospholipids B kit (Wako, Neuss, Germany). Comparison of the migration of disks made from WT apoA-V to that of disks made from the mutant R210E/K211Q-apoA-V on native gradient PAGE (25) demonstrated that the complexes were of similar size.

Recombinant Apolipoprotein E. Recombinant wild type apolipoprotein E was expressed in *E. coli* transformed with an apoE/pET20b plasmid with N-terminal His tag extension and isolated from the solubilized cell pellet by Hi-Trap nickel chelation affinity chromatography. Apolipoprotein CIII was purified from human plasma as described (26).

Receptors, Receptor Associated Protein, Chylomicrons, Heparin, and Albumin. SorLA, LRP, and the receptor associated protein (RAP) were prepared as described (24). ApoC-II-deficient chylomicrons were a generous gift from Drs. Ulrike Beisiegel and Joerg Heeren, Eppendorf University Hospital, Hamburg, Germany. Heparin used for biotinylation was a kind gift of Dr. Per Østergaard (Novo Nordisk, Denmark). Heparin used for the competition studies was purchased from Leo Pharma, Malmö, Sweden. Bovine serum albumin (fraction V) was obtained from Sigma-Aldrich.

Surface Plasmon Resonance (SPR). Binding studies were performed on a Biacore 2000 (Biacore, Uppsala, Sweden) using CM5 sensor chips. LRP or SorLA was immobilized to the sensor chip surface using the Biacore amine coupling kit (Biacore, Uppsala, Sweden). For most of the experiments blocking with BSA was used to reduce nonspecific binding as much as possible. For reference flow cells an equivalent mass of BSA (same level of RU) was bound compared to the total mass of protein in the active flow cell. Binding experiments were performed at 25 °C under continuous flow (5 $\mu\text{L}/\text{min}$) of 40 mM Hepes, pH 7.4, containing 150 mM NaCl and 2.5 mM CaCl_2 , unless otherwise stated. Stock solutions of apoA-V-DMPC disks or lipid-free apoA-V (in 5 M urea), or comparable preparations of other apolipoproteins, were diluted into running buffer just prior to injection. The experiments on immobilized heparin were made as previously described (17). Sensorgrams were analyzed with the BIAevaluation software version 3.0 (Biacore, Uppsala, Sweden). Complete regeneration of the sensor chips, using high salt, acidic or alkaline pH, or detergents was not possible, either for lipid-free or lipid-bound apoA-V. Too harsh regeneration conditions (SDS and other detergents) disrupted the sensor chip surface and denatured the receptors, whereas too mild conditions (high salt, low pH) did not completely break the binding of apoA-V to the receptors.

High Performance Liquid Chromatography. Disks of DMPC, containing either the wildtype (WT) apoA-V or the double mutant R210E/K211Q-apoA-V, were dialyzed into 20 mM sodium phosphate, pH 7.2 and applied to a 1 mL HiTrap Heparin HP affinity column (GE Healthcare). After loading, the column was washed with buffer, and bound proteins were eluted with a linear gradient of NaCl from 0 to 2.0 M, as previously described (17).

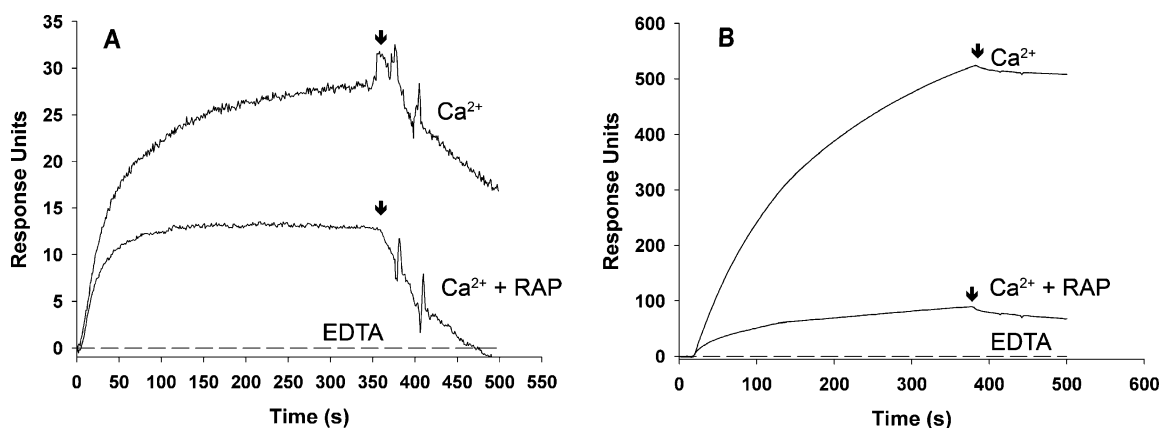


FIGURE 1: Binding of apoA-V-DMPC disks to LRP and SorLA. Sensor chips with immobilized LRP (4.7 fmol/mm²) or with SorLA (11.6 fmol/mm²) together with BSA (61 fmol/mm²) were used. For baseline subtraction a flow cell containing only BSA was used. Binding studies were performed at 25 °C in 40 mM Hepes buffer pH 7.4, containing 150 mM NaCl. (A) Injection of apoA-V-DMPC disks to LRP in the presence of calcium (2.5 mM CaCl_2) or EDTA (10 mM) or after injection of RAP (30 μL of 1.6 μM resulting in 70 RU) with calcium present (2.5 mM). (B) Injection of apoA-V-DMPC disks to SorLA with calcium present, with EDTA present or after injection of RAP (30 μL of 1.6 μM resulting in 440 RU) with calcium present. Binding in the presence of EDTA is indicated with a dashed line because subtraction of the reference flow cell (with BSA only) led to slightly negative values. The arrows indicate the end of the sample injections when the flow was changed to buffer only.

RESULTS

Receptor Binding. Binding of free apoA-V and of apoA-V-DMPC disks to LRP or SorLA was investigated by SPR. The receptors were covalently attached to the dextran matrix of sensor chips, and binding of apoA-V was measured on injection of the protein in the fluid phase. The experiments were performed with receptor-containing flow cells blocked with bovine serum albumin (BSA) to reduce nonspecific binding, and data from a reference flow cell containing immobilized BSA was used for subtraction. Figure 1 panel A shows binding of apoA-V-DMPC disks to LRP in the presence of Ca^{2+} . In buffer with EDTA, no binding was observed, in concert with the requirement of the receptors for Ca^{2+} to attain their native conformation (22). Preinjection of the protein RAP markedly reduced binding of apoA-V. RAP is known to compete for binding of most ligands to the LDL-A repeats in LRP (and SorLA). Taken together these results indicate that apoA-V bound to LRP in a specific manner. In experiments with a second LDLR family member, SorLA (Figure 1, panel B), binding of apoA-V-DMPC disks was competed by RAP and was abolished when apoA-V was injected in the presence of EDTA. As with apoA-V in DMPC disks, lipid-free apoA-V also bound to LRP and the binding was reduced by RAP (data not shown). In contrast to binding of apoA-V-DMPC disks, binding of lipid-free apoA-V to LRP was reduced by only 50% by the presence of EDTA, when the appropriate BSA-coated control was subtracted (data not shown). These results indicate that apoA-V in its lipid-free conformation could bind to denatured receptors.

Specificity of the Interactions. SPR experiments with DMPC vesicles without apoA-V demonstrated that there was no binding to either of the receptors with the empty lipid vesicles (data not shown). Thus, the interaction with lipid disks containing apoA-V must be due to the protein component. In experiments with apoA-V and SorLA-coated, BSA-blocked sensor chips (Figure 2), apoE and apoC-III were used for positive and negative comparisons, respectively, with all three proteins either complexed to DMPC (panel A) or lipid free (panel B). Flow cells with immobilized BSA were used as references. Specific binding was observed

to SorLA-coated, BSA-blocked, flow cells both of apoA-V and apoE, but not of apoC-III.

Receptor and Heparin Recognition Site in ApoA-V. To investigate the possible influence of heparin on binding of apoA-V to LRP and SorLA, apoA-V-DMPC disks were mixed with heparin (10 U/mL) before injection to LRP- or SorLA-coated sensor chips (Figure 3). Heparin abolished the binding to LRP (panel A) and significantly decreased binding to SorLA (panel B), suggesting that the binding site(s) in apoA-V for heparin and for LDLR family receptors are, at least partly, overlapping. It is also possible that bound heparin sterically interferes with binding of apoA-V to the receptors. The effect of heparin was more prominent with LRP than with SorLA.

Bridging to Lipoproteins. To examine if receptor-bound, lipid-free apoA-V could function in bridging of lipoproteins to LDLR family receptors, we studied binding of chylomicrons isolated from an apoC-II-deficient patient (Figure 4). Prebinding of lipid-free apoA-V to receptor-coated sensor chips markedly enhanced chylomicron binding to both LRP (panels A and B) and SorLA (panels C and D), compared to control flow cells with receptors, but without apoA-V. Under the conditions used in panels A and C (with 15 ng of chylomicrons/ μL in the flow phase), binding was enhanced up to 15-fold with LRP and 7-fold with SorLA by the presence of apoA-V. A relatively low surface concentration of apoA-V (4.5 and 5.4 fmol/mm², respectively) was able to bind more than 400 RU of chylomicrons in the case of LRP and about 800 RU in the case of SorLA. The association rates were almost linear during the injection time, indicating a high number of binding sites at the surface. When the concentration of chylomicrons was decreased 10-fold (panels B and D), the association rates decreased only about 5-fold, indicating a tendency for saturation. In all cases, dissociation was very slow after the flow was changed to buffer alone, indicating that stable complexes had been formed. In experiments with chylomicrons, blocking with BSA could not be used because binding of chylomicrons was greater to sensor chips with BSA blocking than without (data not shown).

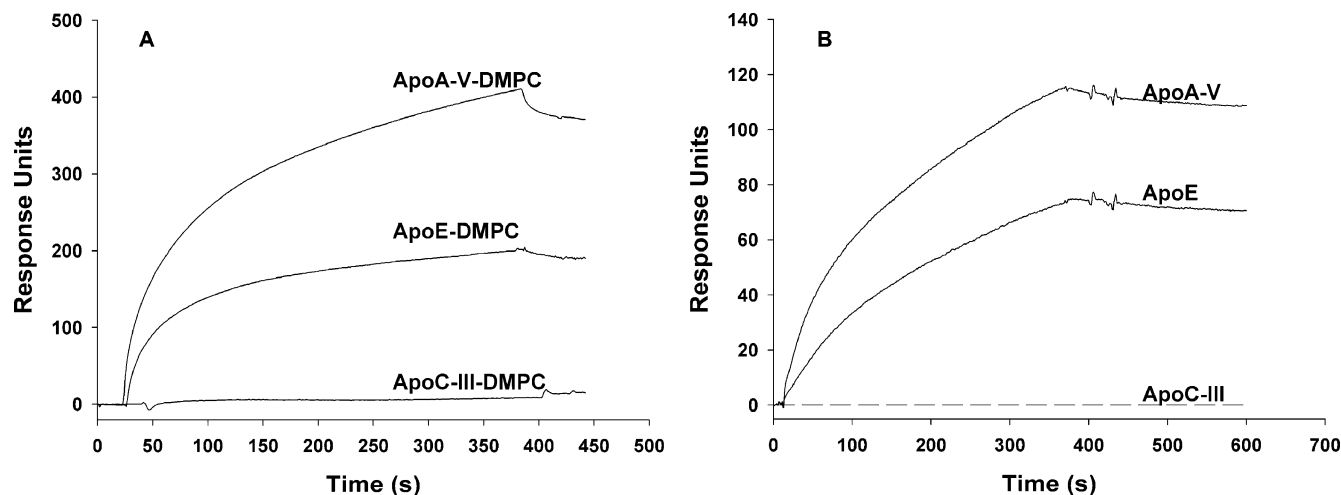


FIGURE 2: Comparison of binding of apoA-V, apoE, and apoC-III, in complex with DMPC or lipid free, to SorLA. Binding studies were performed under the same conditions as in Figure 1, with 2.5 mM CaCl_2 . Sensor chips immobilized with SorLA (8 fmol/mm²) and BSA (30 fmol/mm² in panel A and 60 fmol/mm² in panel B), were used. For baseline subtraction a flow cell containing only BSA was used. Panel A shows injection of disks of DMPC containing apoA-V, apoE, or apoC-III, all at a protein concentration of about 0.1 μM . Panel B shows injection of lipid-free apoA-V, apoE, or apoC-III, all at a protein concentration of about 0.01 μM . In panel B binding of apoC-III was somewhat higher to the reference flow cell than to that with SorLA and BSA. The subtraction led to slightly negative values, and the curve is therefore indicated with a dashed line.

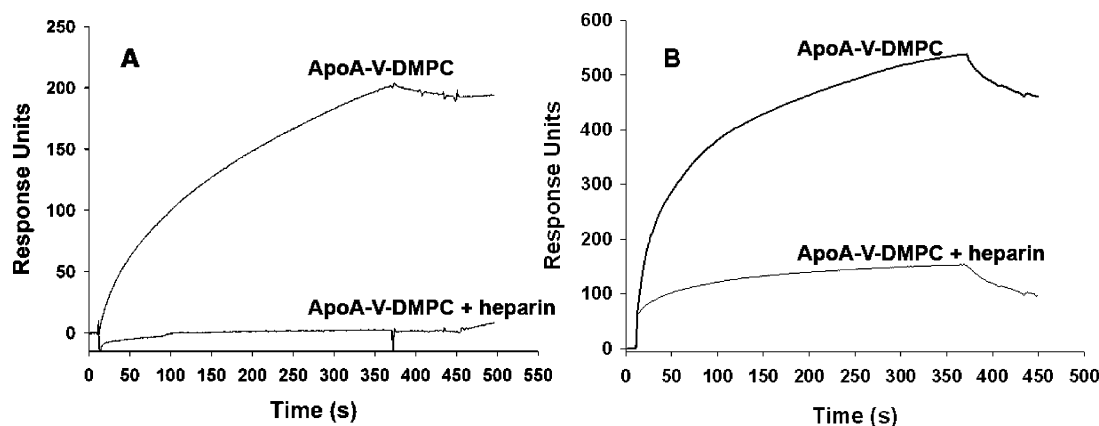


FIGURE 3: Effects of heparin on binding of apoA-V-DMPC disks to LRP and SorLA. Binding studies were performed under the same conditions as in Figure 1, with 2.5 mM CaCl_2 . For baseline subtraction a flow cell containing only BSA was used. ApoA-V-DMPC disks were diluted in running buffer with or without heparin (10 U/mL). (A) The surface concentration of LRP on the sensor chip was 3.7 fmol/mm², and the BSA concentration was 18.5 fmol/mm². (B) Samples corresponding to those used in panel A were injected to a flow cell with SorLA (7.4 fmol/mm²) and BSA (42 fmol/mm²).

Comparison to the Bridging Effect of LPL. LPL binds to proteoglycans and members of the LDL receptor family and mediates binding of lipoproteins to surfaces covered by these agents in a manner similar to that seen with apoA-V (27–29). To mimic the situation at a cell surface, where both LPL and apoA-V may be present simultaneously, we compared apoA-V-dependent chylomicron interaction with LRP and SorLA to that when LPL was present on the receptors. With this experimental design it is not possible to distinguish which mediator is most important for the change in response. Therefore the two mediators (apoA-V or LPL) were not tested together on the same sensor chip. Figure 5 shows binding of chylomicrons to LRP (panel A) and to SorLA (panel B) after preinjection with either apoA-V or LPL. For comparison, care was taken to bind similar amounts of receptors and mediators to the sensor chips. In accord with our previous experiences with LPL-mediated binding it was not possible to find a simple model that could sufficiently well describe the experimental data. The complex kinetics in this case is probably caused by changes of the

size and composition of the chylomicrons due to lipolysis by LPL at the sensor chip surface, illustrated by the comparatively rapid dissociation when the flow was changed to buffer only (28). ApoA-V was able to facilitate chylomicron binding to both types of receptors, but the association was slower than that observed with LPL. Furthermore, dissociation from the receptor-coated chips was extremely slow in the presence of apoA-V. To investigate the possible contribution of nonspecifically bound apoA-V, lipid-free apoA-V was bound to a sensor chip in the absence of receptors and chylomicrons (Figure 5, panel B). As with sensor chips without bound apoA-V (control), there was little specific binding of chylomicrons. This demonstrated that only receptor-bound apoA-V was able to mediate binding of chylomicrons to the surface.

Heparin Binding. Previous studies revealed that apoA-V binds to heparin (17). Sequence analysis identified a stretch of 42 amino acid residues (from 186 to 227) containing eight Arg/Lys residues and three His, but no negatively charged residues. To test the hypothesis that this region of

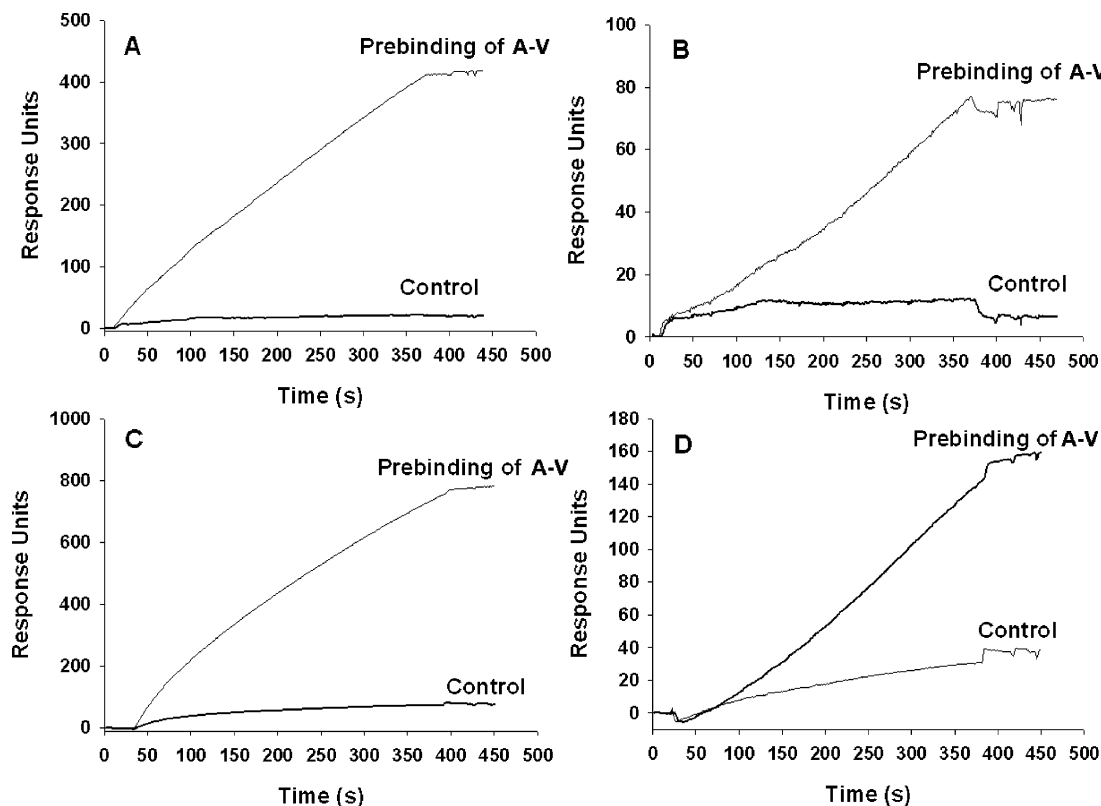


FIGURE 4: Bridging of chylomicrons to LRP or SorLA by apoA-V. Binding studies were performed under the same conditions as in Figure 1, with 2.5 mM CaCl_2 . The chylomicrons were diluted in running buffer. (A) Injection of chylomicrons (15 ng of TG/ μL) to a sensor chip surface with immobilized LRP (7.5 fmol/ mm^2), with and without (control) prebound apoA-V (4.5 fmol/ mm^2). (B) Same as panel A but with chylomicrons diluted to 1.5 ng of TG/ μL . (C) Injection of chylomicrons (15 ng of TG/ μL) to a sensor chip surface with immobilized SorLA (12.8 fmol/ mm^2) with and without (control) prebound apoA-V (5.4 fmol/ mm^2). (D) Same as panel C but with chylomicrons diluted to 1.5 ng of TG/ μL . In this experiment the sensor chips were not blocked with BSA because BSA has affinity for the lipid part in chylomicrons.

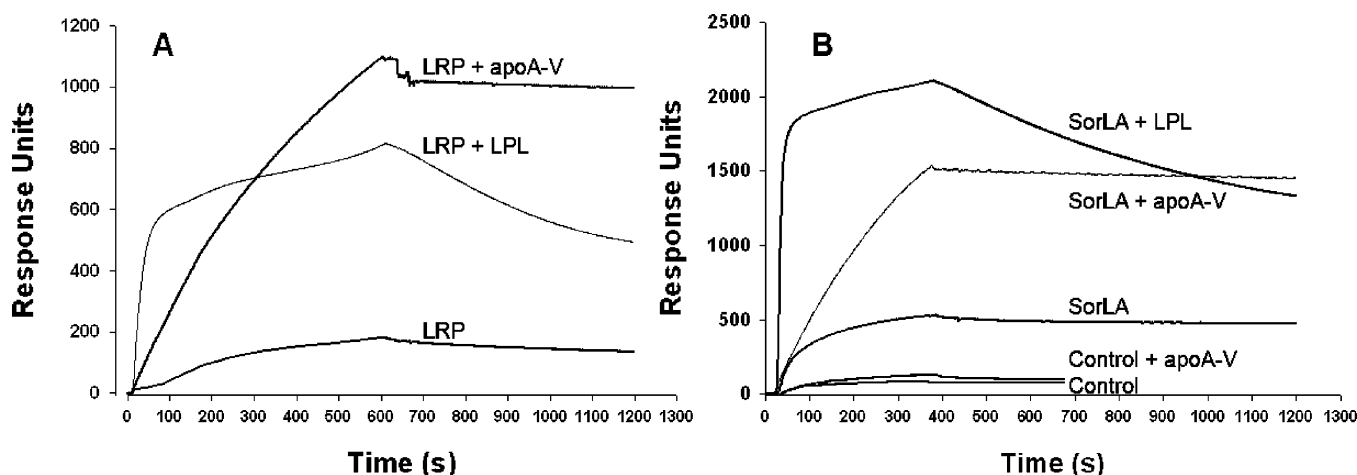


FIGURE 5: ApoA-V bridging of chylomicrons to LRP or SorLA compared to bridging by LPL. Binding studies were performed under the same conditions as in Figure 1, with 2.5 mM CaCl_2 . (A) Injection of chylomicrons (15 ng of TG/ μL) to sensor chips with immobilized LRP (2.3 fmol/ mm^2) and prebound apoA-V (4.9 fmol/ mm^2 at the time for chylomicron injection) or prebound LPL (4.2 fmol/ mm^2 at the time for chylomicron injection) or LRP only (control). (B) Injection of chylomicrons (15 ng of TG/ μL) to a sensor chip surface with immobilized SorLA (6.0 fmol/ mm^2) with prebound apoA-V (5.1 fmol/ mm^2 at the time of chylomicron injection) or with LPL (9.4 fmol/ mm^2 at the time of chylomicron injection) or SorLA only. Flow cells without receptors were used as controls, with and without prebound apoA-V (5.1 fmol/ mm^2) as shown in panel B.

apoA-V is involved in heparin binding, in receptor binding, or possibly in both, site-directed mutagenesis was employed to replace two adjacent positively charged residues with a negatively charged residue and an uncharged, polar residue. The residues chosen for mutation were located in the middle of the positively charged region, and the decision was made based on comparison with known heparin binding sites in other lipoproteins (e.g., apoE). The

resulting double-mutant R210E/K211Q-apoA-V was complexed with DMPC, forming disks of similar size as those made with WT apoA-V. Disks containing the mutant form of apoA-V eluted from a heparin-Sepharose column at a slightly lower salt concentration (0.36 M NaCl) than disks with WT apoA-V (0.40 M), demonstrating a decreased affinity of the mutant apolipoprotein for heparin (Figure 6).

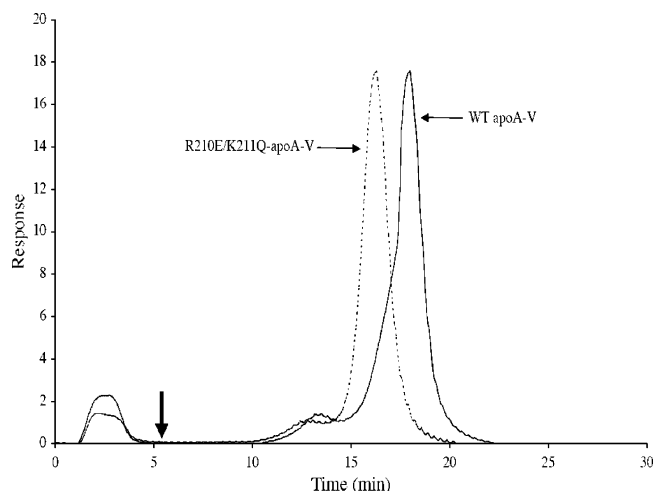


FIGURE 6: Heparin-Sepharose chromatography of apoA-V-DMPC disks. WT-apoA-V or the mutant R210E/K211Q-apoA-V was complexed with DMPC and applied to a Hi-Trap heparin-Sepharose affinity column equilibrated in 20 mM sodium phosphate, pH 7.2. After washing, a linear gradient of NaCl (0 to 2.0 M) in loading buffer was applied. The bold arrow indicates the point at which the NaCl gradient was initiated.

To further compare the heparin interaction of the double mutant apoA-V-DMPC with the WT form, SPR was used for determination of steady-state parameters (Figure 7). Although the apparent equilibrium dissociation constant K_d values did not differ significantly (3.3 $\mu\text{g/mL}$ for WT and 2.3 $\mu\text{g/mL}$ for the double mutant), the binding capacity (number of binding sites) was 8.8-fold higher for WT apoA-V than for the mutant apolipoprotein.

Effect of the Double Mutation on Binding to LRP. To investigate the effect of mutations in the heparin-binding region of apoA-V on receptor binding, we performed studies of steady-state kinetics on the interaction of apoA-V-DMPC disks with LRP (Figure 8). Similarly to the case with heparin binding, there was a higher binding capacity (about 3-fold) for WT apoA-V to LRP than for the mutant R210E/K211Q apoA-V. The calculated K_d value for the double mutant was somewhat lower than for WT apoA-V (1.5 $\mu\text{g/mL}$ compared to 2.8 $\mu\text{g/mL}$).

DISCUSSION

Several hypotheses have been proposed to explain how apoA-V modulates plasma TG levels; recent reports (17, 18, 30) suggest that apoA-V exerts its effect by promoting binding of TG-rich lipoproteins to endothelial heparan sulfate proteoglycans where LPL is located, and where lipolysis occurs. Others have suggested that apoA-V may exert its effects intracellularly by interfering with hepatic TG-rich lipoprotein assembly or secretion (31). In the present study we provide evidence for yet another mechanism. We demonstrate that apoA-V binds to two endocytotic receptors of the LDLR family, LRP and SorLA. Furthermore, apoA-V mediates binding of chylomicrons to LRP and SorLA. Interestingly, Grosskopf et al. found reduced clearance of chylomicron remnants in apoA-V $-/-$ mice and concluded that apoA-V may support receptor interaction (19). Binding of apoA-V to the receptors was markedly decreased in the presence of heparin. It is therefore likely that overlapping binding sites are used for interaction with heparin and the receptors. Experiments with the double-mutant R210E/

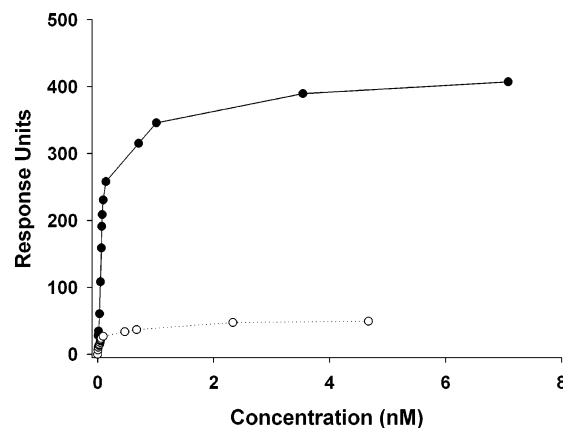


FIGURE 7: Binding of DMPC disks containing WT apoA-V or the mutant R210E/K211Q apoA-V to heparin. Binding studies were performed at 25 °C in 20 mM Hepes, pH 7.4, containing 150 mM NaCl with heparin-coated, streptavidin-containing sensor chips. WT apoA-V-DMPC disks (filled circles) or R210E/K211Q apoA-V-DMPC disks (open circles) were injected at the indicated protein concentrations. Close to maximal binding was recorded after 600 s. A reference flow cell containing streptavidin only was used for baseline subtraction.

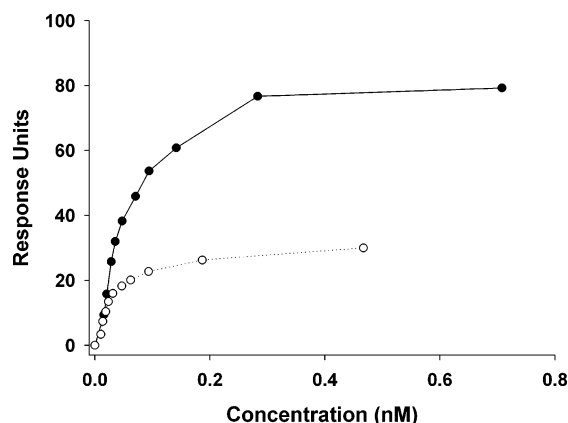


FIGURE 8: Binding of DMPC disks containing WT apoA-V or the mutant R210E/K211Q apoA-V to LRP. Binding studies were performed under the same conditions as in Figure 1, with 2.5 mM CaCl_2 . WT apoA-V-DMPC (filled circles) or R210E/K211Q apoA-V-DMPC (open circles) were injected at the indicated protein concentrations to flow cells with immobilized LRP (6.1 fmol/ mm^2) and blocked with BSA (19.8 fmol/ mm^2). Binding at each concentration was recorded after 600 s. A reference flow cell blocked with BSA only was used for baseline subtraction.

K211Q-apoA-V indicated that these positively charged residues participate in binding both to heparin and to LRP.

We used several approaches to investigate whether binding of apoA-V to sensor chips covered with the different binding partners was specific. For the receptors, binding of apoA-V in DMPC disks was dependent on the presence of Ca^{2+} in the flow phase, and was completely abolished in the presence of EDTA. Additional evidence for specific interaction of apoA-V with the receptors included the findings that binding was competed by RAP, and that DMPC vesicles without protein did not bind to the receptors. Sensor chips with immobilized SorLA bound lipid disks containing apoA-V and apoE, whereas disks with apoC-III, used as a negative control, did not bind. Similar results were found for lipid free apoA-V.

The putative heparin-binding region in apoA-V is localized to a stretch of 42 amino acid residues in the central region

of the molecule, containing eight Arg/Lys residues, three His residues, and no negatively charged residues. Mutation of two positively charged amino acids in the middle of this segment (R210E/K211Q) decreased the apparent affinity of apoA-V for heparin-Sepharose as well as the binding capacity to heparin-coated as well as LRP-coated SPR sensor chips. Simultaneous injection of heparin significantly decreased binding of apoA-V-DMPC disks to both LRP and SorLA, suggesting that the binding sites overlap. The mutations did not abolish either the heparin interaction or the interaction with the receptors, indicating that additional residues must be engaged in the binding. Kinetic analysis revealed that although the double mutation significantly decreased the number of binding sites for apo A-V on heparin and LRP, the apparent K_d values were not much affected. Both heparin and LRP are likely to contain more than one ligand binding site for apoA-V. A possible explanation for our results is that the double mutation abolished binding of apoA-V to low affinity, high capacity binding sites on heparin or LRP, while binding to high affinity binding sites was not changed by the mutations. Individually, and taken together, our findings indicate that the positively charged region in the central region of apoA-V is important for interaction of apoA-V with both lipoprotein receptors and with heparan sulfate proteoglycans. Other apolipoproteins (apoE and apoB) interact with heparin and LDL-receptor family members through similar recognition sites, involving several highly positively charged regions (32).

Receptor-bound apoA-V increased binding of chylomicrons to LRP and SorLA. Compared to the bridging effect of LPL, the dissociation of chylomicrons from both receptors was extremely slow in the presence of apoA-V, suggesting that apoA-V may be even more effective than LPL in tethering chylomicrons to these receptors. ApoA-V is synthesized in the liver and is therefore likely to be present in relatively high concentrations close to receptors such as LRP. The amounts of apoA-V on circulating lipoproteins are low compared to other apolipoproteins (13, 14). This indicates that apoA-V is not easily carried away from the liver on newly synthesized lipoproteins. In contrast, LPL is synthesized in extrahepatic tissues where it mainly acts at the endothelium in adipose tissue, skeletal muscles, and heart. Minor amounts of LPL are carried away on remnant lipoproteins to the liver for degradation (33, 34). In contrast to what was previously suggested (17), based on the local concentrations of LPL and apoA-V in liver and extrahepatic tissues, respectively, it is conceivable that the main TG-lowering effect of apoA-V involves facilitated remnant removal by the liver, rather than stimulated TG lipolysis by LPL in extrahepatic tissues. However, those lipoproteins that carry apoA-V may have a prolonged residence time at the endothelium due to the interaction of apoA-V with heparan sulfate. This may allow longer time for lipolysis by LPL in extrahepatic tissues and possibly also facilitated particle uptake via LDL-receptor family members such as SorLA, LRP, or the VLDL-receptor (35).

Also apoA-V in lipid-free form, at the low concentrations used for SPR, bound to the LDL-receptor family members. This binding was resistant to low pH. Furthermore, experiments in the presence of EDTA demonstrated that binding of lipid-free apoA-V was only reduced by about 50% compared to binding in the presence of Ca^{2+} . Taken together,

these properties of the lipid-free apoA-V could be relevant for recycling of receptor–apoA-V complexes and may allow apoA-V to remain bound after endocytosis through acidic cellular compartments. This binding could also explain why apoA-V levels are low in plasma. Newly synthesized and secreted apoA-V might bind to heparan sulfate proteoglycans or to receptors on the surface of liver cells, like apoE. ApoA-V may, to a large extent, stay in this form, thereby avoiding circulation either as free protein or associated to lipoproteins in the blood. This behavior of apoA-V may be due to its relatively low solubility at neutral pH and its low propensity to dissociate from receptors or HSPG. Several studies have shown that apoA-V has strong effects on plasma TG levels, and, therefore, the mechanism by which it modulates lipid metabolism must be highly effective. The new findings here, and previous studies on the interaction of apoA-V with sulfated glycosaminoglycans (17, 18), can be summarized into two major mechanisms by which apoA-V functions to modulate plasma TG levels.

Heparan Sulfate Proteoglycan-Mediated Lipolysis. For efficient hydrolysis, TG-rich lipoproteins must spend sufficient time at the endothelium to allow contact with lipoprotein lipase, which is located there (36). Although interactions between heparin-binding regions in both apoB and apoE on lipoprotein particles may strengthen binding and prolong lipoprotein residence time at the site of lipolysis, it is conceivable that apoA-V contributes to lipolysis by anchoring lipoproteins to the endothelium (effect on margination). It has also been proposed that apoA-V modulates LPL by direct interaction with the enzyme (15) although we have not been able to repeat this finding in the systems we are using (17). In fact, binding of apoA-V to a sensor chip surface with covalently linked bovine LPL was lower than to an empty sensor chip surface (A. Lookene and G. Olivecrona, unpublished).

Receptor Mediated Internalization. The affinity of apoA-V for heparan sulfate and for lipoprotein receptors could affect receptor-mediated internalization of holo-particles such as remnant lipoproteins. Binding of apoA-V to HSPG, thereby concentrating apoA-V at the cell surface, is likely to assist lipoprotein receptor-mediated endocytosis (37). It is known that chylomicron remnants first bind and concentrate on heparan sulfate proteoglycans in the sinusoids and on the hepatocyte plasma membrane before they are internalized by LRP and possibly other receptors (38). Endocytosis of lipoproteins by cell surface receptors is an important part of their metabolism. Thus, it is possible that apoA-V affects plasma TG levels both at the stage of VLDL secretion from liver (31) and remnant clearance.

We have shown that apoA-V binds to two members of the LDLR family and is capable of mediating binding of lipoproteins to these receptors. Taken together with the affinity of apoA-V for sulfated glycosaminoglycans, we suggest that the TG lowering effects of apoA-V may be attributed to effects on receptor-mediated endocytosis of lipoproteins as well as stimulation of lipolysis at endothelial sites. ApoA-V is expressed solely in the liver. Considering its ability to bind endocytic receptors and to heparan sulfate, it is likely that the main action of apoA-V is in the liver. This could explain why levels of circulating apoA-V are low, and why plasma levels of apoA-V tend to increase with

plasma TG levels and why plasma levels of apoA-V do not change upon heparin injection.

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