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Studies of Intracellular and Extracellular Functions of Apolipoprotein A-V on Triglyceride Metabolism

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Studies of Intracellular and Extracellular Functions of Apolipoprotein A-V on Triglyceride Metabolism

by

Xiao Shu

A dissertation submitted in partial satisfaction of the requirements for the degree of

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in

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in the

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of the

University of California, Berkeley

Committee in charge:

Professor Robert Ryan, Co-chair
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Abstract

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Elevated plasma triglyceride (TG) is a major and independent risk factor for cardiovascular disease. It is generally recognized that apolipoprotein (apo) A-V, a low abundance protein secreted solely by the liver, plays a critical role in TG metabolism. Studies with human APOA5 transgenic (Tg) and gene disrupted mice (apoa5−/−) revealed profound metabolic effects: APOA5 Tg mice exhibited a two-thirds decrease in plasma TG concentration whereas apoa5−/− mice had a four-fold elevation in plasma TG.

Given the very low concentration of apoA-V in plasma, we hypothesized that apoA-V may influence plasma TG levels by affecting the assembly and/or secretion of apoB-containing lipoproteins. When apoA-V was overexpressed in cultured Hep3B cells, neither the amount of apoB secreted nor the density distribution of apoB-containing lipoproteins was affected. Confocal fluorescence microscopy revealed that apoA-V has a unique association with cellular lipid droplets and may be involved in storage or mobilization of intracellular lipids. It was also confirmed in vivo that intrahepatic apoA-V was associated with lipid droplets isolated from livers of wild type and APOA5 Tg mice.

To define the structural requirements for apoA-V lipid droplet association, hepatoma cells were transfected with a series of C-terminal truncated apoA-V variants. Confocal microscopy analysis revealed that apoA-V-(1-146) did not associate with lipid droplets. Ultracentrifugation of conditioned medium revealed that, unlike full-length apoA-V which associates with lipoproteins, apoA-V-(1-146) was present solely in the lipoprotein deficient fraction. These data suggest that the C-terminus of apoA-V is essential for lipid droplet association in transfected hepatoma cells and lipoprotein association in conditioned medium.
On the other hand, we determined whether parenteral delivery of apoA-V is sufficient to lower plasma TG levels in *apoa5*-/− mice. Intravenous injection with apoA-V was found to substantially reduce TG in *apoa5*-/− mice but alterations to the putative heparin binding region of apoA-V attenuated its TG clearing capacity. In addition, parenteral apoA-V had no effect on plasma TG levels in glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) deficient mice. The study suggests a mechanism whereby effective TG clearance requires the interaction of GPIHBP1 and apoA-V.
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CHAPTER 1: Literature review
1.1 Triglyceride (TG) Metabolism and Exchangeable Apolipoproteins (apo)

A recent survey estimated that 30% of the United States adult population exhibits hypertriglyceridemia (HTG) (Ford, Giles et al. 2002). Several epidemiological studies revealed that increased plasma TG concentration is an independent risk factor for coronary heart disease (Cullen 2000; Yuan, Al-Shali et al. 2007). Furthermore, HTG is a hallmark of the metabolic syndrome which is characterized by unfavorable metabolic states such as obesity and insulin resistance (Reaven 1995). The metabolic syndrome confers an increased risk for development of both type 2 diabetes and cardiovascular disease (Reilly and Rader 2003). The precipitating event in the development of the metabolic syndrome is the derailment of energy homeostasis as characterized by changes in fatty acid fluxes and glucose/insulin sensitivity. Elevated plasma free fatty acid levels have long been associated with insulin resistance and have been directly implicated as a primary cause of insulin resistance in muscle (Lewis, Carpentier et al. 2002). It has also been shown that modulation of catabolism of fatty acid derived from lipoprotein TG can directly affect the development of obesity and insulin resistance (Goudriaan, Tacken et al. 2001). Several factors causing HTG such as mutations in the lipoprotein lipase (LPL) gene or in the gene of its co-factor apoC-II have been identified (Breckenridge, Little et al. 1978; Gehrisch 1999). However, the origin of HTG is not always associated with variations of these genes.

1.2 Discovery of ApoA-V

In 2001, a novel protein, apoA-V was discovered and associated with HTG. APOA5 was identified simultaneously by comparative genomic analysis of human and mouse DNA (Pennacchio, Olivier et al. 2001) and by a differential gene expression approach as a gene highly up-regulated during liver regeneration (van der Vliet, Sammels et al. 2001). The former group carried out a comparative genomics study in the region of the apolipoprotein gene cluster (APOA1/C3/A4) on human chromosome 11q23. Comparison with the corresponding mouse sequence revealed a conserved region ~30 kb proximal to the gene cluster, which turned out to be the human APOA5 gene. APOA5 consists of four exons encoding an 1107 bp open reading frame and apoA-V protein is expressed as a 366 amino acid pre-protein, which is subjected to the cleavage of the signal sequence and results the 39kDa mature protein containing 343 residues. ApoA-V is specifically expressed in the liver and the signal peptide directs the protein toward the secretory pathway. Of all known apolipoproteins, apoA-V has the highest homology with apoA-IV (27% identity in humans).

On the other hand, van der Vliet et al. using cDNA subtractive hybridization discovered a gene that is upregulated in regenerating rat liver (van der Vliet, Sammels et al. 2001). The encoded protein, later identified as rat apoA-V, was 367 amino acids with a putative signal peptide of 20 amino acid residues, and expressed exclusively in the liver. ApoA-V mRNA expression was increased from 3 to 12 h after partial hepatectomy with a peak at 6 h. In situ hybridization also confirmed that apoA-V was expressed in hepatocytes. ApoA-V protein was detected in normal rat plasma mainly associated with HDL but its concentration was only about 1 μg/ml. Similar to the change of its mRNA level, apoA-V plasma concentration was increased 5 fold in the early stage of liver regeneration.
1.3 ApoA-V and TG Metabolism in Various Mouse Models

The very first piece of evidence for the role of apoA-V in TG metabolism came from genetically modified mice developed by Pennacchio et al. (Pennacchio, Olivier et al. 2001). Mice overexpressing human APOA5 (APOA5 Tg) had plasma TG levels that were about one third of those from control littermates. On the other hand, apoa5 knockout mice (apoa5-/¬) had about four times higher plasma TG than their wild type (WT) littermates. The level of VLDL was increased in homozygous knockout mice and decreased in transgenic mice compared with controls. VLDL levels in heterozygous knockout mice were intermediate between homozygous knockout and control mice.

Van der Vliet et al. confirmed the TG-lowering role of apoA-V in mice (van der Vliet, Schaap et al. 2002). They showed that adenoviral overexpression of mouse apoa5 decreased plasma TG concentrations by 70%, mainly by decreasing VLDL TG.

The mechanism for the TG lowering effect of apoA-V was investigated in vivo in several other mouse models. However, even though some of the results are supporting with each other, there are still many discrepancies in the field. Fruchart et al. showed that the clearance of [3H] TG-labeled VLDL in APOA5 Tg mice was significantly faster than that of WT mice, but the rate of TG secretion by the liver was equivalent in both mouse lines (Fruchart-Najib, Bauge et al. 2004). After crossing with APOA5 Tg mice, the plasma apoB and apoC-III level decreased significantly. Interestingly, in response to a single bolus of sunflower oil gavage, APOA5 Tg mice showed a smaller postprandial response compared with WT mice. The total apoA-V levels in APOA5 Tg mice were not affected between 0 and 3 h after the gavage. However, the distribution of apoA-V in the plasma was shifted from HDL to VLDL after 3 h.

Merkel et al. confirmed that APOA5 Tg mice have normal hepatic VLDL and intestinal chylomicron production. On the other hand, catabolism of chylomicrons and VLDL was accelerated due to a faster plasma hydrolysis of TG by LPL (Merkel, Loeffler et al. 2005). Similar studies were conducted by Schaap et al. using a adenovirus mediated apoa5 overexpression model (Schaap, Rensen et al. 2004). Along with results reported by Fruchart et al., apoA-V decreased the postprandial TG response in these mice in a dose dependent manner. Increased TG clearance and uptake by skeletal muscle and white adipose tissue were also observed in apoA-V overexpressing mice. However, Ad apoa5 treatment dose-dependently diminished the VLDL-TG production rate without affecting VLDL particle production, suggesting that apoA-V impairs lipidation of apoB. This conflict may be due to differences between the models (genetically modification vs. adenovirus mediated gene transfer). More detailed studies need to be done to solve this problem.

TG metabolism in apoa5-/¬ mice was also studied in the similar manner by Grosskopf et al (Grosskopf, Baroukh et al. 2005). No significant difference in TG production rate was found between apoa5-/¬ and WT control. However, TG rich lipoprotein (TRL) catabolism was affected. Diminished lipolysis of TRL and the reduced rate of uptake of their remnants were observed in apoa5-/¬ mice. Levels of LDL receptor (LDLR) were slightly elevated in apoa5-/¬ mice, consistent with lower remnant uptake rates. VLDL from apoa5-/¬ mice was a poor substrate for LPL, had lower binding activity to LDLR, and showed lower apoE/apoC ratio compared with control VLDL.
1.4 Interaction with LPL, Heparin Sulfate Proteoglycan (HSPG) and Cell Surface Receptors

1.4.1 LPL and HSPG

More efforts have been spent on elucidating the TG lowering mechanism of apoA-V on a molecular level. Since it is well known that LPL is the major enzyme that hydrolyzes TRL in the circulation (Nierman, Rip et al. 2005), it is rational to first hypothesize that apoA-V directly regulates LPL enzymatic activity. As supporting evidence, increased LPL activity (20%) in post-heparin plasma was seen in APOA5 Tg compared with WT mice (Fruchart-Najib, Bauge et al. 2004). LPL activity was also increased (50%) in vitro when using apoA-V enriched human VLDL as the substrate. Direct binding of apoA-V with recombinant or bovine LPL was detected by surface plasmon resonance while binding with HSPG was not detected. Similar result was also reported by Schaap et al (Schaap, Rensen et al. 2004). [3H]triolein-labeled VLDL-like emulsions were used as the substrate to determine the effect of purified apoA-V on LPL-mediated TG hydrolysis in vitro. Preincubation of particles with apoA-V enhanced the lipolysis rate in the presence of apoC-II and apoC-III in a dose-dependent manner. However, apoA-V was ineffective in the absence of apoC-II. It was suggested that apoA-V may act as a stimulatory modulator of apoC-II-induced LPL-mediated TG hydrolysis.

On the other hand, Lookene et al. failed to observe this activation of LPL activity by apoA-V (Lookene, Beckstead et al. 2005). Specifically, no activation was found when VLDL, TG emulsion or dimyristoylphosphatidylcholine (DMPC) liposomes were employed as the substrate. But interestingly, apoA-V-DMPC lipid particles bind heparin-Sepharose and eluted upon application of a NaCl gradient. A closer examination of the binding between apoA-V and heparin was performed by surface plasmon resonance spectroscopy. ApoA-V not only bound to the heparin-coated chip, but also enhanced binding of chylomicrons and VLDL to the chip. This enhancement was not seen for HDL. Moreover, apoA-V-enriched chylomicrons showed increased binding to the LPL-heparin chip. To determine whether a pool of HSPG-bound apoA-V may exist in vivo, apoA-V levels were measured and compared between blood samples before and 10 min after heparin injection. No difference in plasma apoA-V concentration as a function of heparin injection was observed.

The effect of apoA-V on LPL lipolytic activity was also determined using a system that was composed of free or HSPG-bound LPL (Merkel, Loeffler et al. 2005). The hydrolysis of TG-rich lipoproteins by LPL was affected by apoA-V only in the presence of HSPG. A dose-dependent correlation between apoA-V and HSPG-bound LPL-mediated hydrolysis was also found. Direct binding between apoA-V and LPL was confirmed by ligand blotting. To further investigate the interplay between apoA-V and LPL in vivo, a strategy of cross-breeding LPL Tg with apoa5-/- mice and APOA5 Tg to lpl-/- mice was adopted. Increased LPL completely compensated for apoa5 deficiency, whereas apoA-V overproduction only slightly modulated TG in the lpl deficient background, especially given that lpl-/- mice died within 24 h regardless of apoA-V expression. These data suggested that LPL plays the primary role in TG hydrolysis while apoA-V can modulate this process.
1.4.2 Cell Surface Receptors

There is a possibility that apoA-V interacts with cell surface receptors and affects lipoprotein internalization. Nilsson et al. showed specific binding of both free and lipid-bound apoA-V to two structurally distinct LDLR family members, the LDLR-related protein (LRP) and the mosaic type-1 receptor, SorLA (also known as LR11) (Nilsson, Lookene et al. 2007). Preincubation with heparin decreased receptor binding of apoA-V, indicating that overlap exists between the recognition sites for the receptors and heparin. ApoA-V also enhanced the binding of chylomicrons to these receptors. Recently, apoA-V was reported to bind another receptor, sortilin, which has a Vsp10p domain similar to SorLA but lacks LDL-A repeats that SorLA also possesses (Nilsson, Christensen et al. 2008). Receptor-bound apoA-V was rapidly internalized, co-localized with receptors in early endosomes, and followed the receptors through endosomes to the trans-Golgi network. After uncoupling in endosomes, apoA-V was delivered to lysosomes for degradation.

Beigneux et al. (Beigneux, Davies et al. 2007) provided additional evidence for the concept that apoA-V can modulate plasma TG levels by promoting cell surface interactions. These authors reported that apoA-V serves as a ligand for a novel endothelial cell surface protein, termed glycosyl-phosphatidylinositol high-density lipoprotein binding protein 1 (GPIHBP1). Gpihbp1 deficient mice exhibit a striking accumulation of chylomicrons, even on a low-fat diet, resulting in milky plasma with TG levels as high as 5,000 mg/dL. On the basis of these studies, it was concluded that GPIHBP1 plays a critical role in the lipolytic processing of chylomicrons; importantly, it has been shown that apoA-V associates with chylomicrons in human plasma (O'Brien, Alborn et al. 2005). GPIHBP1 is located on the luminal face of the capillary endothelium and its expression in cultured cells conferred an ability to bind both LPL and TRL such as chylomicrons. In studies with GPIHBP1 transfected CHO cells, specific binding to apoA-V containing lipid particles was observed. Mutation of the positively charged heparin-binding domain within apoA-V abolished its ability to bind to GPIHBP1, suggesting that the same region in charge of HSPG and receptor-binding was involved in this interaction (Gin, Yin et al. 2008). These studies provide further evidence that apoA-V may play a role in chylomicron TG hydrolysis.

1.5 ApoA-V and ApoC-III

ApoA-V and apoC-III have opposite effect on plasma TG levels (van Dijk, Rensen et al. 2004). Mice overexpressing apoC-III shows severe HTG, whereas apoc3 deficient mice have decreased plasma TG levels. In contrast (as mentioned above), overexpression of human apoA-V in mice results in lower plasma TG whereas apoa5/- mice have increased plasma TG levels. The effects of apoA-V and apoC-III are also supported by the association between variation in both APOA5 and APOC3 genes and TG levels in human populations (Groenendijk, Cantor et al. 2001; Pennacchio, Olivier et al. 2001)(discussed below).

To tease out the effects of apoA-V and apoC-III on plasma TG level, “double Tg” and “double knockout” mice were generated and studied (Baroukh, Bauge et al. 2004). Both the “double Tg” and “double knockout” mice showed unchanged plasma TG level compared with their WT littermates. In addition, no significant differences in the various plasma particle sizes were observed. These findings suggested that apoA-V and apoC-III independently influence plasma TG concentrations but in an opposing manner. Interestingly, apoA-V plasma protein levels in the “double Tg” mice are about 500-fold
lower than apoC-III level, supporting apoA-V as a potent TG modulator despite its low concentration (discussed below).

The relationship between apoA-V and apoC-III was also studied in a setting in which APOC3 Tg mice overexpressed apoA-V using adenovirus mediated gene transfer (Qu, Perdomo et al. 2007). In response to apoA-V production, plasma TG level was significantly reduced, due to enhanced VLDL catabolism without alternations in VLDL production. Decreased VLDL-cholesterol and increased HDL-cholesterol were also observed. However, the mechanism whereby apoA-V counteracts the effect of apoC-III on plasma TG levels is still not clear.

1.6 Unique Aspects of Human ApoA-V

1.6.1 Hydrophobicity

Human ApoA-V coding sequence predicts a protein consisting of 366 amino acids. A 23-amino acid signal peptide, which is cleaved off the mature protein and directs apoA-V to the secretory pathway, was confirmed by sequencing the N-terminal sequence of human apoA-V in the plasma (Alborn, Johnson et al. 2006). The mature apoA-V (343 amino acids) is predicted to be a highly α-helical protein (Figure 1-1) (Weinberg, Cook et al. 2003). Calculation of mean residue hydrophobicity indicates apoA-V is more hydrophobic than either apoA-I and apoA-IV, while lipid affinities of apoA-V and apoA-I are similar. Indeed, Beckstead et al. provided biochemical data to support these predictions (Beckstead, Oda et al. 2003). Far-UV circular dichroism (CD) analysis of apoA-V revealed about 50% alpha-helix content. Recombinant apoA-V is poorly soluble under neutral-pH (at concentrations greater than 0.1 mg/ml). By contrast, apoA-V is soluble in 50 mM sodium citrate at pH3.0. Upon incubation with bilayer vesicles of DMPC, apoA-V interacts with the phospholipid to form a discrete population of lipid particles sizing from 15 to 20 nm in diameter. Binding with lipid significantly increases the solubility of apoA-V at neutral pH.

1.6.2 C-terminal

There is a unique tetra-proline segment from residue 293 to 296 of mature apoA-V (Figure 1-1). Because the cyclic nature of proline is disruptive to protein secondary structure, the possibility exists that these consecutive proline residues demarcate structural regions in apoA-V. To test this hypothesis, a truncated apoA-V comprising the amino acids before the tetra-proline was generated [apoA-V-(1-292)] (Beckstead, Wong et al. 2007). Far-UV CD analysis of apoA-V-(1-292) showed a similar secondary structure with full-length apoA-V. In guanidine HCl denaturation experiments, both full-length and truncatedapoA-V yielded biphasic profiles consistent with the presence of two structural domains, suggesting that the tetra-proline sequence was not the boundary of these two domains. The change of the denaturation profile of the lower stability component indicated that the lower stability domain was actually the C-terminal domain. Truncated apoA-V displayed an attenuated ability to solubilize DMPC vesicles compared with full-length apoA-V, whereas a peptide corresponding to the deleted C-terminal segment displayed markedly enhanced kinetics. Taken together, it may be concluded that the C-terminal of apoA-V is an important modulator of the lipid-binding activity of the protein.
1.6.3 N-terminal

Computer-assisted sequence analysis and limited proteolysis studies identified an N-terminal fragment as a candidate for the other domain. ApoA-V-(1-146) was generated and studied as a representative of this N-terminal domain (Figure 1-1) (Wong, Beckstead et al. 2008). Unlike the full-length protein, apoA-V-(1-146) is soluble in neutral-pH buffer in the absence of lipid. Guanidine HCl denaturation experiments at pH 3.0 yielded a one-step native to unfolded transition that corresponds directly with the more stable component of the two-stage denaturation profile exhibited by full-length apoA-V. The data suggest the 146 N-terminal residues of apoA-V adopt a helix bundle molecular architecture in the absence of lipid and, thus, likely exist as an independently folded structural domain within the context of the intact protein. It appears that apoA-V may be organized in a manner that is similar to apoE and apoA-I, comprised of a lipid surface seeking C-terminal domain and an alpha helix bundle-forming N-terminal domain.

1.6.4 Heparin Binding Region

Analysis of the amino acid sequence of apoA-V revealed a stretch of 42 residues between 186 and 227 that lacks amino acids with negatively charged side chains and is enriched with eight amino acids with positively charged side chains, such as (three) Lys and (five) Arg, as well as three histidines (Figure 1-1) (Lookene, Beckstead et al. 2005). This highly positively charged region has been shown to be involved in heparin binding, receptor binding (Nilsson, Lookene et al. 2007) and GPIHBP1 binding (Gin, Yin et al. 2008). A double mutant apoA-V (R210E/K211Q), with adjacent positively charged residues replaced by negative and/or uncharged residues, showed decreased binding ability to both heparin and LRP (Nilsson, Lookene et al. 2007). Another mutant with more extensive substitution of positive charges with negative ones (R210E/K211Q/K215Q/K217E) (Figure 1-1) also failed to bind to CHO cells overexpressing GPIHBP1 (Gin, Yin et al. 2008). Taken together, the data suggest that this region is essential for the binding interactions described above. More detailed study on this region needs to be conducted to dissect out the sequence responsible for specific functions, although it is possible that there is overlap between different binding sites.

1.7 ApoA-V plasma concentration

1.7.1 Extremely Low Concentration

The concentration of apoA-V in plasma, compared to other apolipoproteins, is exceptionally low. In rat, the plasma concentration of apoA-V was determined by western blot. When related to the amount of recombinant protein on the blot, it is estimated about 1μg/ml (van der Vliet, Sammels et al. 2001). To determine the plasma concentration in human, O'Brien et al. generated a dual-antibody sandwich ELISA using antibodies against the N- and C-termini of apoA-V (O'Brien, Alborn et al. 2005). Estimates for plasma apoA-V levels range from 114 - 258 ng/ml in normolipemic subjects (Ishihara, Kujiraoka et al. 2005; O'Brien, Alborn et al. 2005; Schaap, Nierman et al. 2006; Talmud, Cooper et al. 2006; Vaessen, Schaap et al. 2006). This corresponds to a 1,000-fold lower concentration than apoB on a molar basis. Since apoA-V was not found on LDL in humans (O'Brien, Alborn et al.
and only 6% of circulating apoB-100 is associated with VLDL, it is estimated that about 1 apoA-V molecule is present per 24 VLDL particles (Merkel and Heeren 2005).

In studies with APOA5 transfected COS-1 cells, control proteins including human serum albumin and apoB6.6 were efficiently secreted during a 3h culture. By contrast, the majority of apoA-V was retained in the cell lysate, with only a small amount recovered in the medium (Weinberg, Cook et al. 2003). The retarded secretion process of apoA-V may partially explain its low concentration in the circulation.

1.7.2 Correlation between Plasma Concentration and TG Levels

Early studies (Pennacchio, Olivier et al. 2001) in APOA5 Tg and apoa5/- mice indicated an inverse relationship between apoA-V and plasma TG levels: in the absence of apoA-V, plasma TG levels were highly elevated whereas overexpression of apoA-V lowered TG compared to controls. Cross-breeding of APOA5 Tg mice with APOC3 Tg and APOE2 Knockin mice efficiently ameliorated the HTG phenotypes (Fruchart-Najib, Bauge et al. 2004; Gerritsen, van der Hooft et al. 2008; Mansouri, Bauge et al. 2008). Adenovirus mediated gene transfer of apoA-V in mice with different genetic backgrounds also resulted in lowering of TG (van der Vliet, Schaap et al. 2002; Schaap, Rensen et al. 2004; Qu, Perdomo et al. 2007), one of which clearly showed a dose-dependent TG lowering effect of apoA-V (Schaap, Rensen et al. 2004). Several truncation mutations of apoA-V in human also indicate that loss of functional apoA-V causes elevated plasma TG levels (Marcais, Verges et al. 2005; Oliva, Pisciotta et al. 2005; Oliva, Pisciotta et al. 2006).

However, various human population studies relating plasma apoA-V and TG do not support the concept of an inverse relationship between these two variables. Several studies with HTG and/or diabetic subjects revealed that apoA-V increased as TG increased (Dallinga-Thie, van Tol et al. 2006; Schaap, Nierman et al. 2006; Talmud, Cooper et al. 2006; Vaessen, Schaap et al. 2006). As well, a positive correlation has also been observed in normolipemic controls (Talmud, Cooper et al. 2006; Vaessen, Schaap et al. 2006). APOA5 Tg mice with normal TG levels are similar to their human counterparts in that there is a significant positive correlation between apoA-V and plasma TG concentrations (Nelbach, Shu et al. 2008), thus establishing that the positive correlation is not confined to human subjects. Recently, Vaessen et al. determined plasma apoA-V levels in mouse models with various TG levels including WT, APOC1 Tg, gpihbp1 KO, apoa2 Tg and apoa2 Tg x apoe KO (Vaessen, Dallinga-Thie et al. 2009). The positive correlation between TG and apoA-V levels was confirmed in these mice. Moreover, when the LPL gene was introduced into LPL deficient mice, both TG and apoA-V levels were decreased. These data indicate that TG levels may actually drive apoA-V levels due to an intimate link between the apoA-V molecule and TRL, comprising both secretion and removal of these lipoproteins.

Although apoA-V has been reported to play an important role in lipolysis (Schaap, Rensen et al. 2004; Grosskopf, Baroukh et al. 2005), several studies provided evidence supporting that plasma apoA-V concentration does not play an acute role in the regulation of plasma TG. A human VLDL kinetics study showed that plasma apoA-V concentration was not significantly associated with VLDL kinetics, while apoC-III concentration, on the other hand, was strongly associated with the delayed catabolism of TG and apoB in VLDL (Chan, Watts et al. 2006). In the postprandial state, no association between apoA-V levels and LPL activity was observed in type 2 diabetic patients. However, a parallel increase of postprandial apoA-V and TG was demonstrated (Pruneta-Deloche, Ponsin et al. 2005; Kahri,
Fruchart-Najib et al. 2007). In another study of type 2 diabetic patients, with or without microalbuminuria, increased postprandial apoA-V and TG levels were associated with microalbuminuria (Tentolouris, Stylianou et al. 2007). Taken together, given the extremely low concentration of apoA-V concentration and its ratio to TRL, it may not directly participate into LPL-mediated TG catabolism as other apolipoproteins, such as apoC-II and apoC-III. However, the concentration of apoA-V may change closely with plasma TG levels and work as a signaling molecule reflecting the demand for TG hydrolysis in the circulation and regulating TG metabolism in a much less acute manner (e.g. regulating gene expression profile).

1.8 Expression Regulation of ApoA-V

1.8.1 Peroxisome Proliferator-Activated Receptor-α (PPARα)

Since APOA5 has been shown in humans and mice to be important in determining plasma TG levels and PPARα agonists are commonly used pharmacologically for lowering plasma TG, it is reasonable to hypothesize that apoA-V expression is regulated by PPARα. Indeed, Vu-Dac et al. showed that Wy14,643 or fenofibrate displayed a strong induction of APOA5 mRNA in human primary hepatocytes (Prieur, Coste et al. 2003; Vu-Dac, Gervois et al. 2003). A PPARα response element consisting of a direct repeat 1 (DR1) located 271 bp upstream of the transcription start site was identified (Prieur, Coste et al. 2003). Moreover, cynomolgous monkeys treated with LY570997 L-lysine (0.3 mg/kg/day) for 14 days showed a 50% decrease in TG and 2-fold increase of serum apoA-V concentration. The apoA-V/apoC-III ratio also increased >2-fold. All these changes were reversible at a 2 week washout of the drug (Schultze, Alborn et al. 2005). Human APOA5 transcripts was also reported associated PPARα and CPT1A1 (a major target gene of PPARα) transcripts (Hahne, Krempler et al. 2008). Until now, the TG lowering effect of PPARα agonists was attributed to the elevated expression of LPL and the decreased expression of apoC-III as well as to a dramatic increase of β-oxidation in the liver (Staels, Dallongeville et al. 1998). The identification of apoA-V as a new PPARα target gene leads to the suggestion that apoA-V could also contribute to the TG lowering effect of fibrates. However, human and murine genes encoding apoA-V are regulated differently in response to fibrates (Prieur, Lesnik et al. 2009). Fenofibrate increased the expression of human APOA5 in Tg mice while repressed the expression of mouse apoa5. It is also consistent with the finding that the functional PPARα response element in human APOA5 promoter is degenerated and non-functional in the corresponding mouse apoa5 sequence.

1.8.2 Farnesoid X-Activated Receptor (FXR)

FXR has also been showed to regulate the expression of genes involved in TG metabolism. Specifically, FXR ligands affect lipid metabolism in the liver and the gastrointestinal tract and lower circulating TG and cholesterol (Edwards, Kast et al. 2002). Prieur et al. showed that bile acids and FXR induced the apoA-V gene promoter activity (Prieur, Coste et al. 2003). An inverted repeat of two consensus receptor-binding hexads separated by 8 nucleotides (IR8) at positions -103/-84 was required for the response to bile acid-activated FXR. However, APOA5 expression was unchanged in hepatocytes after treatment with FXR agonists, suggesting that regulation of APOA5 by bile acids is
complicated. Moreover, the physiological relevance of this regulation remains to be
determined.

1.8.3 Liver X Receptor (LXR)

LXR ligands is known to have lipogenic and hypertriglyceridemic activity and affect
TG metabolism in mice in addition to their anti-atherogenic role (Schultz, Tu et al. 2000). Treatment with the LXR ligand T0901317 revealed a significant decrease of APOA5 mRNA in hepatoma cell lines and APOA5 Tg mice liver tissue. The circulating levels of apoA-V in the Tg mice after treatment were also deceased (Jakel, Nowak et al. 2004). However, the observation that no down-regulation of APOA5 was obtained by LXR-retinoid X receptor (RXR) co-transfection suggests that LXR does not regulate APOA5 promoter activity directly.

1.8.4 Sterol Regulatory Element-Binding Protein 1c (SREBP-1c), Insulin and Glucose

SREBP-1c is a transcription factor with preferential specificity for fatty acid and TG metabolism and is activated by LXR (Yoshikawa, Shimano et al. 2001). In addition, the lipogenic effect of LXR ligands in the liver is mainly attributed to the activation of SREBP-1c. Following this idea, Jakel et al. explored the possible involvement of SREBP-1c in APOA5 down-regulation effect of LXR ligand (Jakel, Nowak et al. 2004). In fact, transfection with active form of SREBP-1c decreased APOA5 promoter activity in a dose-dependent manner. Two putative E-box elements EB1-A5 and EB2-A5, which were able bind to SREBP-1c specifically, were identified in APOA5 promoter at +10/+15 and -76/-81, respectively. Suppression of SREBP-1 mRNA through small interfering RNA (siRNA) interference abolished the decrease of APOA5 in response of LXR ligand T0901317, confirming that the regulation of APOA5 by LXR ligand is through SREBP-1c.

SREBP-1c is also activated by insulin (Foretz, Pacot et al. 1999). Insulin plays a major role in the regulation of carbohydrate and lipid metabolism in liver, adipose tissue and muscle. Besides regulating glucose homeostasis, hepatic fatty acid oxidation, lipogenesis, and protein synthesis are subject to regulation by insulin (O'Brien and Granner 1996). Indeed, Nowak et al. showed that cell lines and mice treated with insulin down-regulate APOA5 expression through decreasing promoter activities in a dose-dependent manner (Nowak, Helleboid-Chapman et al. 2005). However, instead of SREBP-1c, upstream stimulatory factor (USF) 1/2 was demonstrated to bind to the -76/-81 E-box. USFs have been involved in the basal transcription of a number of genes including the FAS gene (Wang and Sul 1995; Wang and Sul 1997; Wang and Sul 1998). Overexpression of USF1 stimulated APOA5 promoter activity and the treatment with insulin reduced the binding of USF1/2 to the APOA5 promoter involving a phosphorylation of the protein through the phosphatidylinositol 3-kinase (PI3K) and P70 S6 kinase pathway. Specifically, phosphorylated USF1 fails to bind to the APOA5 promoter. An insulin clamp study in humans was carried out to analyze the effect of insulin on apoA-V levels in vivo. Heperinsulinemia significantly reduced serum apoA-V protein levels at 3.5 and 8 h by 52% and 72%, respectively. However, no change in TG and apoC-III (a target gene of insulin) levels were observed. These results suggest a potential contribution of apoA-V in HTG associated with hyperinsulinemia.

Glucose has been implicated as an independent signal in homeostasis of carbohydrate and fatty acids for activating the synthesis of glycolytic and lipogenic enzymes. In liver, adipocytes and β-cells, glucose is metabolized to generate an intracellular
signal that allows for transcriptional regulation of metabolic genes (Lefrancois-Martinez, Martinez et al. 1995; Doiron, Cuif et al. 1996; Kahn 1997). Nowak et al. showed that D-glucose activates APOA5 gene expression in a time- and dose-dependent manner in hepatocytes (Nowak, Helleboid-Chapman et al. 2008). This regulation is through an increase of USF1/2 binding to the E-box in the APOA5 promoter. In fact, D-glucose regulates the APOA5 expression via a dephosphorylation of USF1/2 by protein phosphatases 1 and 2A, which are the most abundant phosphatases present in the liver and known to be implicated in the glucose effect. Taken together, these findings may provide a new cross-talk between glucose and lipid metabolism.

1.8.5 3,5,3’-Triiodo-L-Thyronine (T₃)

Elevation of plasma TG is associated with hypothyroidism (Abrams, Grundy et al. 1981; O’Brien, Dinneen et al. 1993). Such elevation has been attributed to low LPL or low hepatic lipase activities. In contract, hyperthyroid patients exhibit elevated rates of dearance of VLDL and normal or decreased circulating TG levels, whereas treatment with thyroid hormones is associated with elevation in both LPL and hepatic lipase activities and concomitantly with a tendency to TG lowering (Tan, Shiu et al. 1998; Mitsuru Ito 2003). Prieur et al. reported that T₃ and a synthetic thyroid receptor β (TRβ) ligand (agonist) CGS-23425 increase apoA-V mRNA and protein levels in hepatocytes (Prieur, Huby et al. 2005). T₃-activated TR directly regulates the APOA5 promoter through a functional direct repeat separated by four nucleotides (DR4) at -98/-113. Interestingly, USF1/2 which bind to an adjacent E-box (-76/-81) cooperate with TR resulting in a synergistic activation of the APOA5 promoter in a ligand-dependent manner. Moreover, apoA-V levels in rats declines with chemically induced thyroid hormone depletion using PTU but returned to normal levels upon T₃ administration. In addition, rats treated with the synthetic TRβ agonist showed increased apoA-V and diminished TG levels. These provided a new potential mechanism whereby thyroid hormones can influence TG homeostasis.

1.8.6 Retinoic Acid Receptor-Related Orphan Receptor-α (RORα)

RORα is a widely expressed receptor that belongs to a subgroup within the family of nuclear receptors consisting of the three genes RORα, β, and γ (Jetten, Kurebayashi et al. 2001). The human RORα gene encodes at least four splicing isoforms, RORα1, α2, α3, and α4 (or RZRα), which differ in their N-terminal domains and display distinct DNA recognition and transactivation properties(Giguere, Tini et al. 1994). RORα has been suggested to be involved in lipid metabolism (Vu-Dac, Gervois et al. 1997; Raspe, Duez et al. 2001; Lau, Nixon et al. 2004). The staggerer mice, which contain a natural deletion within the RORα gene leading to a truncated receptor, exhibit metabolic abnormalities in addition to severe neurological disorders (Sidman, Lane et al. 1962; Hamilton, Frankel et al. 1996; Mamontova, Seguret-Mace et al. 1998). These mice have severe hypoalphalipoproteinemia and atherosclerosis when maintained on an atherogenic diet (Mamontova, Seguret-Mace et al. 1998). Moreover, RORα has already been involved in rat apoA-I and human apoC-III gene transcriptional regulation (Vu-Dac, Gervois et al. 1997; Raspe, Duez et al. 2001). Since APOA5 was identified in proximity to the APOA1/C3/A4 gene cluster and plays an important role in lipid metabolism, Lind et al. and Genoux et al. studied the regulation of APOA5 by RORα (Genoux, Dehondt et al. 2005; Lind, Nilsson et al. 2005). Indeed, overexpression of RORα1 and 4 strongly increase APOA5 promoter activity in a dose-
dependent manner. Three ROR response elements were identified at -279, -260 and -98, one of which overlaps with the previous identified PPARα response element (-271) in the APOA5 gene promoter. Adenoviral overexpression of hRORα in HepG2 cells led to enhanced APOA5 mRNA accumulation. However, the homologous region in mouse apoA5 promoter is not functional. Moreover, in staggerer mice, apoA5 gene is not affected by RORα. Taken together, these data suggest an additional important physiological role for RORα in lipid homeostasis and atherosclerosis.

1.8.7 Hepatocyte Nuclear Factor 4α (HNF4α)

HNF4α (NR2A1) is a highly conserved member of the nuclear receptor superfamily that was initially identified as a transcriptional factor required for liver-specific gene expression, although it is also expressed in kidney, intestine, and pancreas (Sladek, Zhong et al. 1990; Drewes, Senkel et al. 1996). HNF4α has critical roles in hepatocyte differentiation during liver development and in regulating the transcription of genes involved in glucose and lipid metabolism including the APOA1/C3/A4 gene cluster, apoB, apoC-II and PPARα (Li, Ning et al. 2000; Hayhurst, Lee et al. 2001). Given that apoA-V exclusively expressed in the liver, it is reasonable to hypothesize that HNF4α regulates apoA-V expression in the liver. Indeed, Prieur et al. reported that HNF4α directly regulates human apoA-V promoter through DR1 (which was also identified as the PPARα response element) and IR8 (which was previously identified as the FXR response element) (Prieur, Schaap et al. 2005). Inhibition of HNF4α expression by siRNA resulted in down-regulation of apoA-V. Reddy et al. have demonstrated that apoC-III expression can be regulated by signals acting through the Mitogen-activated protein kinase (MAPK) signaling pathway and that this regulation is at least partly mediated by changes in the amount of HNF4α (Reddy, Yang et al. 1999). Based on the same logic, Prieur et al. also showed that the MAPK signaling pathway controls human apoA-V expression and suggested that this regulation is mediated, at least in part, by changes of HNF4α levels (Prieur, Schaap et al. 2005). Incubation with an inhibitor of the MAPK signaling pathway not only induced the levels of both apoA-V and HNF4α, but also robustly increased binding of HNF4α to the two response elements of apoA-V promoter. In contrast, it is known that activation of AMP-activated protein kinase (AMPK) diminishes HNF4α protein levels and inhibits its ability to form homodimers and bind to DNA, and consequently down-regulates the expression of several HNF4α target genes (Leclerc, Lenzner et al. 2001; Hong, Varanasi et al. 2003). An AMPK activator significantly decreased apoA-V mRNA levels as well as apoA-V and HNF4α protein levels. In addition, activation of AMPK dramatically reduced the binding of HNF4α to the apoA-V promoter. Interestingly, sequence comparisons revealed that the human apoA-V DR1 element is not conserved in the mouse apoA-V promoter. HNF4α only binds to human but not to mouse DR1 containing sequence. In the mice lacking hepatic HNF4α expression, no effect on liver or serum protein concentrations of apoA-V was observed. It was suggested that the regulation of apoA-V by HNF4α is species specific.

1.8.8 PPAR-γ Coactivator-1α (PGC1α)

PGC1α is a versatile coactivator for numerous nuclear receptors and is implicated in diverse biological activities including glucose and lipid metabolism. It has been demonstrated that PGC1α stimulates the promoter activity of multiple hepatic genes involved in gluconeogenesis, fatty acid oxidation, bile acid synthesis, and TG metabolism.
through coactivation of HNF4α (Louet, Hayhurst et al. 2002; Shin, Campos et al. 2003; Zhang, Castellani et al. 2004). Prieur et al. showed that PGC1α was capable of stimulating the HNF4α-dependent transactivation of apoA-V promoter (Prieur, Schaap et al. 2005). In human hepatoma HepG2 and Hep3B cells, although HNF4α is expressed at levels as high as in human liver, its activity on target genes including apoA-V and apoC-III is very low. Martinez-Jimenez et al. demonstrated that the low expression of PGC1α may account for the lack of function of HNF4α in those cell lines (Martinez-Jimenez, Gomez-Lechon et al. 2006). Overexpression of PGC1α led to a significant up-regulation of apoA-V as well as other HNF4α-dependent genes. Interestingly, insulin treatment caused repression of PGC1α and down-regulation of a number of HNF4α target gene. Even though the authors did not check apoA-V expression specifically, this finding provides another possible mechanism whereby insulin down-regulates apoA-V expression in addition to USF1/2.

1.9 Genetic Studies and Mutations in Human Population

1.9.1 Single Nucleotide Polymorphisms (SNPs)

Because TG is a known independent risk factor for the development of cardiovascular disease and since apoA-V was identified as a modulator of TG levels, a number of studies have sought to establish a genetic link between apoA-V and TG. Pennacchio et al. (Pennacchio, Olivier et al. 2001; Pennacchio, Olivier et al. 2002) were the first to note that several SNPs in the apoA-V gene, particularly –1131T>C and c.56C>G (S19W), were associated with elevated TG levels. Similar results were obtained by others (Nabika, Nasreen et al. 2002; Vrablik, Horinek et al. 2003; Talmud, Martin et al. 2004; Hodoglugil, Tanyolac et al. 2006). Several approaches have been used to determine whether the rare S19W SNP is functional since it correlates strongly with elevated TG. Based on the fact that this coding SNP occurs in the signal peptide region of the protein, Talmud et al. hypothesized that its secretion efficiency may be affected (Talmud, Palmen et al. 2005). Consistent with molecular modeling studies that predict reduced translocation of this variant across the endoplasmic reticulum, studies in transfected HepG2 cells demonstrated that a fusion protein containing the S19W signal peptide was indeed poorly secreted (50% reduction). The role of the S19W variant was also investigated by precise insertion of a single-copy 19W APOA5 haplotype into the mouse genome (Ahituv, Akiyama et al. 2007). This elegant set of studies revealed that the S19W polymorphism is functional since it resulted in a significant decrease in plasma apoA-V levels.

Since there is a strong correlation between HTG and atherogenesis, Talmud et al. examined atherogenic lesion progression in subjects with the S19W polymorphism (Talmud, Martin et al. 2004). The data showed a trend toward increased atherogenesis in the vessel lumen although it is difficult to draw firm conclusions from these data. Future studies with atherogenic animal models, where variants can be expressed, could provide clues in this area. Interestingly, a recent study using the dyslipidemic apoE2-knockin mouse model demonstrated that expression of human apoA-V not only reduced plasma TG levels but also reduced atherosclerotic lesion formation in apoA-V transgenic mice compared to controls (Mansouri, Bauge et al. 2008). This is perhaps the strongest evidence that apoA-V may have antiatherogenic potential; however, apoA-V metabolism is complex and one needs to take into consideration the contribution of other lipid-regulating factors
such as apoCIII, apoCII, LPL as well as contributions from environmental factors such as diet, obesity, diabetes, etc.

Kao et al. (Kao, Wen et al. 2003) and Tang et al. (Tang, Sun et al. 2006) identified a coding sequence variant c.553G>T in Chinese populations resulting in a cysteine for glycine substitution at position 185 (G185C). This variant was 6.4-fold more common in HTG subjects than normolipemic subjects. Studies on Asian-American patients indicated that carriers of the T allele had plasma TG concentrations twice that of normolipidemic subjects while TT homozygous patients had extremely elevated TG (mean, 2,292 mg/dl) (Pullinger, Aouizerat et al. 2008). The T allele was also associated with a decrease in postheparin LPL activity. This variant is in a highly conserved region of the apoA-V protein which is a potential lipid-binding domain (A171-R245). Furthermore, residue 185 occurs within a predicted Receptor Binding Domain and may therefore have an effect on protein-protein interaction. Recently, Dorfmeister et al. (Dorfmeister, Zeng et al. 2008) showed that recombinant apoA-V G185C protein forms dimers and multimers, which changes the tertiary structure and may influence functionality of the protein, particularly its interaction with LPL. Even though the recombinant G185C showed a slightly decreased HSPG-bound LPL activation in vitro, the binding with receptor LR8 and LRP1 was not affected.

1.9.2 Mutations

Based on mouse knockout models, it was predicted that individuals with rare mutations in APOA5 would present with severe HTG. The first case of mutation associated with HTG was reported by Oliva et al (Oliva, Pisciotta et al. 2005). The phenotype was described as Type I HTG yet LPL and APOC2 mutations had been excluded. Sequencing of APOA5 gene revealed homozygosity for Q148X which resulted in premature truncation of the protein. The mutation was originally designated Q145X due to the miscounting of the start codon ATG. Marcais et al. identified the second proband with APOA5 mutation (Marcais, Verges et al. 2005). Sequencing of APOA5 identified the mutation Q139X, leading a truncated form of the protein (about 38%). Family studies showed that 5 of 9 Q139X carriers had HTG. Interestingly, ultracentrifugation fraction analysis indicated that in Q139X heterozygotes both the WT and the truncated apoA-V was found in the unbound fraction, whereas in normal plasma apoA-V associated with the lipoprotein fraction. LPL activity and mass were dramatically reduced in HTG carriers. During a survey of patients with primary severe HTG without LPL and APOC2 mutation, Oliva et al. identified a novel APOA5 sequence variant (Oliva, Pisciotta et al. 2006). The proband was heterozygous for a g>c transversion in the donor splice site of intron 3 (IVS3 +3g>c, c.161 +3g>c). This mutation led to an exon splice site skipping which would result in the deletion of exon 3, resulting in a frame shift which introduces a premature stop codon with a predicted peptide of 18 amino acids. Decreased post-heparin plasma LPL activity was observed. However, all these case showed that the mutation alone was not sufficient to cause HTG and a second TG-raising allele as well as environmental factors such as increasing age or obesity, seem to be necessary for expression of the HTG phenotype.
1.10 Rationale for My Research

1.10.1 Intracellular

Plenty of data from a variety of studies, including \textit{in vitro} and \textit{in vivo} experiments in different species, showed that apoA-V is a key regulator of plasma TG. However, the molecular basis whereby apoA-V functions to modulate plasma TG is unclear. Two major hypotheses have been proposed: 1. apoA-V has an effect within the plasma compartment to facilitate TG-rich lipoprotein metabolism. 2. apoA-V plays an intracellular role in hepatic lipoprotein biogenesis and/or secretion. Considering some unique aspects of apoA-V we discussed above: the plasma concentration is extremely low (114 - 258 ng/ml); it is expressed only in liver; it is highly hydrophobic and there is significant portion of apoA-V retained intracellularly, it is conceivable that the metabolic role for apoA-V is intracellular rather than extracellular. An intracellular function for apoA-V was suggested by Schaap et al. (Schaap, Rensen et al. 2004), who found that adenovirus-mediated apoA-V expression in mice decreases VLDL-TG production rate in a dose-dependent manner yet has no effect on VLDL particle number, suggesting apoA-V impairs lipidation of apoB but does not impair its secretion. The observation by van der Vliet et al. (van der Vliet, Sammels et al. 2001), that apoA-V mRNA is upregulated during liver regeneration suggests apoA-V serves a function in hepatocyte proliferation. \textbf{We hypothesized that apoA-V may regulate plasma TG levels by affecting the assembly and secretion of apoB containing lipoproteins.} Using the human liver cell line, Hep3B, we examined the effect of apoA-V expression on apoB-100 secretion and lipidation. When apoA-V was overexpressed in cultured Hep3B cells, neither the amount of apoB secreted nor the density distribution of apoB-containing lipoproteins was affected. However, surprisingly, we found that apoA-V does not co-localize with apoB intracellularly but, rather, can be found in association with cytosolic lipid droplets. To follow up with this finding, we further determined the structural elements within apoA-V that are involved in lipid droplet association and apoA-V secretion. We also confirmed \textit{in vivo} that apoA-V indeed associate with lipid droplets and facilitate accumulation of liver TG.

1.10.2 Extracellular

On the other hand, there was also a body of evidence supporting an extracellular role of apoA-V: Previous \textit{in vivo} studies demonstrated that HTG in apoA-V deficient mice can be attributed to decreased chylomicron and VLDL lipolysis and remnant removal (Grosskopf, Baroukh et al. 2005; Merkel, Loeffler et al. 2005). On the other hand, overexpression of apoA-V in mice by adenoviral gene transfer was shown to decrease plasma TG levels confirming a role for apoA-V in TG regulation (van der Vliet, Schaap et al. 2002; Schaap, Rensen et al. 2004; Qu, Perdomo et al. 2007). \textit{In vitro} studies with apoA-V suggest that TG lowering mediated by circulating apoA-V can be explained by its ability to indirectly increase TG hydrolysis by increasing the efficiency of LPL activity (Lookene, Beckstead et al. 2005) and by its ability to contribute to remnant uptake by members of the LDL receptor family (Nilsson, Lookene et al. 2007). Overall, the evidence suggests that apoA-V plays a role in the plasma compartment facilitating TG-rich lipoprotein particle clearance. \textbf{We hypothesize that parenteral delivery of apoA-V is sufficient to lower
plasma TG levels and reverse the HTG phenotype in apoA5-/− mice. Indeed, intravenous injection with apoA-V was found to substantially reduce TG in apoA5-/− mice. When we further mutated the putative heparin binding region of apoA-V, its TG lowering activity was significantly attenuated. In addition, to determine whether GPIHBP1 interact with apoA-V in vivo, we performed the same experiment in gpihbp1-/− mice. Parenteral apoA-V had no effect on plasma TG levels in gpihbp1-/− mice, suggesting that effective TG clearance by apoA-V in vivo requires the interaction with apoA-V and GPIHBP1.
1.11 Figures

**RKGFWDYSFSQ TSGDKGRVEQ IHHQKMAREP ATLKDSLEQD LNMMNKFLEK**

**LRPLSGSEAP RLPQDPVGMK RQLQEEELVE KARLQPYMAE AHELVGNL**

**GLRQQLKPYT MDLMEQVALR VQELQEQRLV VGEDTKAQLL GGVDEAWALL**

**QGLQSRVVHH TGRFKELFHP YAESLVSIG RHVQELHRSV APHAPASPAR**

**LSRCVQVLSR KLTLKAKALH ARIQQNLDQ L REELSRAFAG TGTEEGAGPD**

**PQMLSEEVRO RLQAFRQDTH LQIAAFTRAI DQETEEVQQQ LAPEP**

**PPP GHSA FAPEFQTDS GKVLSKIQAR LDDLWEDITH SLHDQGSHL GDP**

**Figure 1-1. Amino acid sequence of full length human apoA-V without signal peptide.**
The position of the stop codons of apoA-V-(1-146) and -(1-292) are Trp147 (bold) and Pro293 (bold), respectively. The positive charged sequence is between Glu185 and Asp228 (red) and all positive charged residues (including His) within this region are highlighted in blue. The four positive charged residues mutated are highlighted in blue bold.
1.12 References


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CHAPTER 2: ApoA-V association with intracellular lipid droplets
2.1 Introduction

ApоА-V plays a key role in regulation of TG metabolism. Given the very low concentration of apoA-V in plasma, we hypothesized that apoA-V may influence plasma TG levels by affecting the assembly and/or secretion of apoB-containing lipoproteins. When apoA-V was overexpressed in cultured Hep3B cells, neither the amount of apoB secreted nor the density distribution of apoB-containing lipoproteins was affected. Fluorescence microscopy and cell lysate immunoprecipitation studies revealed that apoA-V is not associated with apoB intracellularly, yet immunoprecipitation of apoA-V from the cell culture medium resulted in co-precipitation of apoB. These data suggest apoA-V association with apoB-containing lipoproteins is a post-secretory event. Confocal fluorescence microscopy revealed the presence of apoA-V in distinct cellular structures. Based on Nile Red staining we identified these structures to be intracellular lipid droplets. The data suggest that apoA-V has a unique association with cellular lipids and, therefore, may be involved in storage or mobilization of intracellular lipids.

2.2 Results and Discussion

2.2.1 Effect of apoA-V expression on apolipoprotein accumulation in cells and medium.

Hep3B cells were transfected with a control empty vector or apoA-V plasmid construct. Immunoblot experiments (Figure 2-1) probed with indicated antibodies were performed to determine the relative distribution of apolipoprotein in cell lysates versus conditioned media. In Hep3B cells transfected with an empty vector, apoA-V could not be detected, consistent with the very low concentration of this protein reported by others (O'Brien, Alborn et al. 2005). In apoA-V transfected cells, however, the protein was readily detected with roughly similar amounts present in cell lysate and medium. By contrast, nearly all of the apoA-I and most of the apoE and apoB-100 detected was in the medium. The similar apolipoprotein distribution seen in control vector and apoA-V transfected cells indicates that overexpression of apoA-V does not affect the secretion efficiency of these apolipoproteins. At the same time, however, the difference in apolipoprotein distribution between apoA-V and the other apolipoproteins suggest that its secretion is impaired.

2.2.2 Effect of apoA-V expression on the density distribution of apoB- and apoA-V-containing lipoproteins.

In murine models, apoA-V exerts a strong influence on plasma TG levels. Thus, it is conceivable that, although apoB-100 secretion from Hep3B cells is unaffected by apoA-V transfection, TG-rich lipoprotein particle density distribution may be altered. To examine this possibility, conditioned media from [35S] Met/Cys labeled Hep3B cell cultures were subjected to cumulative rate flotation centrifugation followed by immunoprecipitation with an antibody directed against apoB-100 (Figure 2-2, panel A). In the case of non transfected Hep3B cells, apoB is mainly recovered in the VLDL/IDL density range with a small quantity in LDL. A similar apoB distribution was observed in cell cultures transfected with empty vector or apoA-V vector. We conclude that apoA-V does not influence the lipidation state of apoB secreted from these cells.

Immunoprecipitation of apoA-V (Figure 2-2, panel B) indicated that apoA-V is
associated primarily with particles in the HDL density range. A small amount of apoA-V is found in LDL and VLDL.

2.2.3 ApoA-V is associated with apoB-containing lipoproteins in the medium but not in cell lysate.

In Hep3B cell medium, apoA-V was present in the VLDL/LDL density range, therefore we asked whether apoA-V and apoB are localized on the same lipoprotein particles. Anti-FLAG pulled down apoA-V and apoB from the medium while immunoprecipitation with anti-apoB likewise pulled down apoB and apoA-V (Figure 2-3, right panels). The latter confirms that apoA-V associates with apoB extracellularly and is consistent with the observation that apoA-V is found on VLDL in plasma (O'Brien, Alborn et al. 2005). Hep3B cells expressing apoA-V-FLAG were lysed and the lysate was immunoprecipitated with both anti-FLAG and anti-apoB antibodies. Anti-FLAG antibody pulled down apoA-V but not apoB from the lysate (Figure 2-3, left panels); similarly, apoB antibody pulled down apoB but not apoA-V suggesting that intracellularly apoB and apoA-V are not associated.

2.2.4 ApoA-V and apoB localize to separate subcellular compartments.

Since Hep3B cells have low levels of intracellular apoB (Figure 2-1), McA-RH7777 cells expressing high levels of human apoB-100 were transfected with an apoA-V-GFP vector and used to assess whether apoA-V and apoB-100 traffic together intracellularly. Confocal images (Figure 2-4) of fixed cells probed with anti-apoB followed by an Alexa 594 labeled secondary antibody, confirmed localization of this apolipoprotein to the endoplasmic reticulum (Tran, Thorne-Tjomsland et al. 2002). On the other hand, apoA-V-GFP displayed a very different distribution pattern, clustering around distinct cytosolic structures that display a donut-shaped appearance with non-staining cores. No co-localization of apoB and apoA-V was observed (Figure 2-4, panel A and B) consistent with the immunoprecipitation studies with cell lysate. Based on the distribution of apoA-V and its localization to cytosolic spherical entities, we hypothesized that apoA-V may be associated with intracellular lipid droplets. To test this, lipid droplets were stained with Nile Red while apoA-V was monitored by GFP fluorescence (Figure 2-4, panel C-F). As seen in panel C, apoA-V localized to the perimeter of lipid droplets that stain with Nile Red (Figure 2-4, panel D). The merged images shown in panels E and F confirm co-localization of apoA-V and intracellular lipid droplets. Control studies with native apoA-V and FLAG-tagged apoA-V (Figure 2-5) indicated that the observed localization was not due to the GFP tag. Cells transfected with apoA-I-GFP (Figure 2-6) revealed that, unlike apoA-V, this protein did not localize to lipid droplets but was associated with the ER. The data suggest specificity of apoA-V for lipid droplets.

2.3 Summary and Conclusions

Evidence obtained from genetically engineered mice (Pennacchio, Olivier et al. 2001) and human population studies (Marcais, Verges et al. 2005; O’Brien, Alborn et al. 2005) have revealed that apoA-V plays a role in modulating TG levels. The mechanism whereby apoA-V regulates TG metabolism is not fully understood. One body of evidence suggested that apoA-V functions extracellularly by activating lipoprotein lipase, thereby increasing the efficiency of chylomicron- and VLDL-TG clearance (Schaap, Rensen et al. 2004; Lookene,
Beckstead et al. 2005; Merkel, Loeffler et al. 2005). Given the low concentration of apoA-V in plasma, it is estimated that only a small subfraction of circulating VLDL particles will contain an apoA-V molecule (Olofsson 2005). This raises the possibility that apoA-V’s effects on plasma TG may be intracellular rather than in the plasma compartment.

In the present study we show that apoA-V secretion from cultured Hep3B cells is impaired while its overexpression had no effect on apoB secretion or lipidation. This observation is consistent with that of Weinberg et al (Weinberg, Cook et al. 2003) who noted that apoA-V expressed in COS-1 cells had a low secretion efficiency compared with albumin and a truncated form of human apoB. Inefficient secretion of apoA-V may explain the low circulating concentration of this protein in humans (O’Brien, Alborn et al. 2005). The density distribution of apoB-containing lipoproteins secreted by Hep3B cells transfected with apoA-V was indistinguishable from cells transfected with an empty vector, indicating apoA-V overexpression does not influence apoB-containing lipoprotein particle secretion or lipidation. This result is different from findings reported by Schaap et al. (Schaap, Rensen et al. 2004), who found that VLDL lipidation, but not particle number, was affected by adenovirus mediated apoA-V overexpression in mice.

Whereas it is known that apoA-V associates with VLDL in plasma, we employed confocal immunofluorescence microscopy to determine if an association between apoA-V and apoB occurs intracellularly. Immunofluorescence microscopy and immunoprecipitation of cell lysates showed definitively that apoB and apoA-V are located in separate and distinct cell compartments suggesting apoA-V association with VLDL is a post-secretory event. The latter, together with our observation that most of the newly secreted apoA-V is found in HDL, suggests that apoA-V may be transported from the cell on HDL and exchange onto VLDL post-secretion. Whereas apoB localized to the ER and throughout the secretory pathway, apoA-V-GFP localized in a discrete compartment that did not co-localize with apoB. Interestingly, the confocal microscopy data suggests an intracellular pool of apoA-V exists that escapes the secretory pathway despite the fact that it is synthesized with a secretory signal peptide. In this regard apoA-V is similar to the recently described apolipoprotein, apoO, which is partially retained in cells even though it possesses a signal peptide (Lamant, Smih et al. 2006). ApoO trafficks to lipid droplets within cardiomyocytes. In a similar manner, apoA-V is partially retained in hepatocytes and trafficks to cytosolic lipid droplets. Given that TG is the primary lipid component of intracellular lipid droplets, it is conceivable that apoA-V effects on plasma TG levels are manifest through its interactions with this cellular lipid pool.

2.4 Materials and Methods

2.4.1 Materials

[35S]methionine/cysteine (Met/Cys) was purchased from GE Healthcare. Oleic acid (OA), albumin, monoclonal anti-FLAG M2 antibody and anti-FLAG M2 affinity gel were from Sigma. Minimum essential media (MEM), Dulbecco’s Modified Eagle Medium (DMEM) without Met and Cys, sodium pyruvate solution, MEM non-essential amino acids solution, fetal bovine sera (FBS), horse serum, G418 and trypsin-EDTA were purchased from Gibco. Fluorescent labeled goat anti-mouse Alexa Fluor 594, Nile Red and DAPI were from Molecular Probes. Antibodies used included polyclonal goat anti-apoA-V (Beckstead, Oda et
al. 2003), monoclonal mouse anti-apoE 1D7 (a gift from Dr. Karl Weisgraber), anti-human apoB monoclonal antibody, 1D1, that recognizes only human apoB (University of Ottawa Heart Institute), polyclonal goat anti-apoB (International Immunology Corp), and monoclonal mouse anti-apoA-I (Intracel).

2.4.2 Cell Culture
The human hepatocarcinoma cell line, Hep3B (ATCC), was cultured in MEM containing 10% FBS, 1mM sodium pyruvate and 100 µM non-essential amino acids. Rat hepatoma McA-RH7777-A18 cells stably transfected with human apoB-100 (kindly provided by Dr. Zemin Yao) were cultured in DMEM containing 10% FBS, 10% horse serum and 200 µg/ml G418. Cells were passaged every 4 days. Cells were transfected using Lipofectamine 2000 (Invitrogen).

2.4.3 Construction of Plasmids
To obtain a FLAG-tagged apoA-V (apoA-V-FLAG) expression vector and apoA-V green fluorescent protein (GFP) fusion protein expression vector, PCR was carried out using a plasmid harboring the entire apoA-V coding region (gift from Dr. Len Pennacchio). The amplification product for the FLAG tag was cloned into pFLAG-CMV-5.1 (Sigma) and that of GFP cloned into pEGFP-N1 (CLONTECH) via HindIII and BamHI sites, respectively. Both tags were appended to the C-terminus of the protein.

2.4.4 Immunoprecipitation and immunoblotting
Collected cells were washed with cold phosphate buffered saline and subsequently lysed in a nondenaturing lysis buffer described by Wu et al. (Wu, Zhou et al. 1996). Immunoprecipitation was carried out according to Dixon et al. (Dixon, Furukawa et al. 1991). Protein samples were electrophoresed and imunoblots processed as previously described (Beckstead, Oda et al. 2003).

2.4.5 Pulse-Chase Experiments
Cells were incubated in Met/Cys deficient DMEM for 1 h, then pulsed with [35S] Met/Cys (200 µCi/ml in Met/Cys-free DMEM containing 10% FBS and 0.8 mM OA for 20 min followed by a 3 h chase. Conditioned medium was collected and subjected to cumulative rate flotation centrifugation (Tran, Thorne-Tjomsland et al. 2002). One ml fractions were collected, immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography.

2.4.6 Confocal Microscopy
McA-RH7777-A18 cells were grown on poly-L-Lysine coverslips (BD Biosciences). After transfection with apoA-V-GFP or apoA-V-FLAG, cells were transferred to growth media supplemented with 0.8 mM OA for 6 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and processed as described (Tran, Thorne-Tjomsland et al. 2002). Anti-human apoB monoclonal antibody 1D1 was diluted 1:500 in blocking solution and incubated with the cells for 1 h. Cells were washed with PBS and incubated with AlexaFluor 594 labeled goat anti-mouse IgG. For lipid droplet staining, a Nile Red (Greenspan, Mayer et al. 1985) saturated acetone solution was diluted 1:100,000 in PBS and incubated with cells for 15 min. Images were captured by LSM 510 Meta UV/Vis
confocal microscope.
2.5 Figures

**Figure 2-1. Effect of apoA-V expression on apolipoprotein accumulation in cells and media.** ApoA-V expression vector (ApoA-V) or empty vector (Empty) were transfected into Hep3B cells. Conditioned media were collected and concentrated to the same volume as cell lysates. Equivalent volume loads of cell lysate and conditioned media were subjected to SDS-PAGE followed by Western blotting for apoA-V, ApoA-I, apoE, and apoB. Apolipoprotein standards (20 ng) including apoA-V, apoA-I, apoE and apoB (as human LDL) are shown at the left. The data presented are representative of 3 independent experiments.
Figure 2-2. Effect of apoA-V expression on the density distribution of apoB- and apoA-V-containing lipoproteins isolated from Hep3B media. Hep3B cell cultures transfected with the apoA-V-FLAG expression vector were pulse labeled with $^{35}$S Met/Cys for 20 min followed by a 3 h chase in the presence of 0.8 mM OA. Conditioned media were collected and subjected to cumulative rate flotation centrifugation. Consecutive 1 ml fractions were collected and immunoprecipitated with (A) apoB antibody or (B) anti-Flag antibody for apoA-V and analyzed by SDS-PAGE followed by autoradiography.
Figure 2-3. Apolipoprotein immunoprecipitation from apoA-V transfected cells. ApoA-V-FLAG was transfected into Hep3B cells. Conditioned medium and cell lysate were immunoprecipitated with either anti-FLAG or anti-apoB antibody. Precipitates were analyzed on SDS-PAGE followed by Western blotting with antibodies to apoA-V and apoB as indicated. Left panel: cell lysate; right panel: conditioned medium.
Figure 2-4. Confocal fluorescence microscopy images of apoA-V-GFP, human apoB and lipid droplets in McA-RH7777 cells. Human apoB-100 expressing McA-RH7777 cells transfected with apoA-V-GFP were incubated in the presence of 0.8 mM OA for 6 h, fixed and permeabilized. Panel A is a merged image where apoB was probed with Alexa 594-labeled secondary antibody (red fluorescence) and apoA-V-GFP is seen as green; panel B is an enlarged image. Panels C and D show apoA-V-GFP and lipid droplets stained with Nile Red (red fluorescence), respectively. Panel E presents a merged image of apoA-V-GFP and Nile Red staining; an enlarged image is shown in panel F. Cell nuclei were stained with DAPI (blue) in panels A and E.
Figure 2-5. Effect of sequence tags on the intracellular localization of apoA-V. McA-RH7777 cells were transfected with native apoA-V or apoA-V-FLAG. The upper panel shows the distribution of native apoA-V (apoA-V); cells were fixed, permeabilized and incubated with anti-apoA-V primary antibody for 1 h followed by Alexa 594-labeled secondary antibody (red fluorescence). Lipid droplets (LD) were stained with Bodipy 493/503 (green fluorescence). The merged image shows that native apoA-V associates with lipid droplets in a manner similar to that seen with cells transfected with GFP-tagged apoA-V. The lower panel reveals the localization of apoA-V-FLAG (red) and Bodipy stained lipid (green); the merged image indicates that the FLAG-tagged apoA-V surrounds the lipid droplets. Nuclei were stained with DAPI (blue).
Figure 2-6. Localization of apoA-I in McA-RH7777 cells. McA-RH7777 cells were transfected with GFP-labeled apoA-I and cells were examined by fluorescence microscopy. Left panel, apoA-I-GFP (green); middle panel, lipid droplets (LD) stained with Nile Red (red); and right panel, merged image. The merged image reveals that apoA-I does not colocalize with lipid droplets. Nuclei were stained with DAPI (blue).
2.6 References


CHAPTER 3: Intracellular lipid droplet targeting by apoA-V requires the C-terminal and signal peptide segments
3.1 Introduction

Expression of apoA-V in hepatoma cells results in homing of this protein to intracellular lipid droplets. When hepatoma cells transfected with a full-length apoA-V-green fluorescent fusion protein were cultured in media that was not supplemented with oleic acid, intracellular lipid droplet size and number were reduced compared to that of cells supplemented with oleic acid. Confocal microscopy studies revealed that apoA-V associates with lipid droplets under both conditions. To define the structural requirements for apoA-V lipid droplet association, hepatoma cells were transfected with a series of C-terminal truncated apoA-V variants (Figure 3-1). Confocal microscopy analysis revealed that, in a manner similar to mature full-length apoA-V (343 amino acids), truncation variants apoA-V-(1-292), apoA-V-(1-237), and apoA-V-(1-191) associated with lipid droplets while apoA-V-(1-146) did not. Western blot analysis of the relative abundance of apoA-V in cell lysates versus conditioned medium indicated apoA-V variants that associated with lipid droplets were poorly secreted while apoA-V-(1-146) was efficiently secreted. Ultracentrifugation of conditioned medium revealed that, unlike full-length apoA-V which associates with lipoproteins, apoA-V-(1-146) was present solely in the lipoprotein deficient fraction. Deletion of the N-terminal signal peptide from apoA-V resulted in an inability of the protein to associate with lipid droplets or to be secreted into the medium. Taken together, the data suggest that the C-terminus of apoA-V is essential for lipid droplet association in transfected hepatoma cells and lipoprotein association in conditioned medium while the signal peptide is required for directing the intracellular and extracellular trafficking of this protein.

3.2 Results and Discussion

3.2.1 Effect of oleic acid treatment on apoA-V lipid droplet association

In OA supplemented cultured hepatoma cells, ectopically expressed full-length apoA-V homes to lipid droplets coating their surfaces. Since OA supplementation drives intracellular lipid accumulation and new lipid droplet formation, we sought to determine if OA supplementation is required for lipid droplet association of transfected apoA-V. To examine this question, an apoA-V-GFP fusion protein was expressed in McA-RH cells cultured with or without oleic acid supplementation. Lipid droplet association was then examined by confocal microscopy. As seen in Figure 3-2, in the absence of OA, lipid droplets were fewer in number and smaller in size. Despite this, however, transfected apoA-V-GFP localized to lipid droplets in a manner similar to that seen in cells supplemented with OA.

3.2.2 C-terminal sequence requirements for apoA-V lipid droplet association

Recently, Beckstead et al. (Beckstead, Wong et al. 2007) reported that the apoA-V-(1-292) truncation variant (at the site of a unique tetra-proline sequence) can bind DMPC vesicles but with lower binding efficiency than full length apoA-V. These authors also showed that a 51 amino acid peptide encompassing residues 293-343 (the deleted portion of the protein) was highly efficient in binding to DMPC. Taken together, these results suggested that this region of apoA-V is important in lipid binding. To test whether deletion of these residues, as well as other more extensive C-terminal truncations, alter apoA-V...
association with lipid droplets, plasmid vectors encoding truncated apoA-V-GFP variants were generated (see Figure 3-1). The latter approach provides new insights into how much of the C-terminal domain is required for lipid droplet and lipoprotein association. Following transfection into McA-RH cells, the extent of association with lipid droplets was determined by confocal microscopy (Figure 3-3, panel A). Consistent with our finding in Chapter 2, full-length apoA-V associates with lipid droplets (Figure 3-3, panel A). Homing of apoA-V to lipid droplets is not the result of the GFP fusion protein since we showed in Chapter 2 that without this fluorescent tag the protein still associated with lipid droplets. Upon deletion of 51 amino acids from the C-terminus of apoA-V to generate apoA-V-(1-292)-GFP, lipid droplet association persisted. Three additional apoA-V-GFP truncation variants were constructed, transfected into McA-RH cells and examined for their ability to associate with lipid droplets. The apoA-V(1-237) variant and apoA-V(1-191) variant (data not shown) associated with intracellular lipid droplets while apoA-V(1-146) did not (Figure 3-3, panel A). In the latter case, a diffuse fluorescent protein distribution pattern was observed and lipid droplets were smaller in size and dispersed throughout the cell.

3.2.3 Effect of signal peptide deletion on apoA-V lipid droplet association

The recovery of apoA-V in association with cytosolic lipid droplets raises the question whether the N-terminal signal peptide (SP) is required to direct the protein to cytosolic lipid droplets. To examine whether this 23 amino acid N-terminal sequence (Alborn, Johnson et al. 2006) plays a role in apoA-V post-translational fate, a plasmid construct encoding apoA-V-GFP that lacks a signal sequence, SP-apoA-V-GFP, was transfected into McA-RH cells and the intracellular localization of the protein assessed by confocal microscopy (Figure 3-3, panel A). Interestingly, compared to cells transfected with full-length apoA-V, “lipid droplets” present in the vicinity of SP-apoA-V-GFP fluorescence intensity were amorphous entities.

Adipocyte differentiation-related protein (ADRP), also known as adipophilin, is the major lipid droplet-associated protein in hepatocytes (Fujimoto, Itabe et al. 2004). Thus, we asked whether apoA-V and ADRP co-localize on lipid droplets and whether the ΔSP-apoA-V variant can potentially disrupt this association. Figure 3-3, panel B clearly demonstrates that full length apoA-V and ADRP co-localize on the surface of lipid droplets in McA-RH cells. Control studies with cells incubated in the absence of apoA-V (Figure 3-3, panel B) suggest that overexpression of apoA-V does not alter ADRP trafficking to lipid droplets. Expression the ΔSP-apoA-V variant still results in its co-localization with ADRP (Figure 3-3, panel B) even though lipid droplets are amorphous (Figure 3-3, panel A) suggesting that loss of the signal peptide did not misdirect ΔSP-apoA-V to another intracellular compartment.

3.2.4 Effect of C-terminal sequence elements on the secretion efficiency of apoA-V

In cultured hepatoma cells it is known that apoA-V is not as efficiently secreted as other apolipoproteins, such as apoA-I or apoB. The overall hydrophobicity and potent lipid binding ability of apoA-V may contribute to this phenomenon. The fact that transfected apoA-V-(1-146) does not associate with lipid droplets suggests this variant is less hydrophobic and has a lower lipid surface seeking activity. Based on this, it was hypothesized that apoA-V-(1-146) will be more efficiently secreted from transfected
hepatoma cells compared to full-length apoA-V or apoA-V-(1-292). To test this, full length apoA-V, apoA-V-(1-292) and apoA-V-(1-146) GFP fusion protein constructs were transfected into McA-RH cells. After 24 h culturing in serum free media, conditioned medium was collected and cell lysates prepared. Equivalent aliquots of cell lysate and concentrated conditioned media were then subjected to SDS-PAGE to determine the distribution of apoA-V (Figure 3-4, panel A). Whereas >80% of full-length apoA-V-GFP and apoA-V-(1-292)-GFP were recovered in the cell lysate fraction, a much larger proportion of apoA-V-(1-146)-GFP was recovered in the media. ApoA-V-(1-146) without the GFP tag showed a similar distribution (data not shown). Unlike the C-terminal truncations, 100% of ΔSP-apoA-V was recovered in the cell lysate (Figure 3-4, panel B). In this case, since a GFP tag was not employed, full-length apoA-V lacking a GFP tag was run for comparison and demonstrated that the ratio of protein in cell lysate versus medium was similar to that seen with apoA-V-GFP.

3.2.5 Association of full-length and truncated apoA-V with lipoproteins in media

Since transfected apoA-V-(1-146) does not associate with intracellular lipid droplets, we sought to determine if the secreted protein associates with lipoproteins in conditioned media. Conditioned medium was collected and the d ≤1.21 g/ml (lipoprotein-rich) and d > 1.21 g/ml (lipoprotein-poor) fractions were harvested. Whereas control full-length apoA-V was recovered in association with lipoproteins, apoA-V-(1-146) was exclusively recovered in the lipoprotein-poor fraction (Figure 3-5). The observation that a portion of the full-length apoA-V expressed in transfected hepatoma cells was recovered in the lipoprotein-poor fraction of conditioned media seemingly contradicts previous observations that apoA-V was only found associated with lipoproteins in human (O’Brien, Alborn et al. 2005) and mouse plasma (Nelbach, Shu et al. 2008) and that recombinant apoA-V is not soluble at physiological pH in a lipid-free form (Beckstead, Oda et al. 2003). In the present case, it is conceivable that apoA-V recovered in the d > 1.21 g/ml fraction may contain a small amount of lipid. It is also possible that, owing to the limited lipoprotein secretion efficiency of these cells combined with overexpression of apoA-V, there is insufficient lipid surface area for all the apoA-V to bind to lipoproteins. To address whether the apoA-V recovered in the d > 1.21 g/ml fraction is capable of binding lipoproteins, the apoA-V-containing lipoprotein deficient fraction from conditioned medium was incubated with the HDL fraction obtained from apoa5-/- mice. Subsequent density ultracentrifugation showed that apoA-V transferred to the HDL particles. When the same experiment was performed with conditioned medium containing apoA-V-(1-146), binding to HDL was not observed (data not shown).

3.3 Summary and Conclusions

Lipid droplets are comprised primarily of neutral lipids (TG or cholesteryl ester) and function as a dynamic lipid storage depot (Londos, Brasaemle et al. 1999; Brasaemle 2007). The surface coat of lipid droplets is comprised of a phospholipid monolayer together with several associated proteins. Whereas lipid droplets are most abundant in adipocytes, they can be found in nearly all cell types including hepatocytes. Research on the major lipid droplet associated proteins, Perilipin, ADRP and TIP47 (collectively referred to as “PAT” family proteins) has revealed structural similarities with the class of amphipathic
apolipoproteins (Hickenbottom, Kimmel et al. 2004). Structural analysis of TIP47 provided evidence for a two-domain structure and X-ray crystallography of its C-domain revealed a helix bundle molecular architecture similar to the N-terminal four-helix bundle of apoE. Thus, although lipid droplets are of larger diameter (<1µm in most cells but up to 50 µm in adipocytes) than lipoproteins, both possess a roughly spherical shape, a hydrophobic core and a surface monolayer of phospholipid. Based in this, it is intriguing to consider that the binding interaction of PAT family proteins with lipid droplets may be analogous to that between apolipoproteins and lipoprotein particles. The helix bundle motifs of apoE and apoA-I are postulated to “open” upon lipid interaction, thereby exposing hydrophobic lipid binding sites in the bundle interior (Weisgraber 1994; Ajees, Anantharamaiah et al. 2006). Given the comparable structures of the apoE NT domain and the TIP47 C-terminal domain, a similar conformational adaptation may facilitate its association with the surface of lipid droplets. Apolipoproteins do not normally serve a dual function as lipid droplet- and lipoprotein- associated proteins and, vice versa. An exception may be apoO, a recently identified apolipoprotein component of HDL, reported to associate with lipid droplets in cardiomyocytes (Lamant, Smih et al. 2006). Based on this, it is not unreasonable that apoA-V can associate with cellular lipid droplets.

The observation that apoA-V associates with cytosolic lipid droplets is seemingly contradictory to its expression as a pre-protein containing a 23 amino acid signal peptide. Classically, this N-terminal sequence will direct the newly synthesized protein to the ER for transit through the secretory pathway and, ultimately, export from the cell. While apparently not unprecedented (Lamant, Smih et al. 2006), little is known about how proteins directed to a secretory pathway might escape this fate. In this case, examination of the mechanism of lipid droplet formation may provide a clue. Lipid droplets form rapidly in response to elevated fatty acid. Droplet formation occurs in discrete regions of the ER with neutral lipid accumulating between leaflets of the bilayer. Mature lipid droplets are thought to bud from the ER membrane, forming an independent “organelle”. Given the proximity of this process to the ER lumen, it is conceivable that lipid surface seeking proteins (e.g. apoA-V) within this compartment may associate with nascent lipid droplets and migrate with them as they bud from the membrane toward the cytosol. While no definitive connection exists, it is interesting to note that partial hepatectomy in rats induces lipid droplet formation (Silvia, Mercedes et al. 2006) as well as up-regulation of apoA-V mRNA (van der Vliet, Sannells et al. 2001). Perhaps apoA-V facilitates lipid droplet formation or metabolism.

In the present study we show that lipid droplet association of apoA-V is not contingent upon oleic acid supplementation of hepatocytes but does require the C-terminal portion of the protein. Not only full-length apoA-V, but also C-terminal truncation variants including apoA-V-(1-292), (1-237) and (1-191), retained their ability to associate with lipid droplets following transfection into hepatoma cells. Indeed, loss of lipid droplet homing ability occurred only when the C-terminus was truncated to residue 146 (out of 343 amino acids in full-length apoA-V). Furthermore, the similarity of lipid droplet morphology between full-length and the apoA-V-(1-292) variant suggests that, in the latter case, despite decreased lipid binding activity (Beckstead, Wong et al. 2007), sufficient amphipathic helix elements remain to effect binding of the protein to lipid droplets. On the other hand, the apoA-V-(1-146) variant, wherein a putative highly hydrophobic region (residues 171-241) has been deleted, did not associate with intracellular lipid droplets. Although apoA-V-(1-
146) was efficiently secreted by the cells, it did not associate with lipoproteins consistent with its inability to associate with lipid droplets. The behavior of this variant is similar to a Q139X apoA-V mutant found in humans (Marcais, Verges et al. 2005), where the latter protein was found solely in the lipoprotein-deficient fraction of plasma. Thus, results obtained from studies of apoA-V-(1-146) provide direct evidence that the C-terminal region of apoA-V is necessary for lipoprotein association. At the same time, transfection studies indicate that the absence of the C-terminal sequence does not prevent secretion, again explaining the observation of circulating truncated apoA-V in subjects expressing Q139X apoA-V.

The significant differences in lipid droplet morphology between cells transfected with apoA-V with or without signal peptide suggest this N-terminal sequence may be critical for normal lipid droplet association. The possibility that the signal peptide on apoA-V is defective can be eliminated on the basis of data with apoA-V-(1-146) which contains a signal peptide identical to that present on full-length apoA-V. Results show that the signal peptide functions normally to direct this protein to a secretory pathway. The factors that regulate the cytosolic destination of a portion of the full-length apoA-V in these cells is not known but may be related to the overall hydrophobicity of the protein. In considering the molecular basis for this phenomenon, it is conceivable that signal peptide mediated transit of newly synthesized apoA-V to the ER compartment may result in localization of the protein to the site of lipid droplet genesis. Given that lipid droplet assembly occurs in the ER membrane, ultimately budding into the cytosol and pinching off to create a discrete droplet, apoA-V transiting the ER could make contact with the membrane site of droplet formation. In the absence of a signal peptide, apoA-V appears to alter lipid droplet morphology where the latter appears dispersed. Although speculative, owing to its high lipid binding affinity, ΔSP-apoA-V may disrupt the integrity of the lipid droplets. The ΔSP-apoA-V, however, does not appear to be miss-targeted to another cell compartment since its co-localization with ADRP, a major lipid droplet-associated protein in hepatocytes, is unaltered.

In humans, several common APOA5 polymorphisms are known to be associated with reduced plasma apoA-V and increased plasma TG levels (Pennachio, Olivier et al. 2002). One such polymorphism, cSNP 56C→G (S19W), which is found in the signal peptide domain of the protein, was shown to interfere with the translocation of apoA-V protein (Talmud, Palmen et al. 2005). In the latter study, substitution of Ser 19 by Trp reduced the amount of fusion protein secreted into cell culture medium. In the present study, deletion of the signal peptide abrogated apoA-V’s ability to bind to lipid droplets and to be secreted, suggesting that the signal peptide is required for directing intracellular and extra-cellular trafficking of the protein.

In conclusion, the data presented provide indirect evidence for an intracellular mode of action of apoA-V. The findings are in keeping with the very low plasma concentration of this protein, its relatively poor secretion efficiency as well as the observation that apoA-V mRNA is up-regulated in response to partial hepatectomy. Furthermore, the observation that the C-terminal sequence is necessary for lipid droplet association, as well as lipoprotein association, suggests this region of the protein may be essential for manifestation of TG modulation by this protein. Future in vivo studies with truncated apoA-V variants will permit this hypothesis to be tested.
3.4 Materials and Methods

3.4.1 Materials
OA and albumin were from Sigma. DMEM, horse serum, G418 and trypsin-EDTA were purchased from Invitrogen. FBS was from HyClone. Nile Red and DAPI were from Molecular Probes. Polyclonal rabbit anti-human apoA-V antibody was a kind gift from Dr. Robert J. Konrad (Eli Lilly and Company). Monoclonal mouse anti- ADRP antibody was from Progen Biotechnik.

3.4.2 Cell Culture
Rat hepatoma McA-RH7777-A18 (McA-RH) cells stably transfected with human apoB-100 (kindly provided by Dr. Zemin Yao, University of Ottawa) were cultured in DMEM containing 10% FBS, 10% horse serum and 200 µg/ml G418. Cells were passaged every 4 days. Cells were transfected using Lipofectamine 2000 (Invitrogen).

3.4.3 Construction of Plasmids
DNA amplification was carried out using a plasmid template harboring the entire apoA-V coding region (a gift from Dr. Len Pennacchio, Lawrence Berkeley National Laboratory). Primers were designed to amplify full-length apoA-V and indicated truncations (Figure 3-1). The amplification products were cloned into pcDNA3.1(+) (Invitrogen) or pEGFP-N1 (CLONTECH) in the case of expression of GFP fusions. The GFP tag was appended to the C-terminus of the protein.

3.4.4 Confocal Microscopy
For fluorescence microscopy, McA-RH cells were grown on poly-L-lysine coverslips (BD Biosciences). After transfection with apoA-V or apoA-V-GFP constructs, cells were transferred to growth media supplemented with 0.8 mM OA for 6 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and processed as described (Tran, Thorne-Tjomsland et al. 2002). Cells were washed with PBS and lipid droplets stained with Nile Red as described (Greenspan, Mayer et al. 1985); nuclei were stained with DAPI. To visualize ADRP, fixed cells were permeabilized and subsequently incubated with mouse anti-ADRP (1:10 dilution) followed by incubation with goat anti-mouse Alexa 594 secondary antibody. Images were captured by LSM 510 Meta UV/Vis confocal microscope.

3.4.5 Cell/medium distribution of apoA-V and apoA-V variants
McA-RH cells were grown in poly-L-lysine coated 6 well plates (BD Biosciences). Twenty-four h after transfection, medium was replaced with DMEM plus 10% FBS and 0.8 mM OA. After 16 h, cells were washed with PBS and incubated with serum free medium for 24 h. Subsequently, conditioned medium was collected and cell monolayer rinsed with PBS and the cells lysed with Cell Lysis Buffer (Cell Signaling Technology).

3.4.6 Lipoprotein association of apoA-V and apoA-V variants
To determine whether full-length apoA-V or apoA-V-(1-146) associated with lipoproteins upon secretion into the cell medium, conditioned medium was adjusted to
density 1.21 g/ml by the addition of solid NaBr. The sample was then centrifuged at 100,000 g for 5.5 h in a Beckman TL100 centrifuge. The lipoprotein-rich $d \leq 1.21$ g/ml fraction and $d > 1.21$ g/ml lipoprotein-poor fraction were harvested by aspiration.
3.5 Figures

Figure 3-1. Diagram depicting apoA-V truncation variants generated in this study. The full-length apoA-V schematically shows the signal peptide (SP) region (hatched box) as -23 to 1 and the mature secreted protein as region 1 – 343. The tetra-proline (PPPP) sequence beginning at residue 293 of the mature protein is indicated. Constructs of C-terminal truncation variants used in the present study included the signal peptide together with the region of the mature protein as shown. The deletion of the signal peptide is termed ΔSP.
Figure 3-2. Effect of oleic acid supplementation on apoA-V association with lipid droplets. Confocal fluorescence microscopy images of cultured McA-RH cells transfected with an apoA-V-GFP fusion construct (green fluorescence). Lipid droplets were stained with Nile Red (red fluorescence). Nuclei were stained with DAPI (blue fluorescence). Merged images are shown on the right. Upper panel, no oleic acid supplementation; lower panel, supplementation with OA (0.8 mM for 6 h).
Figure 3-3. Effect of C-terminal and signal peptide truncations on the lipid droplet association properties of apoA-V. Confocal fluorescence microscopy images of cultured McA-RH cells transfected with apoA-V-GFP fusion constructs (green fluorescence). McA-RH cells were incubated in the presence of 0.8 mM OA for 6 h and then fixed. Lipid droplets were visualized with Nile Red (red fluorescence) and nuclei with DAPI (blue fluorescence). 

(A) Intracellular localization of full-length apoA-V, C-terminal truncation variants, signal peptide variant (ΔSP) and lipid droplets; merged images are shown on the right. 

(B) Effect of full length apoA-V and ΔSP-apoA-V deletion on ADRP distribution. Control cells (middle panel) were cells grown in the absence of transfected apoA-V-GFP. ADRP was visualized with goat anti-mouse Alexa594 secondary antibody (red fluorescence).
Figure 3-4. Effect of C-terminal truncation of apoA-V on the secretion efficiency of apoA-V from transfected McA-RH cells. (A) Cells were transfected with apoA-V-GFP fusion protein constructs including full-length apoA-V, apoA-V(1-292) and apoA-V(1-146). After 24 h incubation of cells in serum-free medium, cell lysates (L) and conditioned medium (M) were obtained and equivalent aliquots were applied to gels. (B) compares the ΔSP-apoA-V deletion variant with full-length apoA-V, both transfected into cells without the GFP construct. Western blots were carried out using a polyclonal anti-human apoA-V antibody (α-ApoA-V-NT).
Figure 3-5. Effect of C-terminal truncation on lipoprotein association in conditioned media. McA-RH cells were transfected with full-length apoA-V and apoA-V-(1-146). After incubation of the cells for 24 h in serum free medium, conditioned medium was isolated and the lipoprotein-rich (d ≤ 1.21 g/ml) and lipoprotein-poor (d > 1.21 g/ml) fractions isolated and subjected to SDS-PAGE. Western blots were carried out using a polyclonal anti-human apoA-V antibody.
3.6 References


CHAPTER 4: ApoA-V associates with intrahepatic lipid droplets and influences triglyceride accumulation
4.1 Introduction

ApoA-V, secreted solely by the liver, is a low abundance protein that strongly influences plasma TG levels. In vitro, in transfected hepatoma cell lines apoA-V is largely retained within the cell in association with cytosolic lipid droplets. To evaluate if this is true in vivo, in the present study the amount of apoA-V in the plasma compartment versus liver tissue was determined in APOA5 Tg mice. The majority of total apoA-V (~80%) was in the plasma compartment. Injection of APOA5 Tg mice with heparin increased plasma apoA-V protein levels by ~25% indicating the existence of a heparin-releasable pool. Intrahepatic apoA-V was associated with lipid droplets isolated from livers of WT and APOA5 Tg mice. Furthermore, livers from APOA5 Tg mice contained significantly higher amounts of TG than livers from WT or apoa5-/- mice suggesting the apoA-V influences intrahepatic TG levels.

4.2 Results and Discussion

4.2.1 ApoA-V partitioning between liver and plasma compartment

In general, apolipoproteins mediate their biological effects in the plasma compartment. Their functions include stabilization of lipoprotein structure, modulation of lipid metabolic enzyme activity or serving as ligands for cell surface receptors. Given the extremely low concentration of apoA-V in plasma, its poor secretion efficiency from transfected cells and its ability to associate with intracellular lipid droplets, it is conceivable that apoA-V functions to modulate TG within hepatocytes. An important question related to this is the distribution of apoA-V between liver and plasma in vivo. ApoA-V levels in liver versus plasma were determined in APOA5 Tg mice overexpressing apoA-V. Liver lysates and plasma samples were subjected to a semi-quantitative Western blot analysis using known amounts of isolated recombinant apoA-V as standard. Two independent experiments with pools from 5 mice per study revealed that 80 ± 2% (Mean ± SE) of apoA-V was found in plasma while the remainder was present in liver, the sole tissue that expresses apoA-V.

4.2.2 A heparin releasable pool of apoA-V

ApoA-V contains a stretch of amino acids between residues 186 and 227 that possess a strong positive charge character. Based on studies that show apoA-V binds heparin as well as cell surface HSPG (Merkel, Loeffler et al. 2005; Nilsson, Lookene et al. 2007), it is possible that a pool of HSPG-bound apoA-V exists in vivo. To test this, APOA5 Tg mice were injected with heparin and plasma apoA-V concentration measured as a function of time (Figure 4-1, panel A). A rapid 25% increase in apoA-V concentration was noted after heparin injection while there was a slight decrease of apoA-V concentration in PBS injected controls. Plasma TG levels were also determined for the same time points (Figure 4-1, panel B). As expected (Forte, Krauss et al. 1979), injection of heparin dramatically lowered plasma TG levels compared with PBS.

4.2.3 ApoA-V association with isolated hepatic lipid droplets

In hepatoma cell lines transfected with apoA-V a large proportion of the protein is associated with intracellular lipid droplets (Chapter 2 and 3). Unlike transfected hepatoma cell lines, < 20% of the total apoA-V pool is present in the liver in vivo. Therefore, we sought
to determine whether this pool of apoA-V associates with lipid droplets. Equivalent amounts of apoA-V were recovered in the lipid droplet (together with ADRP marker protein) and microsomal fractions in both APOA5 Tg mice and WT mice (Figure 4-2). Little or no microsomal marker proteins, including calreticulin (CRT) and calnexin (CNX), were recovered in the lipid droplet fraction suggesting that apoA-V identified in this fraction does not arise from microsomal contamination. As expected, no apoA-V was detected in apoa5-/ mouse liver. To confirm apoA-V association with cytosolic lipid droplets in hepatocytes, the experiment was repeated using primary hepatocytes isolated from APOA5 Tg mouse liver. Again, apoA-V was found associated with LDs as well as microsomes (data not shown). Taken together, these data indicate apoA-V associates with hepatic lipid droplets.

4.2.4 Effect of apoA-V on liver TG accumulation

Intracellular lipid droplets are the site of TG storage in hepatocytes. Given that apoA-V associates with lipid droplets in vivo, we hypothesized that apoA-V may affect liver TG levels. To examine this, livers were harvested from APOA5 Tg, WT and apoa5-/ mice and TG levels determined and normalized to liver weight (Figure 4-3). Apoa5-/ and WT mouse livers contained similar amounts of TG while the amount in livers from APOA5 Tg mice was significantly (p<0.05) higher. These data suggest that apoA-V facilitates liver TG accumulation / retention.

4.3 Summary and Conclusions

It is currently accepted that apoA-V plays a role in regulating plasma TG levels. A substantial body of evidence suggests that, extracellularly, apoA-V increases LPL mediated hydrolysis of TG-rich lipoprotein particles thereby contributing to VLDL remnant clearance (Forte, Shu et al. 2009). Notwithstanding its extracellular role, other data suggest that apoA-V plays an intracellular role in regulating plasma TG levels. In rats, following partial hepatectomy, apoA-V mRNA is upregulated over 3-fold (van der Vliet, Sammels et al. 2001). In this physiological setting, regenerating liver tissue may be expected to retain its lipid pool for membrane biosynthesis and energy production such that TG secretion would be inhibited. Furthermore, the findings that apoA-V associates with lipid droplets in transfected hepatoma cells and that C-terminal truncation of apoA-V results in alterations in lipid droplet morphology (Chapter 2 and 3), support the premise that apoA-V may regulate intracellular TG metabolism. The present studies were carried out to assess apoA-V association with lipid droplets in vivo and examine the ability of apoA-V to influence intrahepatic TG content.

Although hepatoma cell lines transfected with apoA-V retained greater than 50% of the expressed protein intracellularly (Chapter 2), the present in vivo studies with APOA5 Tg mice demonstrate that most of the total apoA-V pool is in the plasma compartment. It is conceivable that overexpression of apoA-V in transiently transfected hepatoma cells impairs its secretion efficiency, favoring lipid droplet association. The greater distribution of apoA-V in the plasma compartment in vivo is consistent with its physiological role in mediating hydrolysis and clearance of TG-rich particles (Merkel, Loeffler et al. 2005). This latter observation led us to ask whether some apoA-V is bound to cell surfaces and thus, may not be accounted for in plasma samples. Treatment of mice with heparin induced a
rapid increase in plasma apoA-V levels suggesting a pool of apoA-V exists that is bound to cell surface HS PG or conceivably, GPIHBP1 (Gin, Yin et al. 2008). Although the site of the bound fraction of apoA-V is likely to be endothelial cell surfaces (the site of LPL activity), the possibility that some portion of the released protein is from liver cell surfaces cannot be ruled out.

Using immunofluorescence microscopy, previous studies with transfected hepatoma cells showed that most of the apoA-V localizes to cytosolic lipid droplets (Chapter 2). This observation was unexpected and, conceivably, could result from apoA-V over-expression in these cultured cells. In the present study we unequivocally show that apoA-V is present in lipid droplets harvested from livers of both WT and APOA5 Tg mice indicating apoA-V trafficking to lipid droplet is likely to be physiologically relevant. It is intriguing that apoA-V which is synthesized with a signal peptide that targets it for secretion is found on cytosolic lipid droplets. The signal peptide may be expected to direct newly synthesized apoA-V to the ER compartment which is also the site for lipid droplet genesis (Martin and Parton 2006). Lipid droplets are assembled within the ER membrane and, when it accrues sufficient lipid, buds into the cytosol. It is conceivable that apoA-V, which is very lipophilic, associates with the budding lipid droplets during its maturation resulting in its escape from the secretory pathway. In liver, ADRP is the major lipid droplet associated protein (Brasaemle, Barber et al. 1997) and is a marker for lipid accumulation. It has been shown that ADRP levels correlate with neutral lipid mass in the cell (Brasaemle, Barber et al. 1997; Ducharme and Bickel 2008). Overexpression of ADRP promotes accumulation of neutral lipid and/or lipid droplet formation in cells while, on the other hand, knockdown/knockout of ADRP reduces lipid accumulation, lipid droplet size and number. In the current study we showed that in APOA5 Tg mouse liver, where apoA-V is overexpressed, there was a significant increase of TG content compared with WT and apoa5-/- mice. These data support the premise that apoA-V may facilitate TG accumulation in a manner similar to overexpression of ADRP. We cannot, however, rule out the possibility that the increased TG levels in APOA5 Tg mice may be due to increased liver uptake of TG-rich remnants. Although speculative, the reason why there is no significant difference between liver TG levels in WT and apoa5-/- mice may be that endogenous levels of apoA-V are extremely low and ADRP is sufficient for normal TG accumulation in apoa5-/- mouse.

The association between apoA-V and LD may also indicate that these organelles act as an intracellular storage pool of apoA-V. It was reported previously that apoA-V is very hydrophobic with high lipid binding affinity (Beckstead, Oda et al. 2003; Weinberg, Cook et al. 2003). Thus, it is reasonable to hypothesize that association with LD stabilizes the intracellular pool of apoA-V. Since ADRP is degraded during regression of lipid storage in the cell (Masuda, Itabe et al. 2006), it is possible that, like ADRP, lipid droplet association protects apoA-V from degradation.

In summary, results demonstrating association of apoA-V with liver lipid droplets in vivo indicate this interaction has physiological relevance. Since APOA5 Tg mouse liver had a higher TG level than WT or apoa5-/- mice, it is conceivable apoA-V plays a role in facilitating hepatic TG accumulation and/or LD formation. Future studies on the effect of apoA-V on intracellular TG metabolism may provide insights on how apoA-V regulates TG metabolism.
4.4 Materials and Methods

4.4.1 Materials

Protease inhibitor cocktail, Pefabloc SC, gentamycin sulfate, chloramphenicol, Trolox, Tween 20, and bovine serum albumin Fraction V were from Sigma-Aldrich. Heparin was from Baxter. Primary antibodies included polyclonal goat anti-human apoA-V (Beckstead, Oda et al. 2003), polyclonal rabbit anti-human apoA-V (O'Brien, Alborn et al. 2005), polyclonal guinea pig anti- ADRP (PROGEN Biotechnik), polyclonal goat anti- CRT (kindly provided by Dr. Marek Michalak, University of Alberta) and polyclonal rabbit anti- CNX (Sigma). Secondary antibodies included HRP-conjugated affinity-purified donkey anti-goat IgG, and HRP-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Novex precast 4-20% polyacrylamide gradient gels in Tris-glycine buffer were from Invitrogen. Enzymatic assay kit for determination of TG was purchased from Wako Chemicals, USA.

4.4.2 Mice

Mice used in these studies, including apoa5-/-, APOA5 Tg and WT, have been described (Nelbach, Shu et al. 2008). Studies were carried out on male mice approximately 4 months of age after a 4 h fast. Research on the mice was conducted in conformity with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and was approved by the Children’s Hospital Oakland Research Institute Animal Care and Use Committee.

4.4.3 Immunoblotting

Samples were electrophoresed on 4-20% polyacrylamide gradient gels, transferred to PVDF membranes and processed as previously described (Beckstead, Oda et al. 2003). The polyclonal goat anti-human apoA-V antibody (Beckstead, Oda et al. 2003) used in this study cross reacts with mouse apoA-V in highly concentrated samples, such as isolated lipid droplets.

4.4.4 ApoA-V liver versus plasma distribution

APOA5 Tg mice were used for these studies since apoA-V in liver lysate and plasma from WT mice was too low for detection and reliable quantification. Plasma was prepared from mouse blood collected by submandibular venipuncture. Protease inhibitors and stabilizers were added as previously described (Nelbach, Shu et al. 2008). Liver tissue was obtained from anaesthetized mice after perfusion with Hanks balanced salt solution to remove blood. Tissue was rapidly frozen in cryovials and stored at -80°C until utilized. Separate liver extracts were prepared using weighed portions of individual frozen livers, in 5 volumes of ice-cold Tissue Protein Extraction Reagent (T-PER, Pierce) to which protease inhibitor cocktail and Pefabloc SC had been added. Supernatants were pooled, aliquoted and subjected to immunoblotting with anti-apoA-V IgG. Immunoblot band intensity of plasma and liver homogenate samples was measured by densiometry using the NIH ImageJ software and compared to the corresponding intensity of known amounts of recombinant apoA-V standard on the same blot. Estimation of total apoA-V in the plasma compartment
was made from known weights of animals, assuming plasma volume is 3% of body weight. Calculation of apoA-V in the liver compartment was made from the known weight of the excised liver.

4.4.5 Isolation of lipid droplets

Lipid droplets were isolated from liver tissue as described by Wang et al (Wang, Gilham et al. 2007) with modification. Briefly, liver tissue from APOA5 Tg, WT and apoa5-/mice were homogenized in ice cold homogenization buffer (1:5 w/v) consisting of 250 mM sucrose in 20 mM Tris, pH 7.4 containing 1 mM EDTA, and protease inhibitors. Cellular debris and the mitochondria were separated by centrifugation at 1,000 X g for 10 min and 15,000 X g for 10 min, respectively. The supernatant was centrifuged at 100,000 X g for 1 h to pellet microsomes. Cytosolic lipid droplets were carefully collected from the top of the tube and mixed with glycerol 1:1 (v/v). The lipid droplet fraction was overlaid in the centrifuge tube with equal volumes of homogenization buffer, and subsequently, TBS. The sample was centrifuged in a SW41 rotor at 40,000 rpm for 2 h. lipid droplets were removed from the top of the tube and mixed with protease inhibitors.

4.4.6 Measurement of liver TG content

Lipid extracts of liver tissue were assayed for triglyceride according to the manufacturer’s protocol using the Triglyceride Quantification Kit (BioVision, Mountain View, CA). Briefly, liver tissue was homogenized in 5% Triton-X100 in water (1:10 w/v). Samples were slowly heated to 80°C for 5 min. Insoluble materials were removed by centrifugation. TG concentration in the supernatant was determined by an enzyme based colorimetric assay.

4.4.7 Heparin Injection

APOA5 Tg mice were injected via tail vein with 50 µl heparin (50 units) or PBS. At 0, 1, 3, 8, and 13 min after injection, blood samples were collected by submandibular venipuncture. Mice were anesthetized with Isoflurane during the entire procedure. Plasma was obtained by centrifuging blood at 2,000 X g for 10 min at 4°C and aliquots analyzed for TG and apoA-V (Nelbach, Shu et al. 2008).
4.5 Figures

Figure 4-1. Effect of heparin injection on plasma levels of apoA-V. APOA5 Tg mice were injected with heparin (open squares) or PBS control (filled squares) (8 mice/group) at t=0. Plasma samples collected at t=1, 3, 8, and 13 min post injection were analyzed for (A) apoA-V and (B) TG. Values are presented as percentage of the level at t=0 and expressed as mean ± SE. Student t-test versus respective controls: *, p<0.05; ***, p<0.001.
Figure 4-2. ApoA-V association with liver lipid droplets and microsomes. Livers (2 g) from apoA5 KO, WT and APOA5 Tg mice were harvested and lipid droplets (LD) and microsomes prepared as described in the Methods. Lipid droplets and microsomes were each taken up in 1 ml buffer and 5 μl of each were subjected to electrophoresis. Western blotting was carried out using polyclonal anti-human apoA-V antibody (α-apoA-V), polyclonal anti-ADRP (α-ADRP), polyclonal anti-CRT (α-CRT) and polyclonal anti-CNX (α-CNX). Results are representative of three independent experiments.
Figure 4-3. Liver TG content in different genotypes. Livers were harvested from apoa5-/ (KO) (n=14), WT (n=15) and APOA5 Tg (n=20) mice. Liver TG was extracted and assayed as described in the Methods. Liver TG concentration was normalized to liver tissue wet weight. All data are mean ± SE. One-way analysis of variance was used to test for significance. Post hoc analysis (Tukey-Kramer HSD) examined significance of genotype effects. All analyses were performed using JMP version 7.0 (SAS Institute Inc.). *, p<0.05.
4.6 References


CHAPTER 5: Intravenous injection of apolipoprotein A-V reverses the hypertriglyceridemic phenotype of *apoa5* knockout mice via interaction with GPIHBP1
5.1 Introduction

Elevated plasma TG is a major risk factor for cardiovascular disease. It is recognized that apoA-V, a low abundance protein secreted solely by the liver, plays a critical role in TG metabolism by increasing the efficiency of LPL activity. Humans and mice deficient in apoA-V have severe HTG. The present studies were carried out to determine whether injection of human apoA-V protein into HTG apoa5-/- mice would lower plasma TG. Plasma TG concentration decreased ~60% 4 h post apoA-V injection. Over this time period, ~80% of the injected apoA-V was cleared from the circulation; ApoB-100 was also significantly decreased. VLDL TG and cholesterol concentrations decreased ~60% providing evidence that injected apoA-V increased lipolytic activity and VLDL clearance. Previous in vitro studies showed that apoA-V can bind to heparin and HSPG through a region of positively charged amino acids, and that mutation of these residues (apoA-V heparin mutant) resulted in decreased HSPG binding. When the apoA-V heparin mutant was injected into apoa5-/- mice, TG lowering over 4 h was significantly attenuated compared to WT apoA-V suggesting that this region is essential for the in vivo TG lowering ability of apoA-V. It has previously been shown that GPIHBP1 plays a critical role in TG metabolism and that gpihbp1-/- mice have severe HTG. Injection of apoA-V into these mice failed to lower plasma TG and circulating apoA-V levels were unchanged. These observations suggest that in vivo clearance of TG-rich particles requires interaction between apoA-V and GPIHBP1. Based on its profound TG lowering effect, intravenous administration of apoA-V may have therapeutic value in HTG.

5.2 Results and Discussion

5.2.1 Injection of apoA-V-DMPC lowered plasma TG concentrations

To address the question whether parenteral delivery of apoA-V lowers plasma TG concentrations, apoA-V-DMPC was injected into groups of apoa5-/- mice so that a final plasma concentration of 12.5 μg/ml was achieved. The latter is the average concentration of apoA-V in Tg mice (Nelbach, Shu et al. 2008). Injected DMPC vesicles were used as a negative control. Following apoA-V injection (Figure 5-1, panel A) plasma TG concentrations decreased with time where there was a 25% reduction at 1h and 60% reduction at 4 h; in controls there was a slight reduction (20%) over the 4h period. Like the TG concentrations, plasma cholesterol concentrations in apoA-V-DMPC injected mice also declined with time and were significantly lower than the control group at 2 and 4 h post injection (Figure 5-1, panel B). Consistent with the decrease in TG, the total amount of apoB-100 protein in the plasma dramatically declined over 4 h while ~80% of injected apoA-V cleared from the circulation over the same time period (Figure 5-1, panel C); unlike apoB-100 there was no change in apoB-48. Taken together, the data suggest that injection of apoA-V-DMPC triggers the clearance of TG, cholesterol, apoB-100 and apoA-V.

The above data suggest that delivery of exogenous apoA-V leads to the clearance of VLDL particles in apoa5-/- mice. To examine this further, plasma lipoprotein and apolipoprotein profiles from pooled plasma from apoa5-/- mice before (0 h) and 4 h after apoA-V injection were analyzed by Fast Protein Liquid Chromatography (FPLC). Following treatment with apoA-V-DMPC, there is a substantial reduction (~60%) in both VLDL TG and cholesterol suggesting that the decrease in plasma TG and cholesterol reflects the
clearance of VLDL particles (Figure 5-2, panel A and B). There was no concomitant increase in either LDL or HDL cholesterol concentrations indicating no accumulation of remnant lipids in either of these lipoprotein fractions further boosting the premise that injected apoA-V facilitates VLDL clearance. Changes of apolipoprotein distribution among lipoprotein fractions recovered by FPLC were also determined (Figure 5-2, panel C). Similar to the results obtained for plasma apoB-100, the amount of this protein in the VLDL fraction was vastly diminished after apoA-V injection, while LDL apoB-100 did not change significantly. Post injection, VLDL apoE levels decreased with a paralleled increase in the HDL fraction. Similarly, VLDL apoA-I levels also decreased. Taken together, these data suggest that removal of VLDL in apoA5-/- mice was significantly increased after apoA-V injection.

Exogenous apoA-V distribution among lipoproteins was determined at 1 h and 4 h post injection (Figure 5-2, panel C). The preponderance of apoA-V was found associated with VLDL at 1 h. Within 4 h, most of apoA-V was cleared from the VLDL. These data support the premise that exogenously delivered apoA-V exchanges onto VLDL particles and participates in VLDL catabolism.

5.2.2 Effect of apoA-V dose on TG lowering

To determine the effect of apoA-V dose on TG lowering ability, apoA5-/- mice were injected with half or double the dose found in Tg mice, i.e, 6.25 and 25 µg/ml, respectively. Over this dose range, no difference of TG lowering activity was observed (Figure 5-3). The data suggest that the TG lowering effect of apoA-V is saturated when the plasma concentration is at or above 6.25 µg/ml.

5.2.3 Injection of apoA-V did not increase post-heparin LPL activity

Previous work of others with post-heparin plasma from apoA5-/- mice indicated that LPL activity was significantly lower than that of normal mice thus contributing to HTG (Grosskopf, Baroukh et al. 2005). Additionally, human carriers of the APOA5 Q139X mutation linked with severely elevated TG are also known to have dramatically reduced LPL activity (Marcas, Verges et al. 2005). In the present study we evaluated whether reversing the HTG phenotype of apoA5-/- mice by the injection of apoA-V was due to increased post-heparin LPL activity. Apoa5-/- mice were first injected with apoA-V or DMPC followed by heparin injections 2 h and 4 h later; LPL activity in the plasma was subsequently measured. There was no significant difference in post-heparin LPL activities between apoA-V injected animals and controls (Figure 5-4) thus apoA-V injection had no effect on post-heparin LPL activity.

5.2.4 Mutations within the heparin binding region of apoA-V attenuated its TG lowering activity

ApoA-V contains a stretch of 42 amino acids (residues 186 - 227) that lacks negatively charged residues and is enriched with positively charged ones (Lookene, Beckstead et al. 2005). This positively charged region was shown to be involved in the binding of apoA-V to heparin, LDL receptor family members (Nilsson, Lookene et al. 2007; Nilsson, Christensen et al. 2008) and GPIHBP1 (Gin, Yin et al. 2008). Replacement of positively charged residues by negatively charged ones within this region is associated with decreased binding of apoA-V to both heparin and LRP (Nilsson, Lookene et al. 2007). Moreover, recent in vitro studies
with the apoA-V heparin binding mutant (R210E, K211Q, K215Q, K217E) demonstrated that this protein variant is unable to bind the acidic domain in GPIHBP1 (Gin, Yin et al. 2008). To test whether the latter mutant has decreased TG lowering activity in vivo, wild type (WT) apoA-V or the apoA-V heparin mutant was injected into apoa5-/- mice. The TG lowering effect of apoA-V heparin mutant was significantly less (p<0.01) at 4 h compared with WT apoA-V (Figure 5-5, panel A). The decreased TG lowering effect of the apoA-V heparin mutant was not due to its inability to bind to VLDL because, as noted in Figure 5-5, panel B, comparable amounts of WT and mutant apoA-V associated with VLDL at 1 h. Unlike WT apoA-V, however, a higher proportion of mutant apoA-V remained associated with VLDL at 4 h consistent with delayed clearance of these TG-rich particles. Taken together, these data indicate that the heparin binding ability of apoA-V is crucial for its in vivo TG lowering effect and mutations within this region significantly attenuate this activity.

5.2.5 Injection of apoA-V failed to lower the TG levels of gpihbp1-/- mice

Endothelial cell bound GPIHBP1 has a critical role in TG lipolysis (Beigneux, Davies et al. 2007). It has been reported that gpihbp1-/- mice have extremely elevated plasma TG levels and diminished lipolysis. To determine whether parenterally delivered apoA-V is able to rescue the HTG phenotype of gpihbp1-/- mice, apoA-V-DMPC was injected into these mice and compared to DMPC controls. Following injection of apoA-V there was no significant decrease in plasma TG levels in the gpihbp1-/- mice compared to DMPC controls over a 4 h period (Figure 5-6, panel A). It is noteworthy that even though apoA-V was found associated primarily with VLDL as early as 1 h after injection (Figure 5-6, panel B), its levels did not decrease over time as was the case in apoa5-/- mice (compare Figure 5-2, panel C) consistent with its inability to reduce TG concentrations. Overall, these data suggest that GPIHBP1 is critical not only for the TG lowering activity of apoA-V in vivo, but also for its clearance from the circulation.

5.3 Summary and Conclusions

Although present in plasma at exceeding low concentrations, apoA-V is an important modulator of plasma TG (Pennacchio, Olivier et al. 2001; Forte, Shu et al. 2009). Apoa5-/- mice exhibit elevated TG while APOA5 Tg have reduced plasma TG (Pennacchio, Olivier et al. 2001; Grosskopf, Baroukh et al. 2005; Nelbach, Shu et al. 2008). In addition to increased VLDL levels, diminished lipolysis of VLDL and a reduced rate of remnant uptake were also observed in apoa5-/- mice. VLDL from apoa5-/- mice was a poor substrate for LPL and had low binding to LDLR family members. In the current study, we show that parental delivery of apoA-V into apoa5-/- mice lowered plasma TG concomitantly with reductions in VLDL TG, VLDL cholesterol and apoB-100. These data indicate that parenteral administration of apoA-V can significantly improved VLDL catabolism in HTG apoa5-/- mice.

We have shown that, following apoA-V injection into apoa5-/- mice, total plasma apoB-100 levels dramatically decreased while apoB-48 levels were relatively unchanged. The latter is undoubtedly related to the earlier observation that the apoE/apoB-48 ratio is low in these mice thus diminishing uptake of apoB-48 that requires apoE for clearance (Nelbach, Shu et al. 2008). ApoE associates mainly with VLDL in apoa5-/- mice; however, following apoA-V injection, VLDL apoE levels significantly decreased, which was accompanied by the increased HDL apoE levels. Along with the decrease in of apoB-100 levels,
the transfer of apoE from VLDL to HDL is indicative of increased VLDL lipolysis.

The apoA-V dose employed in the current study was based on the reported average plasma concentration of APOA5 Tg mice, which is 12.5 μg/ml and represents a concentration ~50-100 fold higher than the human apoA-V levels (Nelbach, Shu et al. 2008). When double (25 μg) or half (6.25 μg) the dose was injected into apoa5−/− mice, the TG lowering ability remained unchanged. This suggests that apoA-V in vivo in APOA5 Tg mice is functioning under saturated conditions and that concentrations higher than 6.25 μg/ml will be sufficient to effectively lower TG. The latter is certainly consistent with the well documented observation that plasma apoA-V levels are extremely low but effective in lowering TG in WT mice.

GPIHBP1 is an endothelial cell protein that is required for the lipolytic processing of TRL in the plasma (Beigneux, Davies et al. 2007). In the absence of GPIHBP1, lipolysis of TRL is virtually abolished, leading to severe HTG in gpihbp1−/− mice. GPIHBP1 contains a highly negatively charged domain which is capable of binding proteins with positively charged “heparin-binding domains”. CHO cells overexpressing GPIHBP1 are capable of binding LPL and apoA-V (Beigneux, Davies et al. 2007). In addition, gpihbp1−/− mice showed an abnormal pattern of LPL release after intravenous heparin injection, which confirmed that the interaction between GPIHBP1 and LPL plays a role in lipolysis in vivo (Weinstein, Yin et al. 2008). We hypothesize that the interaction between apoA-V and GPIHBP1 in vivo is also essential for its TG lowering activity. Commensurate with this, injection of apoA-V failed to lower plasma TG levels in GPIHBP1 deficient mice. Moreover, apoA-V clearance was almost negligible in these mice, suggesting that this process requires interaction with GPIHBP1. To our knowledge, this is the first demonstration in vivo that GPIHBP1 and apoA-V are functional partners in facilitating TG lipolysis.

It was previously reported that apoA-V possesses a stretch of 42 residues between amino acid 186 and 227 that is enriched in positively charged amino acids and deficient in negatively charged ones (Lookene, Beckstead et al. 2005). This positively charged region has been shown to participate in heparin binding (Lookene, Beckstead et al. 2005), as well as binding to members of the LDLR family members (Nilsson, Lookene et al. 2007) and GPIHBP1 binding (Gin, Yin et al. 2008). With the mutant used in the current study (R210E/K211Q/K215Q/K217E) where positive charges were substituted with negative or uncharged residues, it was previously demonstrated that the mutant protein failed to bind to CHO cells overexpressing GPIHBP1 (Gin, Yin et al. 2008). These in vitro data are highly suggestive that this region of apoA-V is an important functional element. However, it was not clear from this in vitro approach whether heparin or GPIHBP1 binding interactions are indeed critical for its TG lowering effect in vivo. In the current study we show unequivocally that mutations within this region significantly attenuated the TG lowering effect of apoA-V in apoa5−/− mice. Compared with apoA-V WT which lowered TG by ~60% and DMPC control which lowered TG ~20%, the mutant showed a ~40% TG decrease which represented about half of the TG lowering capacity of the WT. The decreased TG lowering activity of the mutant was not due to the change of lipoprotein binding ability because the mutant was also found on VLDL at 1 h and 4 h after injection. Indeed, the clearance rate of the mutant protein was actually slower than the WT apoA-V. Taken together, these data suggest that the heparin binding region of apoA-V plays a key role in its capacity to lower TG.

Previously, we postulated a mechanism whereby apoA-V facilitates VLDL metabolism
Briefly, it was proposed that apoA-V exchanged from HDL onto VLDL that in turn interacts with HSPG and GPIHBP1 on the surface of endothelial cells where LPL also binds. Coordination between apoA-V, LPL and GPIHBP1 results in accelerated TG hydrolysis. In the current study, we were able to confirm this hypothesis in vivo by showing: (1). ApoA-V was able to rapidly exchange from reconstituted discoidal HDL onto VLDL (2). The interaction between apoA-V and GPIHBP1 is critical for its TG lowering function; (3). The positively charged region of apoA-V is required for this process. In addition, in the current studies, we also discovered that the clearance of apoA-V from the circulation almost completely depended on the presence of GPIHBP1 while mutation of positively charged residues of apoA-V also affected its clearance. Even though we cannot conclude that apoA-V is directly cleared through GPIHBP1, it is likely that apoA-V’s heparin binding region is essential for its interaction with GPIHBP1, which in turn, is essential for TG lipolysis.

Overall, we show that injection of apoA-V has a profound effect on lowering TG levels of apoa5-/− mice. Potentially this may be a useful approach for reversing the HTG phenotype. Given that the effective dose is exceptionally low, parental administration of apoA-V may have potential therapeutic value for treating HTG.

5.4 Materials and Methods

5.4.1 Materials
Primary antibodies included polyclonal goat anti-human apoA-V (Beckstead, Oda et al. 2003), polyclonal goat anti-apoB (International Immunology Corp.), polyclonal goat anti-mouse apoA-I (Abcam), and polyclonal rabbit anti-mouse apoE (Biodesign International). Bis-Tris 4-12% NuPAGE gradient gels were from Invitrogen. Enzymatic assay kits for TG and cholesterol assays were from Wako Chemicals, USA. Heparin was from Baxter. Fluorescent lipase substrate, 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6’-methylresorufin) ester (DGGR), was from Sigma.

5.4.2 Mice
Mice (male, 2-4 months of age) used in these studies, including apoa5-/− and gpihbp1-/− genotypes, were previously described (Beigneux, Davies et al. 2007; Nelbach, Shu et al. 2008). Research was conducted in conformity with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and was approved by the Children’s Hospital Oakland Research Institute and University of California Los Angeles Animal Care and Use Committees.

5.4.3 ApoA-V-DMPC Injection
Human apoA-V recombinant protein was prepared as previously described (Beckstead, Oda et al. 2003). Since apoA-V is not soluble at pH 7.4 the protein was complexed with DMPC as described (Beckstead, Oda et al. 2003) for injection; briefly, DMPC vesicles were generated by extrusion through a 0.05 μm membrane and subsequently complexed with recombinant apoA-V protein by sonication to form reconstituted discoidal HDL. Controls used DMPC vesicles without protein. Mice were fasted 4 h and blood samples were obtained by submandibular vein bleeds before, t=0, and
at 1, 2 and 4 h post-injection. ApoA-V-DMPC was injected by tail vein so as to achieve a final plasma concentration of 12.5 μg/ml which is the apoA-V concentration in APOA5 transgenic mice (Nelbach, Shu et al. 2008). Mice were anesthetized with Isoflurane when manipulated. Plasma samples were rapidly separated and stored at -80°C for further assays.

5.4.4 FPLC

Plasma lipoproteins from pooled plasma were separated by FPLC using a Superose 6HR 10/30 column (Pharmacia LKB Biotechnology). Elution profiles were monitored at 280 nm and 0.5 ml fractions were collected.

5.4.5 Measurement of lipid concentrations

Cholesterol and TG in plasma samples or FPLC fractions were determined by colorimetric assays (WAKO).

5.4.6 Immunoblotting

Concentrated FPLC fractions were electrophoresed on 4-12% Bis-Tris gradient gels, transferred to PVDF membranes and immunoblots processed as previously described (Beckstead, Oda et al. 2003).

5.4.7 Measurement of post-heparin LPL activity

Apoa5-/- mice were injected via tail vein with 50 μl heparin (50 units) 2 h or 4 h after injection with apoA-V or DMPC only. Before and 15 min after heparin injection, blood samples were collected and plasma separated. LPL activities in the plasma samples were determined using fluorometric assay as described (Lee, Desai et al. 2009). Briefly, the lipase activity in the plasma sample was measured as the rate of fluorescence generated from hydrolysis of a lipase substrate DGGR. Two min after mixing plasma sample with DGGR, fluorescent intensity was monitored for 5 min and lipase activity was calculated as relative fluorescent units generated per min (RFU/min). LPL activity was determined by subtracting pre-heparin activity from total post-heparin activity (Dallinga-Thie, Zonneveld-de Boer et al. 2007).
5.5 Figures

Figure 5-1. Effect of apoA-V-DMPC injection on plasma lipids and apolipoprotein levels in apoa5/- mice. Apoa5/- mice were injected with apoA-V-DMPC (squares) or DMPC control (circles). Plasma samples were collected before and at 1, 2 and 4 h post-injection and analyzed for (A) TG and (B) cholesterol. Values are presented as percentage of initial TG concentrations and expressed as mean ± s.e.m (>12 mice/group). Student t-test versus respective controls: *, p<0.05; **, p<0.001. (C) ApoB and apoA-V levels in the plasma samples were determined by western blot.
Figure 5-2. Effect of apoA-V injection on the distribution of plasma lipids and apolipoproteins among lipoproteins. Apoa5/- mice (n=6) were injected with apoA-V-DMPC at t=0. Plasma samples were collected before (diamonds) and 4 h post-injection (squares) subjected to FPLC as described in the Methods. (A) TG and (B) cholesterol concentrations in each fraction were determined. (C) Fractions representing VLDL, LDL and HDL were pooled and apolipoprotein levels in each lipoprotein class were determined by western blot. Results are representative of two independent experiments.
Figure 5-3. Effect of apoA-V dose on TG lowering ability. Apoa5-/- mice were injected at t=0 with different doses of apoA-V-DMPC as indicated (>8 mice/group). Plasma samples collected before (t=0) and at 1, 2 and 4 h post-injection were analyzed for TG. Values are presented as percentage of the initial TG level at t=0 and expressed as mean ± s.e.m.
Figure 5-4. Effect of apoA-V injection on post-heparin LPL activity. Apoa5/- mice were injected with apoA-V-DMPC (white bars) or DMPC control (black bars) followed by heparin (50unit/mouse) injections at 2 or 4 h. Plasma samples were collected 15 min post-heparin. LPL activity was determined as described in the Methods and is presented as Relative Fluorescence Unit (RFU) per second (mean ± s.e.m.). Student t-test indicated no significant differences between groups at either time points (p>0.05, n=6 mice/group).
Figure 5-5. Effect of mutations within the heparin binding region of apoA-V on TG lowering ability. Apoa5/- mice were injected with apoA-V wild-type (WT) (squares), apoA-V mutant (Mut) (triangles) or DMPC control (circles) (>7 mice/group). (A) Plasma samples collected before (t=0) and at 1, 2 and 4 h post-injection were analyzed for TG. Values are presented as percentage of the initial TG concentration and expressed as mean ± s.e.m. One-way analysis of variance was used to test for significance followed by post hoc analysis (Tukey-Kramer HSD) to examine significance between groups. There is significant difference between group a, b and c, p<0.05. (B) Plasma samples at 1 and 4 h post-injection were pooled and subjected to FPLC. ApoA-V level in each lipoprotein class was determined by western blot. Results are representative of two independent experiments.
Figure 5-6. Effect of apoA-V injection on TG and apoA-V metabolism in gpihbp−/− mice. Gpihbp1−/− mice were injected with apoA-V-DMPC (squares) or DMPC control (circles). (A) Plasma samples collected before and at 1, 2 and 4 h post-injection were analyzed for TG. Values are presented as percentage of the initial TG concentration and expressed as mean ± s.e.m (n=5 mice/group). Student t-test indicated no significant difference (NS) between groups for each time point (p>0.05). (B) Plasma samples at 1 and 4 h post-injection were pooled and subjected to FPLC. ApoA-V levels in each lipoprotein class were determined by western blot.
5.6 References
CHAPTER 6: General Discussion, Conclusions and Future Directions
6.1 Project Summary

ApoA-V is becoming an important plasma TG regulator after its discovery in 2001 as one of the newest members of exchangeable apolipoprotein family. However, the underlying mechanism whereby apoA-V affects TG metabolism is still largely unknown. A more basic question related to the mechanism is where apoA-V executes its function. There are currently two major hypotheses addressing this issue: 1) apoA-V functions in the plasma compartment (extracellularly) to facilitate lipolysis/clearance of TRL; 2) ApoA-V plays a role in the liver (intracellularly) to regulate TRL assembly/secretion. Compared with the extracellular mode of action which has been studied more thoroughly, there is not much research about the intracellular role of apoA-V. However, specific evidence, such as its extremely low plasma concentration, its low secretion efficiency and its liver expression pattern, suggested that apoA-V may affect TG metabolism intracellularly.

Transfected hepatoma cell lines were used as models to study the effect of apoA-V on apoB containing lipoprotein assembly and secretion. The results obtained did not support the original hypothesis and showed that expression of apoA-V did not change either apoB secretion or the density distribution of apoB containing lipoproteins. However, we were able to identify a unique intracellular localization of apoA-V, its association with intracellular lipid droplets. This finding opened up a series of new studies: we first determined the structure requirements for apoA-V lipid droplet association. We also confirmed in mice that apoA-V indeed associates with hepatic lipid droplets and influences accumulation of liver TG. Overall, we concluded that apoA-V does not directly affect TRL assembly or secretion in the liver, but it may have a role on intracellular TG accumulation.

Realizing that it is not likely that apoA-V modulates plasma TG levels intracellularly, we started to pursue the extracellular mode of action. To bypass the possible intracellular pathway, we took an approach by directly injecting apoA-V recombinant protein into apoa5-/ mice and monitoring the changes of plasma TG. The TG levels decreased ~60% over 4h after apoA-V injection. Plasma cholesterol and apoB-100 levels also decreased accordingly. Changes in apolipoprotein distributions among lipoproteins suggested increased lipolysis and clearance of TRL. Moreover, we showed for the first time in vivo that the heparin binding region of apoA-V is essential for its TG lowering activity. Most importantly, we discovered that GPIHBP1 is a bona fide apoA-V binding partner in vivo and is required for not only the TG lowering activity of apoA-V but also clearance of apoA-V from circulation. Taken together, these data supported the extracellular role of apoA-V and proposed a comprehensive mechanism by which apoA-V interacts with LPL and GPIHBP1 in concert to facilitate TRL catabolism (see Section 5.3).

Overall, this dissertation has addressed significant questions and provided important insight in the fields of apolipoprotein biology and lipid metabolism.

6.2 Positive Correlation between Apo-A-V and Plasma TG Levels

The positive correlation between apoA-V and plasma TG levels has been shown by several groups (Nelbach, Shu et al. 2008; Vaessen, Dallinga-Thie et al. 2009). It apparently contradicts earlier “all-or-none” observations with apoA-V knockout and human apoA-V transgenic mice. It also contradicts studies in humans revealing that apoA-V truncation mutations associate with severe HTG and apoA-V deficiency. How does one account for this conundrum? To best reconcile this apparent anomaly, it may be useful to consider the
relationship between insulin and glucose levels. A deficiency of insulin results in increased glucose whereas infusion of insulin decreases and normalizes glucose levels. In contradistinction, in insulin resistance, plasma glucose levels increase even as plasma insulin levels increase. Despite the acute effects of insulin (or lack of it), in this physiological setting there is a positive correlation between insulin and glucose. An analogous situation may occur in the case of apoA-V and TG: a complete lack of apoA-V results in elevated TG, whereas acute administration of apoA-V (e.g. injection of apoA-V) results in a decrease in TG levels. On the other hand, in a chronic situation, the correlation between apoA-V and TG levels is positive. It is possible that apoA-V is actually the “insulin” of TG. For instance, when plasma TG metabolism is required, the levels of apoA-V increase to signal the initiation of this process. This hypothesis still needs to be tested experimentally. Compared to the relationship of insulin and glucose, which has been thoroughly studied, there are still many basic questions that need to be answered about apoA-V and TG: besides the question whether apoA-V levels respond to TG changes, what is the mechanism by which apoA-V levels respond to TG changes? What is the apoA-V receptor, GPIHBP1, LDLR or some other unidentified proteins? Is there apoA-V resistance (the counterpart of insulin resistance)? (Very likely given that HTG often associated with increased apoA-V levels in both mice and human(Dallinga-Thie, van Tol et al. 2006; Talmud, Cooper et al. 2006; Henneman, Schaap et al. 2007; Vaessen, Dallinga-Thie et al. 2009)) What is the cause of apoA-V resistance? It may take decades to answer these questions. However, this knowledge would significantly improve our understanding about TG metabolism.

6.3 ApoA-V and Nutritional Status

It was reported that APOA5 expression is regulated by insulin and glucose (Nowak, Helleboid-Chapman et al. 2005; Nowak, Helleboid-Chapman et al. 2008): insulin down regulates APOA5 expression while glucose activates it. Given that insulin and glucose levels are important indicators of nutritional status and regulate APOA5 expression in an opposite manner, it would be interesting to determine how apoA-V responds to fasting and re-feeding, on both a transcriptional and protein level. In an insulin clamp study in human, hyperinsulinemia significantly reduced plasma apoA-V protein levels at 3.5 and 8 h by 52% and 72%, respectively (Nowak, Helleboid-Chapman et al. 2005). It makes sense that during the fasting state when insulin level is low and TG is the major fuel, apoA-V level increases and facilitates TG lipolysis. On the other hand, when re-fed, increased insulin level may down regulate apoA-V and favor glucose uptake and utilization. This hypothesis is worth testing in the future.

6.4 ApoA-V, VLDL and HDL

ApoA-V was found on VLDL and HDL in humans (O'Brien, Alborn et al. 2005). A similar distribution was also found in APOA5 Tg mice (Nelbach, Shu et al. 2008). When fasted 4 h, there is more than 10-fold greater apoA-V associated with HDL than VLDL. However, apoA-V mainly regulates VLDL metabolism instead of HDL. Given that apoA-V is able to rapidly exchange between VLDL and HDL, it is reasonable to hypothesize that HDL may serve as a reservoir for apoA-V. Specifically, when the TG and TRL levels are low in the circulation, apoA-V mainly associates with HDL; when plasma TG levels increase in the
postprandial state, apoA-V rapidly exchanges onto TRL to facilitate lipolysis. Regulation on the transcriptional level would not be efficient enough to respond to the fast change of metabolic state after feeding. This may explain why even under postprandial condition when insulin levels are high and APOA5 expression is suppressed, dietary TG is still rapidly cleared from the circulation. Releasing protein from a storage pool is one of the common strategies to solve this type of issue. For instance, insulin is released from pancreatic \( \beta \)-cells to respond to increased glucose level. It would be interesting to determine how the levels of apoA-V change in postprandial condition between TRL and HDL. Alternatively, Intralipid, an artificial TRL, could be used to test the same hypothesis.

In Chapter 2, we determined that, in hepatoma cells, apoA-V did not associate with apoB intracellularly, which suggested that apoA-V was not on apoB particles when secreted. Given that lipid free apoA-V is not soluble under neutral pH (Beckstead, Oda et al. 2003), it is likely that apoA-V is secreted on HDL or HDL-sized particles. This is in line with the theory that apoA-V is located on HDL after secretion and exchanges onto VLDL when TG levels rise. More extensive study of the apoA-V secretory pathway would reveal interesting details on this topic.

It is well known that apoA-I and apoE adopt different structures and have different receptor binding behaviors when present on particles with different amounts of lipid. As member of the same family, it is likely that apoA-V may have different structure and receptor binding ability on VLDL and HDL. This hypothesis could be addressed by running different size apoA-V containing particles through heparin or receptor coated chips and determining the binding ability using surface plasma resonance. The result may provide insight about the physiological relevance of the exchangeability of apoA-V.

### 6.5 Intracellular or Extracellular

We reported that apoA-V associates with intracellular lipid droplets, which suggested that apoA-V may have its TG lowering activity through an intracellular mechanism (Chapter 2-4). Schaap et al. also reported a decrease in liver TG production rate in mice overexpressing apoA-V using adenovirus mediated gene transfer (Schaap, Rensen et al. 2004). On the other hand, there is a body of evidence indicating that apoA-V performs its TG lowering activity extracellularly (see Chapter 1). We showed in Chapter 5 that injection of apoA-V efficiently decreases plasma TG levels in \( \text{apoa5}^{-/-} \) mice. This experimental approach bypassed the potential intracellular pathway by injecting apoA-V directly into the circulation. In this way, the effect could be attributed to an extracellular mechanism. Even though we could not completely rule out the possibility that apoA-V was taken up by the liver and had an effect inside the liver, given the time scale of the TG lowering activity (< 4 h) and the clearance rate of apoA-V from the circulation, this is not likely to be the case.

However, it does not mean that there is no physiological relevance to the intracellular localization of apoA-V. In fact, we showed that \( \text{APOA5} \) Tg mouse liver accumulates more TG than livers of WT and \( \text{apoa5}^{-/-} \) mice (Chapter 4) and it may due to higher levels of apoA-V associated with hepatic lipid droplets in \( \text{APOA5} \) Tg mice. It was also reported that apoA-V expression is unregulated in rat liver after hepatectomy (van der Vliet, Sammels et al. 2001), under condition wherein liver cells would be expected to reserve lipids for liver regeneration. It makes sense that apoA-V may act as an additional
surface protein to stabilize lipid droplet structure and favor lipid accumulation. There may be other unique aspects of apoA-V on lipid droplets that differentiate it from other known protein from PAT family such as ADRP which mainly plays a structural role. Hepatoma Cell lines with different apoA-V expression levels would be suitable models to address questions such as what is the mechanism that apoA-V facilitates TG accumulation and what is the difference between the function of apoA-V and ADRP on lipid droplets.

6.6 ApoA-V, GPIHBP1, LDLR and Atherosclerosis

It is generally believed that high levels of cholesterol-rich remnants cause atherosclerosis. However, the relevance of TRL and atherosclerosis still remains controversial (Criqui 2007). Recently, Weinstein et al. showed that gpihbp-/- mice, which have severe chylomicronemia as a result of defective lipolysis, develop progressive aortic atherosclerosis (Weinstein, Yin et al. 2010). This study provided more insight about the atherogenic nature of TRL and suggested that lipolysis might not be required to establish the link between TRL and atherosclerosis. Similar to gpihbp1-/- mice, apoa5-/- mice also have elevated TG levels and abolished lipolysis. It would be interesting to determine whether apoa5-/- mice also develop atherosclerosis like gpihbp1-/- mice. If so it would be another atherogenic mouse model independent of hypercholesterolemia. These models would be useful to study the relationship among TRL, lipolysis and atherosclerosis.

We showed that GPIHBP1 is critical for the clearance and TG lowering activity of apoA-V. However, on the molecular level, it is still unclear that how apoA-V is cleared out of circulation through GPIHBP1. It may be through endocytosis, but there is no evidence suggesting that GPIHBP1 can be internalized by any endocytic pathway. Other in vitro experiments have indicated that apoA-V can interact with LDLR family members, which are known endocytic receptors. In addition, apoa5-/- mice have delayed TRL and remnant removal; apoA-V deficient TRL are a poor binding partner for LDLR and the LDLR mRNA levels are up regulated for ~40% in apoa5-/- mice compared with WT (Grosskopf, Baroukh et al. 2005). All together, these data suggest that the interaction between apoA-V and LDLR may also be critical for its TG lowering activity, especially for TG-rich remnant clearance.

To test this hypothesis, LDLR-/- mice could be used as the model to carry out apoA-V injection study. If the clearance of apoA-V is abolished in those mice, it means that LDLR is required for this process. It is possible that the interaction of apoA-V and GPIHBP1 is necessary to dock TRL on the endothelial surface, bringing them into proximity to LPL to initiate lipolysis instead of endocytosis; GPIHBP1 may be also responsible for clustering other related receptors on the cell surface, such as LDLR family members; after lipolysis, the remnants may be immediately endocytosed through receptors in the same cluster, which may or may not require apoA-V specifically since other apolipoproteins such as apoE are also ligands for those receptors. The clustering process triggered by the binding of apoA-V and GPIHBP1 may be critical for the following steps including the internalization of remnants and apoA-V. This may explain the fact that even GPIHBP1 is not directly responsible for the actual endocytotic process, it is absolutely required for the clearance of apoA-V and without it, no clearance was observed. The concept of ligand mediated cell surface receptor clustering is well studied in Wnt/β-catenin signaling pathway (Junge, Yang et al. 2009). It is possible that similar pathways may exist in lipid metabolism. This theory is worth proving experimentally.
The relationship between hypercholesterolemia and atherosclerosis is probably one of those most thoroughly studied topics in the cardiovascular biology field. How to lower atherogenic cholesterol represents a multi-billion dollar question. Pharmaceutical companies are closely monitoring research on cholesterol metabolism and would not want to miss any potential drug target or therapy. Apoe-/- mice is a classic model to study hypercholesterolemia and atherosclerosis. Since injection of apoA-V efficiently increases TRL clearance and lowers plasma TG levels of apoa5-/- mice, it would be an important question for the cardiovascular research community to determine whether injecting apoA-V into apoe-/- mice would lower their plasma cholesterol levels. Actually, Huang et al. was able to show that adenovirus mediated apoA-V overexpression in apoe-/- mice significantly lowered their cholesterol levels (Huang, Bi et al. 2006). The plasma cholesterol in apoe-/- mice is mainly in the form of IDL and LDL which are the TRL remnants and lacking apoE for receptor clearance. If the hypothesis we proposed is true and apoA-V indeed interacts with LDLR family members and facilitates receptor-mediated remnant clearance, a strong cholesterol lowering may be seen in this mouse model. Besides apoe-/- mice, another group reported that apoA-V is atheroprotective by showing that atherosclerotic lesion development was significantly decreased when APOA5 Tg mice were crossed with APOE2 knock-in mice which spontaneously develop atherosclerotic plaques (Mansouri, Bauge et al. 2008). Overall, it is promising that apoA-V may play an important role in cholesterol metabolism in addition to TG metabolism.

6.7 References


