ROLE OF APOLIPOPROTEIN A-V IN TRIGLYCERIDE METABOLISM

BY

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LIST OF ABBREVIATIONS

APO: Apolipoprotein
ER: Endoplasmic Reticulum
HDL: High Density Lipoprotein
IP: Immunoprecipitation
LDL: Low Density Lipoprotein
LPL: Lipoprotein Lipase
MTP: Microsomal Triglyceride Transfer Protein
PCR: Polymerase Chain Reaction
PDI: Protein Disulfide Isomerase
PBS: Phosphate Buffered Saline
SDS: Sodium Dodecyl Sulfate
TLC: Thin Layer Chromatography
TG: Triglyceride
VLDL: Very Low Density Lipoprotein
ROLE OF APOLIPOPROTEIN A-V IN TRIGLYCERIDE METABOLISM

ApoA-V, a 366 amino acid protein synthesized by the liver, was identified in 2001 by comparative sequence analysis. The APOA5 gene is located downstream of the AI/CIII/AIV gene cluster on human chromosome 11q23. Apolipoprotein A-V (apoA-V) plasma concentrations are low (~100-200 µg/L) in comparison to other apolipoproteins, yet exert significant effects on triglyceride homeostasis.

To examine the basis for its low concentration in plasma, the secretion efficiency of apoA-V was measured in stably transfected McA-RH7777 rat hepatoma cells. Pulse-chase analysis revealed that only ~20% of newly synthesized apoA-V was secreted into culture medium within two hours post-synthesis. Similar results were obtained in transfected nonhepatic (CHO) cells. In neither cell system was apoA-V found associated with cell surface heparin sulfate proteoglycans. Similar results were also obtained when endogenous mouse apoA-V was analyzed in primary hepatocytes. In addition to its low secretion efficiency, the electrophoretic banding pattern of apoA-V suggested that the protein is susceptible to intracellular proteolysis with ~60% undergoing presecretory turnover within 2 hours post-synthesis.

To study the regulation of apoA-V by lipid synthesis, stably transfected McA-RH7777 cells were treated with 0.8 mM sodium oleate. The resulting increase in triglyceride synthesis resulted in a dramatic decline in apoA-V
secretion, a corresponding increase in cell-associated apoA-V, but no change in total apoA-V recovery. Reduced secretion was accompanied by movement of apoA-V onto cytosolic lipid droplets, as evidenced by apoA-V's colocalization with ADRP, a lipid droplet marker, and its flotation during sucrose gradient ultracentrifugation.

The possible intracellular role of apoA-V in the regulation of apoB and TG secretion was examined by generating stably transfected doxycycline-inducible McA-RH7777 cells. Upon induction of apoA-V expression, a 32% decrease in apoB-100 secretion and a 21% decrease in triglyceride secretion were observed. In addition, apoA-V expression caused a 57% decrease in the apoA-V in the d<1.006 g/ml VLDL density fraction during equilibrium density gradient ultracentrifugation.

These results indicate that 1) apoA-V inefficiently traffics within the secretory pathway but that its intracellular itinerary may be regulated by changes in TG synthesis and/or accumulation and 2) part of the effect of apoA-V on TG metabolism could be exerted at the level of hepatic TG mobilization and secretion.
Chapter 1

Introduction
General background

Lipids and lipid metabolism are important factors in the etiology of many human diseases, including coronary heart disease (CHD). When lipid metabolism is defective or perturbed, the resulting disorder is referred to as dyslipidemia. One such lipid disorder is hypertriglyceridemia, which has been shown to be an independent risk factor for the progression of CHD (1-5). While there has been controversy regarding the relationship between triglycerides and CHD, one meta-analysis study showed that for every 1 mmol increase of plasma triglyceride the risk of CHD rises by 32% for men and 76% for women (3). Severe hypertriglyceridemia occurs when plasma triglycerides rise above 10 mmol/L (6). Either increased secretion of triglycerides or inhibition of clearance from plasma can result in the development of hypertriglyceridemia.

Because lipids are insoluble in plasma, they are transported through the body as lipoproteins. These protein-lipid complexes are composed of a neutral lipid core, composed of triglycerides (TG) and cholesteryl esters (CE), surrounded by a monolayer of phospholipids and free cholesterol. Several apolipoproteins also occupy the lipoprotein surface; these proteins dictate aspects of both lipoprotein formation and metabolic fate. There are two classes of lipoproteins. The apoB-containing lipoproteins are responsible for the transport of dietary and endogenous TG and cholesterol from the intestine and liver, respectively, to peripheral tissues. The high density lipoproteins (HDL) are thought responsible for the transport of lipids, particularly cholesterol, from peripheral tissues back to the liver for elimination.
Apolipoprotein B and intestinal and hepatic lipid transport

Apolipoprotein B (apoB) is a large amphiathic protein that is essential for the assembly of TG-rich lipoproteins in both the intestine and the liver (7). In the intestines, a truncated form of apoB, containing the amino terminal 48% (apoB48), is formed by a process of mRNA editing (8;9). ApoB48 recruits dietary lipid in the enterocyte forming intestinal chylomicrons. These particles are essential for the transport of dietary lipids across the intestinal epithelium and into the circulation, via the mesenteric lymph. These particles are acted on in plasma by lipases, releasing free fatty acids and glycerol, which are taken up by peripheral tissues, including muscle and adipose (10). The lipolytic modification of these particles yields chylomicron remnants, which are cleared primarily by the liver, via the LDL receptor and LDL receptor-related proteins (LRP)(11). The liver also assembles and secrete apoB-containing lipoproteins in the form of very-low-density lipoprotein (VLDL). In humans, VLDL is formed exclusively by full-length apoB (apoB100), although rodents, including mice, secrete both apoB48- and apoB100-containing VLDL. Hepatic VLDL are also subjected to lipolysis resulting in the formation of successively smaller and higher density particles, referred to as intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). As each chylomicron and VLDL contains a single molecule of apoB (31), which remains irreversibly associated with the lipoprotein during its intravascular metabolism, the plasma concentration of apoB reflects apoB lipoprotein particle number and is a strong positive risk factor for atherosclerosis progression as well as other risk factors associated with the metabolic syndrome (12-15).
Hence, the regulatory mechanisms that control both the production and metabolism of hepatic TG-rich lipoproteins are crucial for the prevention and possible treatment of dyslipidemias associated with obesity, type 2 diabetes, and cardiovascular disease.

**Intracellular assembly of VLDL**

The assembly of apoB-containing VLDL particles occurs in the liver as a two-step process, which begins with the co-translational lipidation of apolipoprotein B by the microsomal transfer protein (MTP) (16). MTP is an ER-localized heterodimer composed of a unique 97-kDa subunit complexed with the ubiquitous ER folding enzyme, protein disulfide isomerase (PDI). MTP was first identified based on its capacity to transfer neutral lipids, including TG and CE, between donor and acceptor membrane vesicles, in vitro. MTP is abundant in liver and intestine, suggesting a possible essential role in apoB-containing lipoprotein formation. This prediction was validated by the discovery that the absence of apoB-containing lipoproteins in the human genetic disorder, abetalipoproteinemia, is caused by mutations in the gene for MTP (17). The resulting severe lipid and fat soluble vitamin malabsorption phenotype associated with genetic ablation of MTP is due to the absence of chylomicron formation in the intestine and the absence of apoB-containing lipoproteins in the plasma (18-20). The exact mechanism by which MTP facilitates the initial lipidation of apoB is not understood in detail, although the ability of MTP to transfer lipids to apoB during its translation and translocation into the ER is probably important.
Additionally, a number of groups have demonstrated a direct physical interaction between MTP and apoB (21), suggesting a possible chaperone-like folding function of MTP during early stages of VLDL assembly.

After completion of apoB translation and the first step in lipoprotein formation, it is believed that a relatively small, dense (~20 nm diameter) precursor lipoprotein particle is formed. Unlipidated and underlipidated apoB is vulnerable to rapid presecretory degradation and considerable effort has gone into elucidating the bases for this form of co- and posttranslational gene regulation (16). The ubiquitin/proteasomal pathway of protein degradation is responsible for a large percentage of presecretory apoB turnover, although other mechanisms have also been proposed (22). Once sufficient lipid is added to apoB during first-step assembly, it becomes relatively stable within the secretory pathway and escapes most degradation pathways. The nascent particle is then capable of undergoing enlargement during the second step in VLDL assembly.

The second step of particle assembly begins with the fusion of the precursor lipoprotein with apoB-free lipid droplets resulting in the formation of a mature VLDL. The intracellular site of second-step assembly is controversial with groups proposing that particle maturation occurs either in the ER (23) or the distal Golgi (24). In either case, an essential prerequisite for second-step particle maturation is the creation of so-called secretion-coupled lipid; that is, lipid that has been targeted for VLDL assembly via its translocation from the cytosol into the ER lumen. Although most of the enzymes responsible for TG formation have been identified and characterized (25), the mechanisms by which lipids are
mobilized or secreted via transport into the ER are not well understood. However, convincing data has emerged to suggest that MTP plays a critical role in this process, as genetic or pharmacological ablation of MTP activity reduces the neutral lipid content of the microsomal fraction (26-28).

**Mobilization of TG for VLDL assembly and regulation of VLDL production**

The formation of hepatic VLDL particles depends on triglyceride availability. Intracellular reserves of triglyceride serve as a primary source of triglycerides making up VLDL. In fact, approximately 70% of VLDL-TG comes from this pool while 30% is derived from de novo triacylglycerol synthesis (29). Mobilization of cytosolic lipid droplets for VLDL assembly is believed to require a cycle of TG lipolysis and reesterification. (30). Triacylglycerol hydrolase (TGH) is one enzyme that has been proposed to play a role in TG mobilization. Dolinsky *et al.* (31) propose a mechanism for TGH in which the enzyme hydrolyzes cytosolic lipid droplet TG that is ER associated. The resulting diglyceride products are then reesterified by either DGAT1 or DGAT2 and then shuttled into the VLDL assembly pathway.

A balance between hepatic TG production and its secretion as VLDL is essential. Hepatic over-production of triglyceride-rich VLDL is a hallmark of insulin resistance and type 2 diabetes (32-33) and is caused by both an increased flux of free fatty acids, arising from unregulated adipocyte lipolysis, as well as increased hepatic de novo lipogenesis. It has also been proposed that FoxO1-mediated upregulation of MTP is partially responsible for VLDL
overproduction under conditions of insulin resistance (34). Although VLDL production can be positively regulated to some extent, the ability of the VLDL pathway to maintain liver lipid homeostasis under some metabolic conditions is insufficient, leading ultimately to nonalcoholic fatty liver disease (NAFLD) and hepatosteatosis, a condition also observed when the secretion of VLDL is blocked (27;35).

**Metabolism of VLDL**

The mouse liver secretes approximately 25 to 50 mg of triglyceride per day, which is primarily delivered to muscle, heart, and adipose tissue (36). The enzyme lipoprotein lipase (LPL) plays a primary role in the delivery of VLDL-derived FFA to the periphery during TG-rich lipoprotein metabolism (37). LPL is localized to the capillary endothelium by virtue of its interaction with heparin sulfate proteoglycans (38;39). ApoC-II, a component of VLDL, activates LPL promoting the lipolytic removal of TG from the VLDL core and its conversion to IDL. Individuals with genetic defects of apoC-II display high levels of plasma TG that are comparable to individuals with LPL deficiencies (40-43). Another VLDL apolipoprotein that regulates LPL activity, in this case negatively, is apoC-III (44). The potency of apoC-III as an LPL inhibitor is observed upon its overexpression in transgenic mice, which results in a ~10-fold increase in fasting TG concentrations (45). Hepatic lipase (HL) is another lipolytic enzyme responsible for the lipolysis and remodeling of VLDL and its metabolic products. In addition to its lipolytic properties, HL, by virtue of its localization within the sinusoids of the
liver, also plays a critical role in the hepatic uptake of VLDL remnants, a process that may be independent of its catalytic activities (46). The ultimate clearance of the triglyceride rich lipoprotein particles and remnants occurs by receptor mediated endocytosis in the liver, via either LDL receptor or LRP (47).

**General information on apoA-V**

Apolipoprotein A-V is a 39 kDa protein expressed exclusively in the liver. This apolipoprotein was discovered in 2001, a late discovery in comparison to the majority of other apolipoproteins, many of which were discovered in the early to mid 20th century. The delayed discovery of this protein can be accounted for by its extremely low plasma concentration. ApoA-V plasma concentration ranges between 114 and 258 ng/ml in normolipidemic individuals (48-51), while the concentration of apoA-I is ~1 mg/ml (49). The identification of apoA-V occurred simultaneously by two different groups. Van der Vliet *et al.* identified apoA-V as an apolipoprotein that was associated with the early phase of liver regeneration in rats (52). At the same time, Pennacchio *et al.* revealed this novel protein by comparative sequence analysis. Comparison of human and mouse genomic DNA sequences, revealed that the apoA-V gene was located 30 kb downstream of the APOAI/CIII/AIV gene cluster on human chromosome 11q23 (53). There is 71% identity between human and mouse apoA-V (53) and 42% identity between human and avian apoA-V (54).
Genetics of apoA-V

Variations within the APOAI/CIII/AIV/AV gene cluster have been shown to result in differences in plasma TG levels in humans (55-71) (57;72-75). In 2002, the APOA5 gene was reported to increase the risk of high plasma triglycerides in patients with familial combined hyperlipidemia (69). A rare heterozygous Q139X truncation mutation in APOA5 was shown to increase the risk of late-onset hyperchylomicronemia attributed to severely reduced lipoprotein lipase activity (76). Two A5 genetic variants were examined in 483 pregnant women (77) and it was found that these variants led to increased triglyceride concentration. In other studies, two polymorphisms of the APOA5 gene were found to be linked to coronary artery disease (CAD) in Taiwanese Chinese (78). An association between APOA5 gene variants and common carotid artery intimal medial thickness (CCA IMT) was found in a study involving 2,273 participants (79). CCA IMT was identified as a measurement of atherosclerosis risk. These genetic-based population studies provide further evidence for the role that the APOA5 gene plays in the regulation of triglyceride metabolism and CAD.

Regulation of APOA5 gene expression

Human hepatic APOA5 mRNA expression level has recently been reported to be independently associated with plasma free fatty acids (80). PPARα ligands are shown to increase APOA5 mRNA in both primary liver cells and hepatic cell lines (81-82). Vu-Dac et al. have discovered the class of hypolipidemic drugs known as fibrates to be activators of PPARα. PPARα
agonists reduce plasma triglycerides by increasing lipolytic removal of TG, via increased LPL (83) and decreased APOC3 (84) expression, and by increasing expression of enzymes involved in fatty acid oxidation. The increase in apoA-V mRNA levels induced by PPARα agonists, suggests that this may account, in part, for the hypolipidemic effects of this class of compounds (81). Hahne et al. also identified a strong positive association between PPARα mRNA and APOA5 (80). Furthermore, Schultzze et al. have shown that in cynomolgus monkeys PPARα agonists increased serum apoA-V (85).

Prieur et al. have identified a farnesoid X receptor or FXR response element in the APOA5 gene (82). While they were able to establish that FXR ligands activated the APOA5 promoter, they saw no corresponding increase in APOA5 expression. The LXR ligand T0901317 decreased APOA5 mRNA in HepG2 and Huh7 cells, as well as, apoA-V protein in liver and serum of APOA5 transgenic mice (86). The transfection of nuclear SREBP1c in HepG2 cells has been shown to reduce A5 promoter activity (86). An orphan nuclear receptor known as RORα has been shown to activate APOA5 gene expression (87;88).

**APOA5 and APOC3 and triglyceride metabolism**

Located in the same gene cluster, apoA5 and apoC-III, both influence triglyceride metabolism albeit in opposite directions. Hence, one explanation for the relationship between apoA-V and TG may be due to its indirect effects on apoC-III. Baroukh et al. generated mice that express both A5 and C3 or KO of both apo genes (44). They found that these genes influence the concentration of
plasma triglycerides independently of each other. Gao et al. generated another transgenic line that supported Baroukh et al. data (89). They created a transgenic line carrying the 116-kb human apoAI/CIII/AIV/AV gene cluster and also a mutant transgenic line in which the apoC-III enhancer was deleted. Here they demonstrated that the apoC-III enhancer did not regulate apoA-V expression. Talmud et al. show that variants of APOA5 influence plasma TG independently of C3 (55). Olivier et al. also showed that the APOA5 effects on plasma triglyceride concentrations is independent of APOC3 in humans (74). The APOC3 enhancer is a common regulatory element for the AI/CIII/AIV/AV gene cluster, however Gao et al. found that the only apolipoprotein in this cluster that it does not affect was the newly discovered APOA5 (89).

**ApoA-V and regulation of triglyceride metabolism**

Recombinant lipid-free apoA-V is insoluble at pH 3.5-9, unless it is associated with lipids (90). Additionally, computational analysis of apoA-V revealed a large number of α-helical secondary structures and a high overall hydrophobicity (91). These data strongly suggest that apoA-V is an avid lipid binding protein. In humans, apoA-V has been found associated with VLDL, HDL and chylomicrons (49). In mice, apoA-V is mainly associated with HDL (52;92), although studies have also shown that apoA-V can readily exchange between VLDL and HDL (92).

Initial studies of apoA-V function revealed that transgenic overexpression of human apoA-V in mice caused an ~70% decrease in plasma triglycerides,
relative to wild type littermates (53). Conversely, apoA-V knockout mice demonstrated a ~4-fold increase in plasma TG (53). Other studies using adenoviral over-expression of apoA-V in mice also demonstrated a significant reduction of plasma triglycerides (93). Overall, these data suggest an inverse correlation between apoA-V and triglyceride levels in the plasma, although subsequent studies have indicated a more complex relationship between apoA-V and plasma TG, as discussed below.

ApoA-V and cholesterol metabolism

Fruchart-Najib et al. showed a 35% decrease in plasma cholesterol in hyperlipidemic hAPOA5 mice that have been inter-crossed with hAPOC3 mice (94). In mice that overexpress apoA-V by adenovirus injection, Van der Vliet et al. showed a reduction of cholesterol with the greatest reduction in the HDL fraction (93). Grosskpf et al. however, noted a 30% increase in plasma HDL cholesterol in apoAV−/− mice (95) whereas Pennacchio et al. saw no change in cholesterol in either the knockout or transgenic mice (53).

Controversy regarding the relationship between apoA-V and plasma triglyceride

It is clear that apoA-V is an important factor in the regulation of triglyceride metabolism; however, as time passes the role that apoA-V plays has become associated with more complexity and controversy. Initial studies by Pennacchio et al. in mice revealed a clear negative correlation between apoA-V and plasma
triglyceride (53). Likewise, Schaap et al. showed a negative correlation when apoA-V was over-expressed by adenovirus mediated expression (96). Ishihara et al. utilized a sandwich enzyme-linked immunosorbent assay for human plasma to determine that apoA-V is negatively correlated with plasma triglycerides (48). O’Brien et al. also used ELISA methods and showed a negative correlation between apoA-V and plasma triglycerides in normolipidemic individuals (49).

Although numerous studies point to an inverse relationship between apoA-V and plasma TG levels, more recent studies have identified either no correlation or a positive correlation between apoA-V and plasma triglycerides (50;51;97-99). For instance, Merkel et al. discovered a trend of increased plasma triglyceride in apoA-V transgenic mice (100). Positive correlations were also seen in human population studies involving diabetic and hypertriglyceridemic individuals (50;97-99), as well as in normolipidemic individuals (51;99). To further examine these surprising findings, Nelbach et al. examined the correlation between plasma apoA-V and plasma triglycerides in transgenic mice expressing various levels of human apoA-V in a mouse apoA-V null background (92). Using ELISA methods, they determined a strong positive correlation between apoA-V and plasma TG, essentially refuting the initial findings of Pennacchio et al. While clearly, more work is required to resolve these apparent discrepancies, recent studies by Vaessen et al. have proposed an explanation for the positive correlation between apoA-V and TG levels in humans (101). They suggest that apoA-V levels follow TG levels due to a close link between apoA-V and TG-rich lipoproteins. They hypothesize that increased plasma apoA-V levels is a
response to an increased demand for plasma TG hydrolysis under physiological conditions (101).

**Proposed extracellular functions of apoA-V in TG metabolism**

Several studies have provided evidence that apoA-V functions in the circulation to promote TG clearance. Fruchart-Najib *et al.* implemented *in vitro* recombinant apoA-V to demonstrate a physical interaction of apoA-V with LPL, which resulted in enhanced lipolytic activity (94). Schaap *et al.* have proposed a dual mechanism for apoA-V, involving both a reduction in VLDL-TG production (see below) and an increase of LPL-mediated triglyceride hydrolysis (96). They specifically proposed that apoA-V may facilitate the access of LPL to the triglyceride molecules within the core of lipoprotein particles and, in-line with Fruchart-Najib *et al.*, suggested that apoA-V could also increase triglyceride hydrolysis by stabilizing the LPL dimer, perhaps via a physical interaction. In another study by Merkel *et al.*, the presence of apoA-V enhanced plasma hydrolysis of triglyceride-rich lipoproteins associated with heparin sulfate proteoglycan-bound LPL (100). ApoA-V is a heparin-binding protein that binds as a lipid complex. Based on this, apoA-V associated TG-rich lipoproteins may have enhanced access to cell surface heparin sulfate proteoglycans and therefore, greater access to LPL and increased lipolysis (102). Grosskopf *et al.* used apoa5<sup>−/−</sup> mice to show that in the absence of apoA-V the resulting hypertriglycerideremia was due to decreased lipolysis and clearance of VLDL (95). In this system, they saw no effect of apoA-V on triglyceride production. Sun *et al.*
used deletion mutants to establish a region of apoA-V that was necessary for both lipid and LPL binding (103).

Recent studies have also shown that apoA-V serves as a ligand for some LDL receptor family members (104;105). Avian apoA-V was found to bind the LDL receptor homologue LR8 (104). Apoa5−/− mouse VLDL demonstrated lower affinity for LDLr compared to wild type control (95). A recent study by Nilsson et al. (106) revealed that that apoA-V is endocytosed by sortilin, a receptor from the Vps10p domain gene family. This data provides another possible avenue for apoA-V-mediated clearance of VLDL and its remnants.

**Intracellular hypothesis for the mechanism of apoA-V**

ApoA-V is synthesized with a signal peptide; however, human plasma has extremely low concentrations of apoA-V, ranging between 24 and 406 µg/L (49). This low concentration would result in a stoichiometry of one apoA-V molecule per 1000 VLDL particles (107). This stoichiometry is difficult to reconcile with the proposed roles of apoA-V in clearance of VLDL. Both the low plasma concentration and liver specific expression suggest that apoA-V’s primary function may be carried out within the liver. Based on apoA-V’s structural characteristics, a mechanism has been proposed whereby apoA-V may function within the cell to act as an intracellular brake on the assembly and secretion of VLDL-TG (91). Beckstead et al. proposed that apoA-V binds lipids and cellular membranes and therefore could decrease the assembly of VLDL (90).
Cytosolic lipid droplets and apoA-V mechanism

Lipid droplets are made up of a neutral lipid core and a monolayer of polar lipids and proteins, including ADRP, perilipin, and TIP47 (108). These droplets emerge from the endoplasmic reticulum into the cytosol (108;109). In hepatocytes, cytosolic lipid droplets are thought to be a source of triglycerides for VLDL assembly (30). Shu et al. have observed that apoA-V associates with lipid droplets (110) and they subsequently identified the C-terminus as being responsible for this intracellular lipid association (111). ApoA-V is not the only apolipoprotein known to associate with lipid droplets. In fact, apolipoprotein O, has been shown to co-localize with the lipid droplet associated protein, perilipin (112). The mechanism by which apoA-V can escape the ER to associate with cytosolic lipid droplets is unknown. However, this novel intracellular localization further suggests an unusual trafficking itinerary for apoA-V and provides further support for the idea that apoA-V may function intracellularly. Together, its low plasma concentration, high hydrophobicity, and affinity for cytosolic lipid droplets support an intracellular role of apoA-V in modulating TG metabolism.
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Chapter 2

Biogenesis and Post-transcriptional Regulation of Apolipoprotein A-V

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ABSTRACT

Apolipoprotein A-V (apoA-V) plasma concentrations are low in comparison to other apolipoproteins, yet exert significant effects on triglyceride homeostasis. To examine the basis for its low concentration in plasma, the secretion efficiency of apoA-V was measured in stably transfected McA-RH7777 rat hepatoma cells. Pulse-chase analysis revealed that only ~20% of newly synthesized apoA-V was secreted into culture medium within 2 hours post-synthesis. Similar results were obtained in transfected non-hepatic (CHO) cells. In neither cell system was apoA-V found associated with cell surface heparin sulfate proteoglycans. The electrophoretic banding pattern of apoA-V suggests that the protein is susceptible to intracellular proteolysis with ~60% undergoing complete presecretory turnover within 2 hours post-synthesis. To study the regulation of apoA-V by lipid synthesis, stably transfected McA-RH7777 cells were treated with 0.8 mM sodium oleate. The resulting increase in triglyceride synthesis resulted in a dramatic decline in apoA-V secretion, a corresponding increase in cell-associated apoA-V, but no change in total apoA-V recovery. Reduced secretion was accompanied by movement of apoA-V onto cytosolic lipid droplets. To examine the potential intracellular role of apoA-V in the regulation of apoB and triglyceride secretion, a stably transfected doxycycline-inducible McA-RH7777 cell line was generated. Upon induction of apoA-V expression, a 32% decrease in apoB-100 secretion and a 21% decrease in triglyceride secretion were observed. These results indicate that 1) apoA-V inefficiently traffics within the secretory pathway but that its intracellular itinerary may be regulated by changes in TG synthesis and/or accumulation and 2) part of the effect of apoA-V
on TG metabolism could be exerted at the level of hepatic TG mobilization and secretion.
INTRODUCTION

Apolipoprotein A-V (apoA-V) is a potent regulator of plasma triglyceride homeostasis. When overexpressed in transgenic mice, apoA-V reduced plasma TG levels by 65% whereas apoA-V gene inactivation increased plasma triglycerides by 4-fold (1). In humans, the relationship between apoA-V and plasma TG concentrations is more complex. Several alleles of apoA-V are associated with elevated TG, including apparent loss-of-function and null mutations (2;3). On the other hand, the relationship between apoA-V and TG concentrations is variable, with studies revealing both positive and negative correlations between apoA-V and plasma TG concentrations (4-6). Most recently, a study of human apoA-V expression in transgenic apoA-V knockout mice revealed a positive correlation between apoA-V and plasma TG concentrations (7).

Despite the impact of apoA-V on plasma triglycerides, its mode of function is still poorly understood. The preponderance of current literature indicates that apoA-V expression affects plasma TG turnover by stimulating LPL-mediated lipolysis either directly or indirectly (6;8-11). It has also been noted that apoA-V is a ligand for LDL receptor family members and other potential lipoprotein receptors, perhaps reflecting a role in catabolism of TG-rich lipoproteins and their remnants (12-15). While apoA-V can clearly impact plasma TG levels, a difficulty in explaining its function arises from its extremely low mean concentration in plasma of 157 µg/L, corresponding to ~ 0.001 molecule of apoA-V per VLDL (16). While apoA-V could still function in plasma at such low concentrations
relative to TG-rich lipoproteins, there have also been suggestions that it might function within the hepatocyte to directly modulate hepatic TG metabolism and secretion (17). Although no significant effects of apoA-V on TG production were observed in apoA-V transgenic mice, Schapp et al. (18) reported reduced hepatic TG production upon adenovirus-mediated expression of human apoA-V in mouse liver. The discovery of apoA-V as a gene that is upregulated during liver regeneration (19), with the potential to reside on cytosolic lipid droplets (20), supports the possibility that apoA-V responds to and perhaps modulates aspects of intracellular hepatic TG metabolism.

In the current report, the secretory trafficking of apoA-V was examined in both hepatic and non-hepatic cells under basal conditions and conditions of oleate-stimulated TG synthesis. These studies suggest that the low plasma concentrations of apoA-V may be due, in part, to its inherently inefficient exocytic trafficking and that TG accumulation within hepatoma cells is further antagonistic to apoA-V secretion. Interestingly these studies also revealed that in an inducible cell line, apoA-V affects the secretion of both apoB and TG.
MATERIALS AND METHODS

Cell culture

COS-1 and McA-RH7777 cell lines were grown in DMEM containing high glucose (4.5 g/l), 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). CHO-KI cells were maintained in F1/2 containing 10% FBS and antibiotics. Stable McA-RH7777 and CHO-KI cells were maintained in media containing 250 µg/ml G418. Inducible cells were maintained in media containing 25 µg/ml hygromycin and 250 µg/ml G418. All cells were kept at 37°C with 5% CO₂.

Transfection and selection of stable clones

Constitutive clones were created as follows: McA-RH7777 and CHO-KI cells in 100 mm dishes were transfected at 30% confluence with 16 µg of apoA-V and 2 µg of a neomycin resistance plasmid (18 µg total DNA) using the FuGENE 6 (Roche Molecular Biochemicals) method as described (21). Twenty-four hours post transfection, cells were subjected to selection with complete DMEM (10% FBS) supplemented with 750 µg/ml G418 (Cellgro). Selection media was replaced every 48 hours for 10 days. Individual clones were isolated, expanded, and maintained in 250 µg/ml G418. Additionally, McA-RH7777 cells transfected with only the neomycin resistance plasmid were generated to use as a control, the cells are referred to as McA-Tet.

Inducible clones were created as follows: McA-RH7777 cells in 100 mm dishes were co-transfected at 30% confluence with 10 µg of AVpTRE and 10 µg
of Tet-on plasmid (BD Biosciences). Twenty-four hours post transfection, cells were subjected to selection with complete DMEM (10% FBS) supplemented with 750 µg/ml G418 (Cellgro) and 50 µg/ml hygromycin. Selection media was replaced every 48 hours for 10 days. Individual clones were then isolated and expanded in 250 µg/ml G418 and 25 µg/ml hygromycin and were maintained at this concentration of G418 and hygromycin for the remainder of the experiments. To induce apoA-V expression, cells were incubated for 48 hours with 1 µg/ml of doxycycline (BD Biosciences). Individual clones were analyzed for inducible expression by immunoprecipitation and two high apoA-V expressers were then selected (Clone #4 and #47).

**Metabolic radiolabeling and immunoprecipitation**

Transfected cells were incubated in DMEM containing 10% FBS and 0.8 mM oleic acid complexed to 1.5% BSA for 2 hours. Cells were then incubated in Met/Cys deficient media for 20 minutes followed by a 4 hour radiolabel with [\(^{35}\)S] Met/Cys (100 µCi/ml; Perkin Elmer) in Met/Cys-deficient DMEM also supplemented with 10% FBS and 0.8 mM oleic acid and 1.5% BSA. After labeling, cells and media were harvested and adjusted to lysis conditions as described (22). Cells were immunoprecipitated with rabbit anti-human apoA-V or goat anti-human apoB (Academy Bioscience). The anti-human apoA-V antibody used in these experiments was a polyclonal antibody raised in rabbit, against the apoA-V protein lacking the last 30 C terminal amino acids.
For pulse chase experiments, cells were incubated with or without oleic acid supplementation for 2 hours. Cells were then pulse radiolabeled with $[^{35}\text{S}]$ Met/Cys for 10 min and then chased with media containing 2.5 mM Met and 1 mM Cys and 10% FBS with or without 0.8 mM oleic acid complexed to 1.5% BSA for the indicated times. ApoA-V from cell lysate and media was immunoprecipitated and fractionated by SDS-PAGE. Before gel loading, samples were boiled in SDS-PAGE sample buffer containing 100 mM DTT. Dried gels were exposed to BioMax MS (Eastman Kodak, Rochester, NY) film using a Biomax TransScreen-LE intensifying screen (Eastman Kodak) at – 70 °C. Band intensities were then quantified by exposing the gel to BAS-MS (FUJI FILM) imaging plate at room temperature and BAS-5000 phosphorimager (FUJI FILM) analysis.

**Immunofluorescence of intracellular apoA-V**

Stably transfected McA-RH7777 cells were plated on poly-L-Lysine coated coverslips and 24 hours later cells were fixed in 3% paraformaldehyde in PBS for 20 minutes and blocked for 1 hour with buffer containing 0.2 M glycine, 0.1 mg/ml saponin, and 30 mg/ml bovine serum albumin in PBS. Cells were then incubated for 1 hour in primary antibody buffer containing the appropriate antibody, 0.1 mg/ml saponin and 1 mg/ml BSA in PBS. The following dilutions were used: rabbit anti-apoAV, 1:300; mouse anti-ADRP (Fitzgerald), 1:50. Cells were then incubated with a secondary antibody for 1 hour at the following dilutions: rhodamine-conjugated goat anti-rabbit IgG, 1:200 (Jackson ImmunoResearch);
and FITC-conjugated goat anti-mouse IgG, 1:200 (JacksonImmunoResearch).
Cells were post-fixed with 10% paraformaldehyde, mounting solution (ProLong Gold antifade reagent, Invitrogen) added, and viewed using a Zeiss Axioplan 2 microscope with a x 63 oil objective.

**Quantification of secreted triglycerides**

Cells were plated into 100 mm dishes at 50% confluency and the experiment was performed the next day. Cells were labeled for 24 hours in 3 mls media containing 0.8 mM oleate complexed to 1.5% BSA in complete DMEM and 10 µCi/ml $[^3H]$oleic acid (Perkin Elmer Life Sciences). Following label, media samples were transferred to Centricon YM-30 for concentrating. Samples were concentrated to 500 µl at 4,000 x g at 4°C. Samples were then subjected to Bligh-Dyer extraction as described (23 - 24). Thin layer chromatography was performed and triglyceride bands collected and analyzed by liquid scintillation counting.

**Isolation of VLDL apoB**

Cells were oleate stimulated for 2 hours with 0.8 mM oleate and 1.5% BSA. Cells were then radiolabeled with $[^35S]$ Met/Cys for 4 hours. Media was transferred to a Beckman TLA 100.3 tube with 3.5 ml volume capacity and spun at 100,000 rpm for 18 hours at 15°C. Tubes were then sliced into a top 1 ml (d ≤ 1.006 g/ml) and bottom 2 ml (d ≥ 1.006 g/ml) fraction using a tube slicer (Beckman Instruments). Samples were then modified to lysis conditions and subjected to immunoprecipitation with anti-apoB antibody (5 µg), as described
Band intensities were quantified using a BAS-5000 Phosphorimager (FUJIFILM).

**Isolation of lipid droplets**

Cells were oleate stimulated for 24 hours before cells were scraped and pelleted in cold PBS, and re-suspended in a hypotonic lysis buffer as described by Brasaemle *et al.* (25). A Potter-Elvehjem tissue homogenizer was used to homogenize the cells. Supernatant and floating fat layer were then collected after a 10 min low speed spin at 1000 x g at 4 °C and adjusted to 20% sucrose. The sample (1 ml) was then loaded into the bottom of a 13.2 ml ultra-centrifuge tube for a SW41Ti swinging bucket rotor. Overlaying the sucrose-adjusted sample were 5 mls 5% sucrose and 6.5 ml hypotonic lysis buffer. Following a 30 minute spin at 28,000 x g at 4 °C (Beckman L8-70 ultracentrifuge), tubes were then fractionated from the top using an Autodensiflow Gradient Fractionator (Labconco Industries). Samples (1 ml) were TCA precipitated and subjected to Western blot analysis.

**Lipoprotein fractionation of apoA-V and apoB in McA-RH7777 cells**

McA-RH7777 cells were pre-incubated in 0.8 mM oleate and 1.5% BSA for 24 hours. Cells were then radiolabeled with [35S] Met/Cys as described previously. Media was collected and adjusted to a density of 1.10 g/ml with solid KBr and loaded onto the bottom of a Beckman SW40 centrifuge tube. The sample was then overlaid with the following KBr solutions in 1X PBS: 3 ml of
d=1.065 g/ml, 3 ml of d=1.02 g/ml, and 2 ml of d=1.006 g/ml. Samples were then ultracentrifuged at 35,000 rpm at 4 °C for 18 hours. Tubes were fractionated with an Autodensiflow Gradient Fractionator (Labconco Industries) into 1 ml samples. Densities were then determined gravimetrically and samples were subjected to immunoprecipitation and SDS-PAGE analysis. Each gradient is representative of media collected from one 150 mm dish of transfected cells.
Results

Stable transfection of McA-RH7777 and CHO-KI cells with human apoA-V

McA-RH7777 and CHO-KI cells were stably transfected with human apoA-V and a neomycin resistance plasmid. Individual clones were selected and grown to confluency. Transfected cells were then metabolically radiolabeled with 100 µCi/ml [35S] Met/Cys for 3 hours. Cell lysates were then immunoprecipitated with anti-apoA-V antibodies. We observed several clones with high apoA-V expression (Figure 1). McA-RH7777 clones numbered 5, 6, and 16 were selected and maintained as high expressers. McA-RH7777 clone number 14, a low apoA-V expresser was selected as a control. CHO-KI clone number 6 was selected as an apoA-V high expresser (data not shown).

Comparison of apoA-V secretion kinetics in hepatic and non-hepatic cells

Previous analyses revealed inefficient secretion of apoA-V from transiently transfected COS-1 cells undergoing continuous metabolic radiolabeling with [35S] Met for 3 h. (17). As endogenous apoA-V expression is predominantly limited to hepatocytes, we compared the secretory behavior of apoA-V in stably transfected rat hepatoma cells (McA-RH7777) and CHO-KI cells. As observed in Figure 2A, during a four-hour pulse, little newly synthesized apoA-V was observed in media fractions from either cell type, although the cellular content of apoA-V was abundant. To examine secretion parameters more quantitatively, pulse-chase analyses were performed. After a 10 min pulse and 120 min chase, only 20% of newly synthesized apoA-V was secreted from McA-RH7777 cells.
and 38% from CHO. In addition to limited recovery of apoA-V in media, little cell-associated apoA-V remained after the 120 min chase (5-10%). Hence, in addition to its limited secretion, ~60% of newly synthesized apoA-V appeared to undergo pre-secretory turnover. These studies indicate that apoA-V is inefficiently secreted in cells of both hepatic and non-hepatic origin and undergoes considerable intracellular turnover.

ApoA-V displayed a unique banding pattern in several cell types including COS-1, CHO-K1, and McA-RH7777 cells. This is not likely due to glycosylation as apoA-V contains no N-linked glycosylation sites. To determine if the observed banding pattern is an experimental artifact or if it was occurring intracellularly, stably transfected McA-RH7777 cells were labeled for 1, 2, 5, and 10 minutes (Figure 3). The ratio between the bands is fixed, independent of label time, with the two major bands appearing at each labeling time point. If the banding pattern developed in a time dependent fashion after synthesis then there would be a precursor - product relationship detected. This would be evident with one band appearing in the 1-2 min and the appearance of the double band between 5-10 min. The fixed ratio between the bands suggests that the clipping occurs during lysis and immunoprecipitation or that it may be occurring in vivo, co-translationally.

Avid binding to the cell surface after secretion could explain the limited recovery of apoA-V in media fractions, a possibility consistent with apoA-V’s potential to associate with heparin sulfate proteoglycans (9). To determine if a population of apoA-V associates with heparin releasable binding sites on McA-
RH7777 cells, transfected cells were radiolabeled with \[^{35}\text{S}]\text{Met/Cys}\) in the presence and absence of heparin. The distribution of cell associated and media apoA-V was then examined. As seen in Figure 4, the relative distribution of apoA-V present in cell and media fractions was not affected significantly by the presence of heparin at concentrations up to 10 Units/ml.

**Effect of oleate on apoA-V secretion efficiency and subcellular localization**

As apoA-V plays a role in TG metabolism, we explored whether apoA-V intracellular trafficking might be affected by alterations in cellular TG synthesis and accumulation. Stably transfected McA-RH7777 cells were incubated with and without 0.8 mM oleate prior to and during pulse-chase analyses. As observed in Figure 5A and B, inclusions of oleate caused a marked (~46%) reduction in the secretion of apoA-V from stably transfected McA-RH7777 cells and a corresponding (~36%) increase in cell-associated apoA-V (Panel C). The oleate-induced alteration in secretion occurred exclusively at the level of trafficking and subcellular localization, as no change was observed in the recovery of newly synthesized apoA-V in the absence or presence of oleate after a 120 min chase (Panel D).

Shu et al.\(^{(20)}\) demonstrated that an apoA-V-GFP fusion protein and native apoA-V can associate with cytosolic lipid droplets in oleate treated McA-RH7777 cells. Presumably, this localization arises from the retrotranslocation or other means of movement of apoA-V from the ER lumen into the cytosol, where association with lipid droplets can occur \(^{(26)}\). We therefore hypothesized that the reduced secretion of apoA-V observed upon oleate treatment may be caused by...
a corresponding increase in delivery of apoA-V to lipid droplets. To explore this possibility, stably transfected McA-RH7777 cells were incubated with oleate for 24 hours. The addition of oleate promoted increased synthesis and accumulation of neutral lipids as assessed by an increased intensity of Nile red staining (Figure 6A). Indirect immunofluorescence microscopy demonstrated that oleate treatment was accompanied by increased apoA-V localization on lipid droplet structures (Figure 6B), which were identified based on colocalization with the lipid droplet binding protein, ADRP. The oleate-induced re-localization of apoA-V onto lipid droplets was further confirmed biochemically by isolating lipid droplets via sucrose density gradient centrifugation as described (Figure 6C) (27). Relative to controls, oleate treatment resulted in a ~16-fold increase in TG contained in the lipid droplet fraction (fraction 1; 5.42 versus 88.18 µg TG/mg cell protein in control and oleate treated cells, respectively). When gradient fractions were subjected to SDS-PAGE and immunoblotting, the apoA-V content in fraction 1 was also increased ~15-fold in oleate versus control cells. These data indicate that the movement of apoA-V onto lipid droplets may directly compete with the exocytic trafficking of apoA-V.

Triglyceride turnover in apoA-V transfected McA-RH7777 cells

The overexpression of human APOA5 in mice has resulted in dramatically decreased plasma triglyceride levels. Fruchart-Najib et al. reported that TG turnover is faster in human APOA5 transgenic mice, and that this strongly correlated with increased LPL activity (8). These data indicated that apoA-V may
induce a decrease in VLDL size by activating lipolysis and increasing VLDL clearance (8).

To see if there are differences in TG turnover between apoA-V expressing cells and non-transfected McA-RH7777 cells, we used pulse chase analysis (Figure 7). Non-transfected and apoA-V stably transfected McA-RH7777 cells were incubated in oleate and BSA supplemented media, then radiolabeled with 20 µCi/ml [3H] oleic acid for 4 hours and chased for 0 -16 hours. For the chase, media was removed and cells then incubated with media containing just BSA (no oleate, no serum). Media was subjected to Bligh-Dyer extraction; while lipids in cells were extracted directly from the dish after BCA analysis. Extracted lipids from both cell and media were separated by thin layer chromatography and bands corresponding to triglyceride were cut out and quantified by liquid scintillation counting. These analyses revealed no alteration in [3H] TG in control versus apoA-V cells, indicating that TG turnover is unaffected by apoA-V in this system.

**Generation of apoA-V inducible McA-RH7777 cells**

ApoA-V inducible McA-RH7777 cells were generated as described in Materials and Methods section. Individual high apoA-V expressing clones were selected and maintained. We wanted to determine the maximum rate of synthesis with doxycycline incubation. To examine this, cells were split into 100 mm dishes in duplicate and were induced with 1 µg/ml doxycycline for 6, 12, 24, 48 h followed by metabolic radiolabeling for 4 h with [35S] Met/Cys (Figure 8). Cell
lysates were immunoprecipitation with anti-apoA-V antibodies. Twelve hour induction with 1 µg/ml doxycycline appeared to give the maximum rate of synthesis. Experiments were performed after 48 h induction with doxycycline or as otherwise indicated.

**Effect of apoAV expression on apoB and triglyceride secretion**

Based on its low concentration in plasma and relatively high abundance in cells, the possibility that apoA-V affects intracellular aspects of hepatic TG metabolism has been discussed (16-18). Hence, we explored whether the expression of apoA-V in stably transfected McA-RH7777 cells could affect apoB or TG secretion. In pulse-chase studies the apoA-V transfected McA-RH7777 cells utilized in Figures 1-7 displayed a ~20% reduction in apoB secretion relative to mock transfected controls (data not shown); however, due to possible phenotypic variability associated with clonally selected cell lines we sought a more controlled system to study the relationship between apoA-V and TG secretion. To this end a stably transfected McA-RH7777 cell line was generated enabling maximum inducible expression of apoA-V with 12 h doxycycline incubation as described (Figure 8). As seen in Figure 9A, one of the cell lines identified displayed doxycycline-dependent induction of apoA-V with undetectable basal expression. To examine the consequences of apoA-V expression on cellular TG, cells were induced for 24 h and TG content measured. Induction with doxycycline was associated with a significant ~20% increase in cellular TG (Figure 9B). Parallel effects on apoB secretion were observed during continuous 4 h radiolabeling of
cells with [\textsuperscript{35}S] Met with and without doxycycline. ApoA-V expression resulted in a 32 % reduction in media apoB (Figure 9C), an effect that appeared specific for apoA-V as doxycycline had no impact on the secretion of another endogenous proteins, transferrin (Figure 9D).

We explored further the effects of apoA-V expression on apoB and TG secretion by subjecting both McA-RH7777 cells and inducible apoA-V cells to treatment with and without doxycycline for 12 h, followed by 24 h label with [\textsuperscript{3}H] oleate. Doxycycline treatment of McA-Tet cells had no impact on TG secretion whereas the apoA-V expressing cells displayed a ~20% reduction in [\textsuperscript{3}H] TG secretion (Figure 10A). These experiments were done using control McA-Tet cells to demonstrate that this effect is specific for apoA-V expression.

In addition, the density distribution of apoB lipoproteins was examined to determine if apoA-V expression has an affect on lipidation of apoB containing lipoproteins in the inducible McA-RH7777 cells (Figure 10B). Media from these dishes was subjected to ultra-centrifugation and the top 1ml (d \leq 1.006 g/ml) and bottom 2ml (d \geq 1.006 g/ml) fractions were isolated and then subjected to immunoprecipitation with anti-human apoB antibody. In the presence of doxycycline, there is a ~ 57% decrease in the top: bottom ratio of apoB; indicating a decrease in apoB lipidation.

**Distribution of apoA-V in McA-RH7777 cells**

In human plasma, apoA-V is found on VLDL, HDL, and chylomicrons but not LDL (28). In mouse plasma, apoA-V was present on HDL but not VLDL or LDL.
To examine the distribution of apoA-V in rat hepatoma cells, stably transfected McA-RH7777 cells were oleate treated for 24 hours followed by a 4 hour radiolabel. The samples were then subjected to potassium bromide density gradient ultra-centrifugation. After ultracentrifugation, 1ml fractions were collected and immunoprecipitated for either apoA-V or apoB. A similar distribution was found between apoA-V and apoB, both being found in the VLDL fractions (Figure 11).
Discussion

The possibility that apoA-V can impact cellular aspects of hepatic TG secretion has been highly discussed. When apoA-V was overexpressed using adenovirus vector a significant reduction in VLDL-TG secretion was observed; however, this finding was not corroborated in transgenic mice overexpressing apoA-V in liver. Likewise, Shu *et al.* reported that apoAV overexpression failed to affect either apoB or TG secretion in stably transfected Hep3B cells (20).

Despite being synthesized with a 23 amino acid signal peptide, which directs it towards the secretory pathway, apoA-V is largely retained within the cellular compartment. ApoA-V is present in human plasma at concentrations from 24 to 406 µg/L compared to apoA-I plasma concentration which is approximately 1 g/L (28). The triglyceride lowering mechanism remains unclear and data exists supporting both intracellular and extracellular modes of action. However, the extremely low plasma concentration points toward an intracellular mechanism. In addition, studies performed in transfected cells have shown that there are minimal levels of apoA-V secreted into the media. Weinberg *et al.* demonstrated poor secretion efficiency with the transient transfection of human apoA-V into COS-1 cells (17). We were able to support this finding in stably transfected hepatic and non-hepatic cell types. Using pulse-chase methods we demonstrate that apoA-V is inefficiently secreted from stably transfected hepatic and non-hepatic cells.

We were interested to examine the post-transcriptional effects of increased triglyceride synthesis on apoA-V. The secretion of VLDL-TG ApoB
containing particles is dependent on oleate stimulation in McA-RH7777 cells. We observed that oleate treatment of stably transfected McA-RH7777 resulted in a significant decrease in the secretion efficiency of apoA-V. Additionally, there was an increase in the percentage of apoA-V that was cell associated compared to minus oleate. As the data indicates, it appears that oleate treatment results in the intracellular accumulation of apoA-V.

The assembly of VLDL occurs as a two step process, beginning with the translocation of apoB followed by the incorporation of bulk lipid shuttled from cytosolic lipid droplets. Availability of cytosolic lipid is a factor in the amount of VLDL secreted into the plasma (29). Studies have shown that 70% or more of hepatic VLDL is derived from cytosolic lipid droplets in a process involving lipolysis and re-esterification (30). The diacylglycerol derived from these lipid droplets are re-esterified into triglyceride to form the mature VLDL particle (31). Formation of these cytosolic lipid droplets has been proposed to occur by the budding off from the endoplasmic reticulum (32). Studies by Shu et al. have shown by immunofluorescence methods that apoA-V-GFP co-localizes with cytosolic lipid droplets in transfected McA-RH7777 cells (20). ApoA-V could be associating with the ER membrane and cytosolic lipid droplets to regulate the lipid trafficking toward VLDL production. When lipid droplet fractions are isolated from McA-RH7777 cells by sucrose density gradient we saw that oleate stimulation resulted in a shift of apoA-V into the lipid droplet fraction. This may support the hypothesis that apoA-V functions at the intracellular level to limit the
mobilization of lipid for VLDL assembly. However, we saw no significant effect on triglyceride turnover when apoA-V was overexpressed.

To study apoB and triglyceride secretion, we employed the use of a stably transfected inducible cell line. Sodium oleate stimulation was used to increase the secretion of apoB containing lipoprotein particles (33). In our system, we saw that over-expression of human apoA-V resulted in an increase of intracellular triglyceride. Our studies revealed that apoA-V over-expression resulted in a decrease in cell associated and secreted apoB suggesting that apoA-V causes a reduction in VLDL-TG secretion. This is different than data obtained from Shu et al. which showed that apoA-V expression has no effect on either the lipidation or secretion of apoB (20). To further support our hypothesis, apoA-V overexpression in our inducible cell line showed a significant decrease in secretion of \([^{3}H]\) oleic acid labeled triglyceride. Furthermore, apoA-V expression was shown to cause a significant decrease in the secretion of apoB-VLDL triglyceride rich particles.

ApoA-V is found to be associated with HDL, VLDL, and chylomicrons but not LDL in humans (28). APOA5 transgenic mice have the highest apoA-V levels on HDL but was shown to re-distribute to VLDL when incubated with apoa5/- VLDL (34). In our study, we saw that after secretion from McA-RH7777 cells apoA-V was found present in the triglyceride-rich apoB associated VLDL fractions.

ApoA-V has been shown to traffic inefficiently within the secretory pathway and is poorly secreted. While the mechanism whereby apoA-V reduces plasma
triglycerides remains to be further elucidated, this data provides evidence that apoA-V expression results in reduced secretion of VLDL-TG particles. One possible explanation for this is that apoA-V associates with cytosolic lipid droplets to limit the amount triglyceride available for the assembly of apoB-containing lipoprotein particles.
Figure 1. Generation of human apoA-V stably transfected McA-RH7777 cell lines. McA-RH7777 cells were transfected with 16 µg human apoA-V and 2 µg of a neomycin resistance plasmid. Clones were selected for with 750 µg/ml G418 and grown to confluency. Individual clones were then metabolically radiolabeled with 100 µCi/ml [35S] Met/Cys for 4 h. Cell lysates were harvested and immunoprecipitated with rabbit anti-human apoA-V. High apoA-V expressers (clone # 5, 6, and 16) and one low apoA-V expresser (clone # 14) were expanded and maintained in 250 µg/ml G418.
Figure 2. Kinetics of apoA-V secretion in stably transfected McA-RH7777 and CHO-KI cells. A) Human apoA-V stably transfected McA-RH777 and CHO-KI cells were radiolabeled with $[^{35}\text{S}]$ Met/Cys for 4h, cell lysate (C) and media (M) were collected and immunoprecipitated for human apoA-V. B) Stably transfected CHO-KI (left) and McA-RH7777 (right) cells were pulse radiolabeled with $[^{35}\text{S}]$ Met/Cys for 10 min without oleate stimulation and chased 0-120 min as indicated. After each chase time, the percentage of protein recovered in cells and media was quantified by immunoprecipitation, SDS-PAGE, and phosphorimager analysis. Each time point is representative of the percentage of remaining radiolabeled apoA-V in media and cells per dish. The lower panel represents the SDS-PAGE analysis of cell and media at 0 and 2 h.
Figure 3. Electrophoretic banding pattern of apoA-V in McA-RH7777 cells.

Stably transfected McA-RH7777 cells were radiolabeled with $^{35}$S Met/Cys for 1, 2, 5, and 10 minutes in duplicate 100 mm dishes, followed by immunoprecipitation of the cell lysate with anti-apoA-V antibodies. Samples were then analyzed by SDS-PAGE and autoradiography.
Figure 4. ApoA-V membrane association. A) ApoA-V stably transfected McA-RH7777 cells were plated into 100 mm dishes in duplicate. Twenty-four hours later cells were radiolabeled with 50 µCi/ml $[^{35}\text{S}]$ Met/Cys; after 4 h, various concentrations of heparin (0-10 units/ml) were added for a total of 4 h followed by immunoprecipitation of cell lysate (C) and media (M) with anti-apoA-V antibody. B) Media: Total ratio was calculated; quantitation of data by phosphorimager analysis. No statistically significant differences were observed (ANOVA, $p=0.6546$).
Figure 5. Effect of oleate on apoA-V secretion kinetics, intracellular accumulation, and degradation. A) Stably transfected McA-RH7777 cells were radiolabeled for 10 min followed by a two hour chase and percent secretion calculated over time. B) Percent secretion of apoA-V with the addition of oleate. C) Percent cell associated. D) Percent degradation. Phosphorimager analysis was performed to quantitate SDS-PAGE.
Figure 6. Oleate treatment increases the amount of apolipoprotein A-V in the lipid droplet fraction. A) Cells were incubated minus or plus oleate for 24 hours followed by lipid droplet staining with Nile Red. B) McA-RH7777 cells were pre-incubated with or without 0.8 mM oleate for 16 h prior to fixation with 3.7% formaldehyde and immuno-stained as described under Materials and Method Section. All images were visualized under a x 63 objective using a confocal microscope. (a) apoA-V minus oleate; (b) ADRP minus oleate; (c) merged figure; (d) apoA-V plus oleate; (e) ADRP plus oleate; (f) merged figure. C) McA-RH7777 cells were incubated minus or plus oleate for 24 hours. Lipid droplets were isolated by sucrose density gradient ultra-centrifugation. Fractions were collected, subjected to SDS-PAGE, and probed with human apoA-V antibodies for Western blot analysis. Each gradient is representative of one 150 mm dish.
Figure 7. Triglyceride turnover in non-transfected and apoA-V transfected cells. Control and apoA-V expressing McA-RH7777 cells were split into 60 mm dishes and labeled with 20 µCi/ml [³H]-oleic acid in oleate and BSA supplemented media for a 4 hour pulse, followed by chase times of 0, 8, 16 hours. Media was subjected to Bligh-Dyer extraction lipids were extracted from cells on the dish. Extracted lipids from both cell and media were separated by TLC and bands corresponding to TG were cut out and quantified by liquid scintillation counting (ANOVA; p=.0773 ns). White bars, non-transfected McA-RH7777 cells; black bars, apoA-V transfected McA-RH7777 cells.
Figure 8. Synthesis of apoA-V in stably transfected inducible McA-RH7777 cells. ApoA-V inducible McA-RH7777 cells were generated as described in Materials and Methods Section. Individual high apoA-V expressing clones were selected and maintained in 250 µg/ml G418 and 25 µg/ml hygromycin. Cells were split into 100 mm dishes in duplicate and were induced with 1 µg/ml doxycycline for 6, 12, 24, 48 h followed by metabolic radiolabeling for 4 h with [$^{35}$S] Met/Cys. Cell lysates were immunoprecipitated with anti-apoA-V antibodies and analyzed by SDS-PAGE.
Figure 9. Impact of apoA-V expression on apoB in stably transfected inducible McA-RH7777 cells. A) Inducible cells were generated by co-transfection with AVpTRE and Tet-On plasmids. ApoA-V expression was induced by 48 h incubation with 1 μg/ml doxycycline. B) Measurement of intracellular triglycerides per mg cell protein was determined by enzymatic assay. C) Inducible cells were radiolabeled 4 h with or without 1 μg/ml doxycycline and immunoprecipitated for apoB-100 or D) for transferrin. Phosphorimager analysis was performed to quantitate band intensities observed in C and D. Statistically significant differences were observed in cellular apoB-100 (T test; p= .0408; n=3) and media: total apoB-100 (T test; p=.0029; n=3) with the expression of apoA-V.
Figure 10. Impact of apoA-V expression on triglyceride and triglyceride-rich lipoprotein particles in stably transfected inducible McA-RH7777 cells.

A) Cells labeled for 24 h with 10 µCi/ml $[^3]H$ oleic acid in media supplemented with 0.8 mM cold oleic acid and 1.5% BSA. Secreted $[^3]H$-oleic acid triglyceride in McA-Tet media (left panel) and apoA-V inducible media (right panel) was concentrated to 500 µl. Lipids were extracted by the Bligh-Dyer method and triglycerides collected by thin layer chromatography. B) Inducible cells were radiolabeled for 4 h, media was then harvested and spun at 100,000 rpm for 18 hrs at 15 °C. Top 1 ml ($d \leq 1.006$ g/ml) and bottom 2 ml ($d \geq 1.006$ g/ml) fractions were collected and subjected to immunoprecipitation for apoB-100 (upper panel) and results were quantitated by phosphorimager analysis (bottom panel). Statistically significant differences were observed in secreted $[^3]H$ labeled triglyceride (T test; $p=.0361$; n=3) and $[^35]S$ labeled apoB (T test; $p=.004$; n=3) with the induction of apoA-V expression.
Figure 11. Distribution of apoA-V and apoB-100 in stably transfected Mc-RH7777 cells. Cells were pre-incubated for 24 h in 0.8 mM oleate and 1.5% BSA followed by a 4 h radiolabel with 100 µCi/ml \(^{35}\)S Met/Cys also containing oleate. Media was harvested and adjusted to 1.10 g/ml with KBr and loaded into a Beckman SW40 centrifuge tube for the swinging bucket rotor. Sample was overlaid with the following KBr solutions in 1x PBS: 3 ml d=1.065 g/ml, 3 ml d=1.02, 2 ml d=1.006 g/ml. After ultracentrifugation at 35,000 rpm at 4 °C for 18 h, 1 ml fractions were collected and immunoprecipitated for apoA-V or apoB and analyzed by SDS-PAGE and autoradiography.
Reference List


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Chapter Three

Liver Specific Over-expression of Human Apolipoprotein A-V in Mice
Introduction

Apolipoprotein A-V (apoA-V) plays an important role in triglyceride metabolism. Recent studies have proposed that apoA-V mediates this function by modulating the assembly or secretion of VLDL from the liver. VLDL production by the liver has been shown to influence plasma lipid levels. Furthermore, plasma lipid levels influence the development and progression of cardiovascular disease (1;1). It has also been suggested that apoA-V functions to enhance lipoprotein lipase activity and increase the removal of VLDL remnants. Pennacchio et al. generated both human apoA-V over-expressing and knockout mice (2). They observed a 70% decrease in plasma triglyceride in the transgenic animals when compared to control mice; an increase in plasma triglyceride was seen in knockout mice. Genetic associations between APOA5 single nucleotide polymorphisms and plasma triglyceride levels in humans, provide further evidence for the link between apoA-V and triglycerides (3-10).

While the mechanism of apoA-V remains unknown, studies by Schaap et al. have shown that this apolipoprotein may play a role in reducing the production of VLDL-triglyceride (11). Triglyceride production was examined in these mice after injection of Triton WR1339 to block lipolysis (12). These investigators found a significant reduction in VLDL-TG production and proposed that the effect was specific to apoA-V overexpression, not a result of adenoviral overexpression. However, other studies have shown no effect of apoA-V on VLDL-triglyceride secretion rates between hAPOA5 and wild type mice (13;14).
For our studies liver specific human apoA-V transgenic mice were generated and the effects of apoA-V overexpression on the production of triglyceride were examined by a liver perfusion method (15). Additionally, primary hepatocytes isolated from transgenic mice were utilized to examine at the effect of apoA-V on apoB and triglyceride production. These data further support the hypothesis that apoA-V reduces plasma triglycerides by attenuating VLDL production.
Materials and Methods:

Transfections, metabolic radiolabeling, and immunoprecipitation

COS-1 cells were transiently transfected at 50-60% confluency by the FuGENE 6 method (Roche Molecular Biochemicals) using a 2:1 (volume to mass; µl:µg) ratio of FuGENE 6 to DNA. Cells were incubated 24 hours post transfection in Met/Cys deficient media for 20 minutes followed by a 4 hour radiolabel with [$^{35}$S] Met/Cys (100 µCi/ml; Perkin Elmer) in Met/Cys- deficient DMEM. After labeling, cells and media were harvested and adjusted to lysis condition as described (16). Protein from cell lysate and media were immunoprecipitated with the following antibodies: mouse anti-FLAG M2 monoclonal antibody used at 10 µg/ml; anti-mouse apoA-V antibody raised in rabbit to entire mouse apoA-V sequence (animal #05410); human apoA-V antibody raised in rabbit against mature apoA-V lacking the C terminal 30 amino acids (animal #2466). For Western blot analysis, the following antibodies were used: anti-rabbit IgG (1:6000 dilution); anti-mouse IgG (1:4000 dilution); anti-goat IgG (1:4000 dilution)(Sigma).

Production of human apoAV transgenic mice

With the assistance of the Dr. Liqing Yu and the Transgenic Core Facility (Wake Forest University), we developed liver-specific human apoA-V transgenic mice. Our construct was part of a liver specific vector which has a 5' human apoE promoter region and a 3' hepatic control region, including a poly-linker cloning region. The enzymes MluI and Clal were used to clone human apoA-V into this
vector giving rise to an 11 Kb construct. After removal of plasmid backbone sequences, the apoA-V construct was microinjected into pronuclei of fertilized mouse embryos. Three founders were generated and one transgenic line was characterized for the following experiments.

**Characterization and breeding of human apoA-V transgenic mice**

The initial genotyping of mice was performed by PCR analysis of tail DNA and presumptive genotypes were assigned based on inheritance of the human apoA-V transgene. For PCR genotyping, two sets of primers (Oligo # 601, 602, 603, and 604) were implemented for checking transgene integrity: a hepatic control region primer set which produces a PCR product of 271 base pairs and a human apoE promoter region primer which produces a PCR product of 423 base pairs. To verify the initial genotyping assignment Western blot analysis was performed on plasma samples of mice. Mice were analyzed for protein expression by Western blot of plasma samples obtained by tail bleed. Samples (1 µl) were fractionated by 12.5% SDS-PAGE, and the proteins were then transferred to a PVDF membrane. Anti-human apoA-V was the primary antibody used at a 1:500 dilution; the HRP-conjugated secondary antibody, goat anti-rabbit IgG, was used at a 1:6000 dilution. After screening for the expression of apoA-V, positive founder pups were bred with B62DF1 wild type mice. Offspring were also screened for transmission of the transgene by PCR and protein expression analyzed by Western blotting. Male mice with high levels of apoA-V
expression were backcrossed to B62DF1 mice to generate a working stock of animals.

**Membrane preparation of liver tissue**

Frozen liver samples (0.2 g) were added to membrane buffer (1.2 ml) containing 20 mM Tris-HCl at pH7.5, 2 mM MgCl₂, and 0.25 M sucrose, adjusted to 1 mM PMSF, 10 µg/ml pepstatin A, and 10 µg/ml leupeptin. Samples were then homogenized for ~1 min (Polytron) and placed on ice followed by centrifugation at 3,500 rpm in a table top micro centrifuge for 10 minutes at 4 °C. The supernatant was removed and spun in a TL100 ultracentrifuge using the TLA45 rotor at 45,000 rpm for 1 hour at 4°C. Pellets were resuspended in 50 mM Tris-HCl, 1% SDS, and 1 mM EDTA. Protein concentration was determined by BCA assay and samples were subjected to Western blot analysis.

**Liver perfusion of pLIV-hA5 mice**

To examine the effect of apoA-V on hepatic apoB and triglyceride accumulation rates *in vivo*, liver perfusion studies were performed on the human apoA-V transgenic mice (15). These liver perfusion studies were conducted on male mice that were maintained on a regular chow diet and not fasted prior to the procedure. One day prior to the liver perfusion, approximately 1 ml per mouse of red cells was collected by cardiac puncture. The blood was then spun at 1,100 x g for 20 min at 4 °C. Red blood cells were washed with 0.9% NaCl and 0.01% D-glucose to remove white blood cells and then with perfusion media to remove saline and D-glucose. A 10% hematocrit of red cells was obtained by the addition of
a perfusate medium consisting of Krebs-Ringer bicarbonate buffer containing amino acids, penicillin and streptomycin (p/s), insulin and hydrocortisone as described (17). In preparation for the surgery, the mice were injected with 2.5 mg ketamine and 0.5 mg xylazine. The bile duct was cannulated to monitor the health of the liver. The portal vein was then exposed and cannulated with a 22 gauge BD Insyte Autoguard shielded I.V. catheter (Becton Dickinson). Next, the liver was flushed with oxygenated perfusate medium at 1 ml/min using a peristaltic roller pump. The vena cava was then cannulated. To begin the perfusion there was a 15 min flushing period. Afterwards, a volume of 10 ml of fresh medium containing red cells was perfused through the liver at a perfusion rate of 1 ml/min for a total of 180 minutes. This perfusate medium was oxygenated with 95% Oxygen, 5% CO₂ through a silastic tubing lung. During the perfusion, the color of the perfusate changes from red to blue as it passes through the liver indicating that the liver is healthy and utilizing the oxygen supply. Every 30 min 1.5 ml of perfusate was collected. At the final time point, the remainder of the 10 ml was collected.

Following the liver perfusion, the liver was collected, weighed, and frozen in liquid nitrogen to be analyzed for apoA-V expression by Western blot and for hepatic triglyceride concentration by enzymatic assay. From these time points total cholesterol (TC), free cholesterol (FC), triglyceride (TG), and phospholipid (PL) accumulation rates were determined by quantitating the amount of lipid in each time point after Bligh-Dyer lipid extraction (18). In addition, bile accumulation rates were determined. Total perfusion volume, hematocrit, leakage during perfusion, and liver weight were recorded and taken into account when analyzing the data.
Accumulation rates were calculated as µg/min/g liver weight. Our liver perfusions were performed with six wild type and six overexpressing pLIV-hA5 mice.

**Plasma lipid measurements**

Plasma samples were obtained by tail bleeding. TC, TG, and PL concentrations were analyzed by enzymatic assays (19), phospholipids (Wako Lipids), total cholesterol and triglycerides (Roche Diagnostics).

**Isolation of primary hepatocytes**

To isolate primary hepatocytes, tubing was filled with a pre-warmed, perfusion buffer (1x HBSS without Ca or Mg/10 mM Hepes, pH 7.4) supplemented with 0.5 mM EDTA and the portal vein was then exposed and cannulated with a 21-24 gauge needle and pumped at a flow rate of 3-4 ml/min. After the mouse was anesthetized, the inferior vena cava was then cut to drain the blood. After 7-10 minutes of perfusion the pump was stopped and switched to the digestion buffer (1x HBSS without Ca or Mg/10 mM Hepes, pH 7.4) that was supplemented with 5 mM CaCl$_2$, and 0.3 mg/ml collagenase. Mice were perfused with digestion buffer for 6-8 minutes until the liver visually degraded. The liver was then excised, with the gall bladder removed, and snipped with scissors in the digestion buffer. The tissue was further dissociated by pipetting up and down in William’s E wash medium that was supplemented with 10% FBS, L-glutamine (L-glu), penicillin (100 units/ml), streptomycin (100 µg/ml) (p/s), and 0.1 nM insulin. The digest was filtered through a 100 µm cell strainer. Hepatocytes were then
pelleted (in William’s E medium) and the viability of primary hepatocytes determined by trypan blue. Cells were plated on collagen-coated plates and incubated at 37°C in DMEM containing low glucose (1g/l), 10% FBS, and p/s.

**Pulse-chase analysis of primary hepatocytes**

The pulse chase protocol for primary hepatocytes was adapted from experiments performed by Chen et al. (20). After one hour of attachment, the cell monolayer was washed and incubated for an additional 3 hours. Cells were then washed three times with PBS and chased in Met- and Cys-free DMEM for 30 minutes to deplete the cellular pool of Met and Cys. Medium was replaced with 1 ml of Met and Cys-free DMEM containing 200 µCi of [35S] Met/Cys. Cells were pulsed for 45 minutes and incubated in 1 ml of DMEM containing 10 mM methionine and 3 mM cysteine for the specified time period. After each chase time point, the media was removed and microfuged at 1,000 rpm to remove cell debris and 200 µl 5x lysis buffer was added and then adjusted to 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 2.5 mg/ml BSA, and appropriate antibody (mouse albumin, mouse apoA-V or human apoA-V). After washing, 1 ml of 1x lysis buffer containing protease inhibitors was added to the cells. After ~5-10 minutes, cells were scraped and centrifuged at 15,000 rpm for 10 minutes. Supernatant was then collected and adjusted to 2.5 mg/ml BSA and appropriate antibody added. Samples were incubated with antibody overnight at 4 °C and then centrifuged at 15,000 rpm. Next, 25 µl protein G-Sepharose was added for 1.5 h with inversion. The protein G-Sepharose beads were then removed by
centrifugation and supernatants were transferred to fresh tubes for
immunoprecipitation.

**Analysis of radiolabeled triglyceride in primary hepatocytes**

Cells were labeled for 24 hours in 3 mls media containing 0.8 mM oleate
and 1.5% BSA in DMEM and 10 µCi/ml [³H] oleic acid. Following labeling, media
samples were transferred to Centricon YM-30 and spun at 4,000 x g at 4 °C.
Samples were exchanged into PBS and concentrated to a final volume of 500 µl.
Samples were then subjected to Bligh-Dyer extraction as described (21). Thin
layer chromatography was performed and triglyceride bands collected and
subjected to liquid scintillation counting.
Results

Reactivity of human and mouse apoA-V antibodies

Amino acid sequence analyses show a high level of identity between human and mouse apoA-V; they display 71% amino acid identity and 78% similarity. The anti-human apoA-V antibody used was a polyclonal antibody, raised in rabbit against apoA-V protein lacking the last 30 C terminal amino acids. Based on the high level of sequence identity, one would assume that human apoA-V would have shared epitopes with mouse apoA-V and that our anti-human apoA-V antibody would recognize mouse apoA-V. To address this question we FLAG tagged mouse apoA-V with a C-terminal flag. COS-1 cells in 100 mm dishes were transiently transfected with 15 µg of AP, human apoA-V, and mouse apoA-V-FLAG (Figure 1). Next, 20% of cell lysates were TCA precipitated and proteins were separated by electrophoresis and transferred to a PVDF membrane. The primary antibody for the first blot was anti-human apoA-V antibody and followed by the secondary antibody, anti-rabbit IgG. The anti-human apoA-V antibody reacted strongly with human apoA-V while it displayed low reactivity with mouse apoA-V. The primary antibody for the top blot was mouse anti-FLAG M2 monoclonal antibody; confirming abundant expression of apoA-V. These results demonstrate that the anti-human apoA-V antibody used has low reactivity to mouse apoA-V.

To characterization our mouse apoA-V antibody, COS-1 cells were transiently transfected with alkaline phosphatase (AP), human A-V FLAG, or
mouse A-V FLAG (Figure 2). Cells were then radiolabeled for 3 h with $^{35}$S Met/Cys and subjected to immunoprecipitation with anti-FLAG M2 and mouse apoA-V antibodies and analyzed by SDS-PAGE and autoradiography. As indicated by these transfections, the mouse apoA-V antibody used shows strong reactivity with mouse apoA-V but does not appear to react with human apoA-V.

**Liver specific expression of human apoA-V**

A liver specific vector was used to create the pLIV-hA5 construct that was subsequently microinjected into mice for the over-expression of human apoA-V (Figure 4A). PCR for initial genotyping of pLIV-hA5 mice tail snips was done with primers for a hepatic control region which recognize a PCR product of 271 base pairs and the primers for the apoE region recognizes a PCR product of 423 base pairs. Numbers 272, 276, 277, and 278 represent mice positive for transgene incorporation (Figure 3). Overall, mice demonstrated approximately 50% inheritance of the human apoA-V transgene.

After initial genotypic analysis by PCR, plasma samples obtained from these mice were examined by Western blot analysis to determine relative protein expression. When human apoA-V was overexpressed in the liver it appears in the plasma. We also analyzed a variety of tissues including liver, heart, kidney, spleen, lungs, stomach, and intestines in both wild type and transgenic mice (Figure 4B). From these blots, it is clear that human apoA-V expression is liver specific and furthermore that our human apoA-V antibody does not recognize endogenous mouse apoA-V. To further characterize these wild type and
transgenic mice, plasma lipids were assessed by enzymatic assay (Figure 4C). Plasma was collected from a total of 10 wild type and 10 transgenic mice. There was a significant reduction of plasma triglycerides seen in the apoA-V transgenic mice compared to that of the wild type mice, 22.01 ± 2.54 mg/dL and 51.02 ± 4.04 mg/dL respectively. These results indicate that human apoA-V is being over-expressed specifically in the liver and that the transgenic mice demonstrated the predicted phenotype of reduced plasma triglycerides.

Characterization of apoA-V expression in transgenic and wild type mice

Liver membrane preps and plasma samples from both control and transgenic mice were subjected to Western blot analysis (Figure 5A). First, the blot was probed with a mouse apoA-V antibody to examine endogenous mouse apoA-V. After the blot was stripped, it was then re-probed with human apoA-V antibody. The results show that the mouse antibody does not recognize rat apoA-V or that McA-RH7777 cells do not express apoA-V. When the mouse antibody was used we saw a strong band, representative of endogenous mouse A-V present in liver extract with no apoA-V visible in the plasma samples. As expected, there was no difference in endogenous expression between wild type and transgenic mice. We then compared the blot probed with human apoA-V antibody. ApoA-V over-expression is evident in the inducible human apoA-V expressing McA-RH7777 cell lysate that was used as a control. As anticipated, human apoA-V was observed in transgenic tissue samples. When human apoA-V was over-expressed in these mice there was also evidence of apoA-V present.
in the plasma. In Figure 5B, we probed a blot with tissue samples from both wild type and transgenic mice with mouse apoA-V antibody. Interestingly, we see endogenous mouse apoA-V in liver, heart, and kidney. Also, as observed in both figures, apoA-V demonstrates a unique banding pattern similar to that seen in transfected cells.

**ApoA-V and apoB secretion kinetics in primary hepatocytes**

Primary hepatocytes were isolated from transgenic mouse liver to examine apoA-V and apoB secretion (Figure 6). After a one hour pulse with $^{35}$S methionine/cysteine followed by a two hour chase the percent secretion of apoB was $33.23\% \pm 6.50 \, (n=3)$ and $7.5\% \pm 2.87 \, (n=3)$ for apoA-V. Overall, these data suggest that the endogenous mouse apoA-V secretion efficiency in primary hepatocytes is similar to that seen in transfected cells. The results were also similar when we looked at the secretion kinetics.

**Triglyceride secretion in primary hepatocytes**

Primary hepatocytes were isolated from both wild type and transgenic mouse livers. Cells were labeled for 24 h in 0.8 mM oleate and 1.5% BSA with $^3$H oleic acid. Media was then concentrated and subjected to Bligh-Dyer extraction and TLC. When radiolabeled triglyceride secretion in the primary hepatocytes was measured observed $990.7 \pm 125.1 \, \text{DPM x}10^3/\, \text{mg cell protein} \, (n=3)$ of $^3$H triglycerides in wild type mice and $650.7 \pm 55.00 \, (n=3) \, \text{DPM x}10^3/$
mg cell protein in transgenic mice (P=.068) (Figure 7). This result represents a trend towards decreased triglyceride secretion when apoA-V is over-expressed.

**Accumulation rates of lipid during 3 h of liver perfusion**

To determine the effect of human apoA-V over-expression on the production of liver triglycerides, liver perfusion studies were carried out on wild type and liver specific human apoA-V transgenic mice (Figure 8). Our results were calculated as the accumulation rates (µg/min/g liver) of phospholipid, total cholesterol, or triglycerides during a 3 hour liver perfusion. The data show that there were no significant changes in phospholipid or total cholesterol levels. There was a trend towards a reduction in triglyceride accumulation rate in the apoA-V transgenic mice. Wild type mice had a triglyceride accumulation rate of 4.67 ±2.29 µg/min/g liver (n=6) versus 1.30 ±.41 µg/min/g liver (n=6) in transgenic mice.
Discussion

Human apoA-V transgenic mice were generated using a liver specific vector. In these mice we have shown that human apoA-V over-expression reduces plasma triglycerides levels while having no significant effect on total cholesterol or phospholipid levels. Van der vliet et al. saw a 40% reduction in cholesterol with adenoviral overexpression of apoA-V (22) while Pennacchio et al. saw normal cholesterol in apoA-V transgenic mice (2). Both studies observed reduced triglycerides when apoA-V was overexpressed.

Primary hepatocytes were isolated from wild type and transgenic mice in order to examine the effect apoA-V over-expression had on apoB and triglyceride production. Unfortunately, the transgenic hepatocytes lost much of the expression of human apoA-V seen in liver membrane preps. Pulse-chase experiments were difficult to execute due to this loss of over-expression. However, we were able to analyze the secretion kinetics of apoB and endogenous mouse apoA-V. Secretion efficiency of apoA-V in primary hepatocytes was similar to what was seen in transfected McA-RH7777 cells.

Schaap et al. looked at the effect of low dose of adenoviral expressing apoA-V in mice (23). They observed that even low expression of apoA-V had a significant effect on plasma triglycerides. Similar to data in transfected cells, there was a trend toward decreased triglyceride secretion in the primary hepatocytes of human transgenic apoA-V mice.
In our liver perfusion studies, we have shown a trend toward decreased triglyceride accumulation in the human apoA-V transgenic mice. Over the course of a three hour liver perfusion, triglyceride accumulation was linear over time. In these studies, the mice were on a chow diet; however, it is probable that a high fat diet would make the effect of apoA-V over-expression more visible. Future studies involving liver perfusion should perhaps be performed with the mice on a high fat diet. As mentioned previously, Schaap et al. also looked at the effect of apoA-V on hepatic VLDL production (23). In their studies they used adenoviral expression of apoA-V. It may be argued that adenoviral over-expression interferes with the results. Many of the studies that examined the effect of apoA-V on VLDL-TG production are triton-based methods. We therefore used a non-triton based method to examine liver triglyceride production. We did see a trend toward a decrease in triglyceride accumulation in our human apoA-V transgenic mice.

While the mechanism of apoA-V remains unclear these results help to support the hypothesis that apoA-V functions to reduce hepatic VLDL-TG secretion.
**Figure 1: Reactivity of human anti-apoA-V antibody.** COS-1 cells were transiently transfected with 15 µg of AP, human apoA-V, and mouse apoA-V-FLAG in 100 mm dishes. 20% of lysate was TCA precipitated and proteins were separated by electrophoresis and transferred to a PVDF membrane for Western blot analysis. The primary antibody for the upper blot was mouse anti-FLAG M2 monoclonal antibody (Sigma). The primary antibody for the lower blot was anti-human apoA-V antibody (animal #2466) followed by the secondary antibody, anti-rabbit IgG at a 1:6000 dilution.
Human AV
Mouse AV FLAG

M2 Ab.

Human AV  Ab.

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Figure 2. Characterization of mouse apoA-V antibody. COS-1 cells were transiently transfected with 15 µg alkaline phosphatase (AP), human A-V FLAG, or mouse A-V FLAG. Cells were radiolabeled 3 h with $[^{35}\text{S}]$ Met/Cys and subjected to immunoprecipitation with 10 µg/ml M2 or 5 µl mouse apoA-V antibodies (animal #05410) and analyzed by SDS-PAGE and autoradiography.
M2 Ab.

Mouse AV Ab.
(#05410)
Figure 3. Genotypic analysis of pLIV-hA5 mice tail DNA. Primers for a hepatic control region recognized a PCR product of 271 base pairs and the primers for the apoE region recognizes a PCR product of 423 base pairs (Oligo # 601, 602, 603, and 604). Numbers 272, 276, 277, and 278 represent mice positive for transgene incorporation.
Figure 4. Liver specific expression of apoA-V. A) Liver specific vector used to create the pLIV-hA5 construct and subsequently microinjected into mice for the over-expression of human apoA-V. B) Various tissues (0.2g) were isolated from wild type and transgenic mice and subjected to Western blot analysis. C) Plasma triglycerides (mg/dL) of wild type (n=10) and transgenic mice (n=10) analyzed by enzymatic assay. Results are displayed as mean ± SEM; p < .0001.
Figure 5. Characterization of apoA-V expression in wild type and transgenic mice. Tissue extracts and plasma samples were obtained from wild type and transgenic mice. ApoA-V inducible McA-RH7777 cell lysate was used as a control. Samples were prepared in SDS-lysis buffer and subjected to Western blot analysis. A) Liver tissue and plasma samples were probed with mouse apoA-V antibody (top gel) at a 1:500 dilution followed by anti-rabbit IgG secondary antibody (1:6000 dilution). The blot was then stripped and re-probed with human apoA-V antibody (bottom gel) at a 1:500 dilution, followed by anti-rabbit secondary antibody. B) Tissue sample blots from both wild type and transgenic mice were probed with 1:500 dilution of mouse apoA-V antibody to examine endogenous apoA-V.
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Mouse A-V

Human A-V
Figure 6. Secretion kinetics of mouse apoB and mouse apoA-V in primary hepatocytes. Primary hepatocytes were isolated from transgenic mice as described in Materials and Methods Section. A) Cells were pulsed for 45 minutes with $^{35}$S Met/Cys and chased for 2 h. The percentage of each protein secreted during the 2 h chase was expressed as a mean percentage (±SD, n=3). B) Cells were pulsed for 1 hour with $^{35}$S Met/Cys and chased for 0 - 120 minutes as indicated. For both A and B, cells and media were collected and subjected to
immunoprecipitation with anti-mouse apoA-V or anti-apoB antibodies followed by SDS-PAGE and phosphorimager analysis.
Figure 7. Triglyceride secretion in primary hepatocytes. Primary hepatocytes were isolated from wild type and transgenic mice as described in Methods. Cells were radiolabeled for 24 h with 10 µCi/ml [3H] oleic acid in the presence of 0.8 mM oleic acid. Media was collected and triglycerides extracted by the Bligh-Dyer method and analyzed by TLC. Experiment was done in triplicate, results are displayed as mean ± SEM (p=.0676 ns).
Figure 8. Accumulation rates of lipid during a three hour liver perfusion of apoA-V transgenic mice on chow diet. Wild type and transgenic mice were subjected to liver perfusion as described in Materials and Methods. Perfusate samples were drawn at 0, 30, 60, 90, and 120 minutes and lipid (phospholipid, total cholesterol, and triglyceride) accumulation was measured by enzymatic assay and expressed as µg/min/g liver weight. Results were calculated and displayed as a mean ± SEM (n=6).
Reference List


Chapter Four

Summary and Conclusions

Many studies in mice and humans have clearly demonstrated that apoA-V plays a role in triglyceride metabolism; however, it is less apparent how apoA-V performs this function. Current studies on apoA-V have revealed several possible mechanisms for the role of apoA-V. Schaap et al. proposed a dual effect of apoA-V in the regulation of VLDL-TG (1). In their studies, apoA-V inhibited the
production rate of VLDL-TG and also increased the hydrolysis of TG through the activation of lipoprotein lipases. Other studies have shown that apoA-V expression does not affect VLDL production and that its primary function is extracellular in nature. Merkel et al. suggested that apoA-V may influence TG metabolism by guiding VLDL-TG to proteoglycan-bound LPL (2). Another proposed mechanism involves apoA-V mediated clearance of lipoprotein remnants by the liver.

The plasma concentration of apoA-V is less than 1% of apoA-IV and 0.1% of apoA-I. Based on a minimal plasma concentration and structural studies by Weinberg et al. (3), it was proposed that apoA-V functions at the intracellular level to modulate hepatic lipoprotein biogenesis and secretion. We wanted to examine the basis for the low levels of apoA-V in the plasma and to determine if it was due to low expression, an increase in turnover, or a reflection of the secretion efficiency. The secretion of apoA-V was initially examined in transfected COS-1 cells (3). Our lab then generated stably transfected McA-RH7777 and CHO-KI cells to examine the secretion efficiency of apoA-V. As a comparison, in our study of apoA-V trafficking behavior, we utilized apoA-V stably transfected CHO-KI cells, a cell type that does not form lipoproteins. CHO-KI cells do not express endogenous apoB-100 or MTP, two requirements for the formation of lipoproteins (4). The comparison of lipoprotein-forming and non-lipoprotein forming cell types allowed us to determine if the behavior of apoA-V is related to the formation of lipoproteins.
By studying the trafficking pattern of apoA-V with a variety of methods, we hoped to gain insight into the possible intracellular role that apoA-V plays in triglyceride metabolism. The secretion kinetics and efficiency of apoA-V were assessed by pulse chase methods; quantitative measurements of the percent secretion and degradation of apoA-V were then calculated. When we compared apoA-V between hepatic and non-hepatic cell types, we observed ~10-20% of newly synthesized apoA-V secreted from McA-RH7777 cells and similar secretion efficiency and kinetics displayed in the non-hepatic cell type. Because they are similar, it is possible that the trafficking behavior of apoA-V is not responsive to the formation of lipoproteins but some intrinsic property of apoA-V as the secretion efficiency of apoA-V is low in non-hepatic cells including COS-1(3) and CHO-KI, and hepatic cells such as McA-RH7777 cell types. Furthermore, when human apoA-V secretion kinetics was examined in primary hepatocytes we saw similar rates of secretion. The electrophoretic banding pattern of apoA-V, in both stably transfected cells and mouse liver, suggests that the protein is susceptible to intracellular proteolysis. A study of the secretion efficiency and kinetics supports the hypothesis that apoA-V functions intracellularly.

ApoA-V has been shown to be a heparin-binding protein (5). The heparin binding domain of apoA-V consists of a cluster of positive residues. Studies have proposed that apoA-V alters plasma TG by enhancing cell surface interactions with heparin sulfate proteoglycans. We were curious to see if human apoA-V in McA-RH7777 cells was associating with the cell membrane after secretion via
HSPGs, perhaps explaining the apparent low secretion rate. In our studies, we saw that apoA-V does not appear to associate with the cell membrane. The poor secretion efficiency of apoA-V could explain the low concentration in circulation in humans.

Triglyceride availability has been shown to regulate the assembly and secretion of apoB-containing particles (6). To understand how the behavior of apoA-V was affected by alterations in cellular triglyceride synthesis and accumulation we investigated intracellular stability, trafficking, and secretion of apoA-V in the presence of oleic acid. This mono-unsaturated fatty acid is a substrate for triglyceride synthesis and is often used to increase the secretion of apoB triglyceride-rich lipoproteins in cultured cells (8). Oleate has also been shown to increase second-step particle maturation, increasing the size of lipoprotein particles secreted from the cell. Moreover, oleate incubation produces an accumulation of lipid droplets within the McA-RH7777 cells. We proposed that increasing triglyceride synthesis would have a stabilizing effect on intracellular apoA-V and decrease the secretion of apoA-V in hepatocytes. In our studies, oleate stimulation was shown to promote the retention of cellular apoA-V by ~36% while the secretion of apoA-V decreased ~46%. These results suggest that the trafficking of apoA-V may be regulated by increased triglyceride synthesis.

Triglycerides within cytosolic lipid droplets undergo lipolysis and are re-esterified to triglyceride before they are incorporated into triglyceride-rich VLDL. In hepatocytes, lipid droplets are a storage form of neutral lipids that may be
used for production of VLDL (9). Droplet formation occurs in discrete regions of the ER, presumably, where neutral lipid synthesis occurs. Recent studies by Shu et al., found that apoA-V is somewhat retained in hepatocytes and traffics to cytosolic lipid droplets. A GFP-tagged version of apoA-V was found to co-localize with ADRP, a known lipid droplet binding protein (10;11). This is an interesting result considering that apoA-V is synthesized with a 23 amino acid signal peptide purposed for secretion. They propose that apoA-V's effects on plasma triglyceride levels are apparent through its interactions with cytosolic lipid droplets. Our model of oleate treated McA-RH7777 cells supported these results. Furthermore, when lipid droplet fractions were isolated from these cells by sucrose gradient ultracentrifugation, we saw an increase of apoA-V in the lipid droplet associated fraction in the presence of oleate. These data suggests that cellular triglyceride accumulation promotes the movement of apoA-V onto cytosolic lipid droplets. We therefore hypothesized that the reduced secretion of apoA-V observed upon oleate treatment may be caused by a corresponding increase in delivery of apoA-V to lipid droplets. ApoA-V’s association with cellular lipids may indicate involvement with the storage or mobilization of intracellular lipids. It is possible that apoA-V functions to decrease the secretion of VLDL-TG by limiting mobilization of TG.

ApoB is the main protein component of LDL and TG-rich VLDL particles and has an essential role in the intracellular assembly of TG-rich lipoproteins in the intestine and liver. As an integral component of triglyceride metabolism, the regulation of apoB-containing particles affects the development of
atherosclerosis, diabetes, and obesity (12). Currently there are two main theories for the function of apoA-V in the reduction of plasma triglycerides and controversial evidence surrounding both. While there have been many reports on increased LPL activation in the presence of apoA-V it is important to recall that apoA-V circulates at very low plasma concentrations suggesting an intracellular function.

The assembly of VLDL occurs as a two-step process beginning with the intra-hepatic lipidation of apoB (13). The second step involves the transfer of lipid to form a mature VLDL particle. Studies of the structural properties of apoA-V led Weinberg et al. to propose that apoA-V inhibits the secretion of VLDL (3). Based on the physical properties of apoA-V, Beckstead et al. also supported this hypothesis (14). We hypothesized that apoA-V has a role in lipoprotein assembly and wanted to examine the effect of apoA-V on apoB secretion, which may explain decreased TG secretion. Preliminary data in transiently transfected COS-1 cells revealed that apoA-V expression effected the secretion of B34 (data not shown). We wanted to explore the hypothesis that apoA-V effects plasma VLDL-TG by modulating hepatic apoB assembly. Rat hepatoma McA-RH7777 cells, which express endogenous apoB-100 and apoB-48, were stably transfected with human apoA-V in a pCMV5 plasmid to study the effect on apoB-100 and triglyceride secretion. Our preliminary studies with this cell line revealed a decrease in the secretion of both apoB and triglyceride. To examine this even further, our laboratory generated an apoA-V stably transfected cell line under the control of a tetracycline inducible promoter. Inducible cells were incubated for 12
- 48 hours in doxycycline to stimulate the expression of apoA-V; the maximum rate of synthesis was observed with 12 hours doxycycline incubation. ApoA-V was significantly over-expressed in this cell line and there was no leaky basal expression of the protein. The data revealed that induction of apoA-V expression resulted in a ~32% decrease in the secretion of apoB and 20% decrease in triglyceride. ApoA-V expression also resulted in a 57% reduction in top: bottom ratio of apoB, suggesting a decrease in apoB associated VLDL. The data from the above experiments support the hypothesis that apoA-V modulates the production and or secretion of triglyceride rich lipoprotein particles.

Similar to initial studies, we observed an inverse relationship between apoA-V and triglyceride in our stably transfected cells and transgenic mice. There have been controversial results showing both negative and positive correlations between apoA-V and plasma triglycerides. Many recent studies have shown positive correlation between apoA-V and triglycerides in humans (15-21). The mechanism by which apoA-V lowers plasma triglycerides is complex and many questions remain unanswered.

It is known that after secretion from the liver, apoA-V is found primarily on HDL and VLDL but not with LDL (22;23). Van der Vliet et al. also noted that apoA-V was mainly associated with HDL in mouse plasma (23). Interestingly, apoA-V and apoCIII have similar lipoprotein distribution patterns. When we analyzed the density distribution of apoA-V in McA-RH7777 cells we found it to correspond to the distribution of apoB-100 present in the VLDL fractions. McA-RH7777 cells do not have high levels of HDL so it is not surprising that apoA-V is
not present in the higher density fractions. It remains undetermined in what form apoA-V is secreted from the liver. It is possible that in circulation apoA-V exchanges between HDL and VLDL. Whether apoA-V is secreted with TG-rich VLDL or its binding to VLDL occur post-secretion remains to be determined.

Our laboratory generated and characterized liver specific human apoA-V mice to examine its effect on rates of triglyceride secretion by liver perfusion. Previous studies using a Triton WR-1339 method to examine triglyceride production found no difference in triglyceride production in the presence or absence of apoA-V (24;25). In this case, triton may be affecting the results of this experiment. We therefore examined triglyceride production using a non-triton based method. Other studies have used adenoviral overexpression of apoA-V to examine triglyceride production. Here Schaap et al. observed a dose-dependent reduction in secretion of VLDL-TG in mice expressing apoA-V (1). However, the adenoviral overexpression of apoA-V may produce changes in liver metabolism and not portray accurate results. Using a liver perfusion method, we analyzed the triglyceride accumulation in wild type and apoA-V transgenic mice and found a trend towards a reduction in triglyceride accumulation. It is possible that the mice may not have been challenged properly and should have been placed on a high fat diet to enhance VLDL-TG secretion. Furthermore, we saw a reduction in secreted triglycerides in transgenic primary hepatocytes. When we looked at hepatic triglyceride in the liver of these mice we observed that apoA-V expressing mice had increased hepatic triglyceride compared to wild type.
In summary, unusual trafficking behavior and low secretion of efficiency of apoA-V has been demonstrated in both hepatic and on hepatic cell types. These experiments have demonstrated that apoA-V may be regulated by changes in triglyceride synthesis and or accumulation. Based on these studies, the mechanism of apoA-V may be exerted at the level of hepatic VLDL production.

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