IMPACT OF HEPATIC APOLIPOPROTEIN A-IV EXPRESSION ON VLDL PARTICLE EXPANSION, TRIGLYCERIDE SECRETION, AND STEATOSIS

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LIST OF ABBREVIATIONS

ABCA1 – ATP-binding cassette transporter A1
ACC-1 – Acetyl-CoA carboxylase
Apo – Apolipoprotein
ATGL – Adipocyte triglyceride lipase
BSA – Bovine serum albumin
ChREBP – Carbohydrate-responsive element binding protein
CGI-58 – Comparative Gene Identification-58
CM – Chylomicron
COS – African green monkey kidney cells
Dox – Doxycycline
CREB-H – Cyclic AMP-Responsive Element-Binding Protein H
DSR5 – delta-5-desaturase
ELOVL – Elongation of very long-chain fatty acids
EM – Electron microscopy
ER – Endoplasmic reticulum
FAS – Fatty Acid Synthase
FBS – Fetal bovine serum
FFA – Free fatty acids
GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase
HDL – High density lipoprotein
HFHC – High fat/high cholesterol diet
HNF4-α – Hepatocyte Nuclear Factor 4 – alpha
HSA – Human serum albumin
HSL – Hormone sensitive lipase
LCAT – lecithin-cholesterol acyltransferase
LDL – Low density lipoprotein
LDLr – Low density lipoprotein receptor
LPL – Lipoprotein lipase
LRP – Low density lipoprotein receptor-related protein
McA-RH7777 – McArdle rat hepatoma cells
mRNA – Messenger ribonucleic acid
MTP – Microsomal Triglyceride Transfer Protein
NAFLD – Nonalcoholic fatty liver disease
NASH – Nonalcoholic steatohepatitis
PBS – Phosphate buffered saline
PLTP – Phospholipid Transfer Protein
PPARα – Peroxisome Proliferator-Activated Receptor – alpha
PYY – Peptide YY
IPEC-1 – Pig intestinal epithelial cell model
qPCR – Quantitative polymerase chain reaction
SCD-1 – Stearoyl-CoA desaturase-1
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR-B1 – Scavenger receptor BI
SREBP-1a – Sterol responsive element binding protein 1-a
TG – Triglyceride
VLDL – Very low density lipoproteins
ABSTRACT

Melissa A. VerHague

IMPACT OF HEPATIC APOLIPOPROTEIN A-IV EXPRESSION ON VLDL PARTICLE EXPANSION, TRIGLYCERIDE SECRETION, AND STEATOSIS

Dissertation under the direction of
Gregory S. Shelness, Ph.D., Professor of Pathology

Several apolipoproteins (apo) can impact triglyceride (TG) transport by modulating VLDL assembly and secretion. Here we explore the impact of apoA-IV on VLDL particle expansion. Previous studies demonstrated that apoA-IV expression promotes apoB lipoprotein-mediated TG secretion in transfected enterocytes and hepatoma cells. We therefore examined the impact of apoA-IV expression on VLDL particle dynamics in stably transfected McA-RH7777 hepatoma cells. Expression of apoA-IV caused an increase in TG secretion that was attributed to 10.1 nm increase in VLDL₁ particle diameter. While these data suggest that apoA-IV can directly impact VLDL particle expansion, there is no current evidence indicating that apoA-IV can promote lipid transport by this or any other mechanisms, in vivo. To explore the role of apoA-IV in vivo, we assessed the impact of both apoA-IV deficiency and overexpression on hepatic VLDL-mediated lipid efflux in two different mouse models of hepatic steatosis.
Hepatic steatosis induced by either a high fat diet or enhanced *de novo* lipogenesis, caused by transgenic overexpression of a constitutively active form of SREBP-1a (SREBP-1a^Tg), was associated with a robust induction (up to 43-fold) of hepatic apoA-IV mRNA and protein levels. In both models, a positive linear correlation between hepatic TG content and apoA-IV mRNA abundance was observed (r^2 = 0.8965). To examine whether induction of apoA-IV affected hepatic TG secretion, SREBP-1a^Tg mice were crossed with apoA-IV knock out mice (A4KO). With Triton blockade of peripheral lipolysis, SREBP-1a^Tg/A4KO mice demonstrated a 24% reduction in hepatic TG secretion rate, relative to SREBP-1a^Tg controls, but no change in apoB production. Negative stain electron microscopy revealed a 33% decrease in the abundance of secreted large VLDL particles with diameters ≥120 nm. Conversely, mice infected with a recombinant human apoA-IV adenovirus demonstrated a 38% increase in hepatic TG secretion rate and a 39% reduction in liver TG content relative to LacZ controls, associated with a 43% increase in large diameter VLDL particles and no change in apoB secretion. In conclusion, hepatic steatosis in mice induces hepatic apoA-IV expression, which, in turn, promotes lipoprotein particle expansion and reduces hepatic lipid burden without increasing the number of secreted atherogenic apoB-containing lipoprotein particles.
CHAPTER I
INTRODUCTION

Significance

Obesity and its associated pathological conditions, such as metabolic syndrome, have reached epidemic proportions, causing an increase in nonalcoholic fatty acid disease (NAFLD). It is now estimated that one-third of the adult population in developed countries is affected by NAFLD. While NAFLD is characterized by abnormally high lipid content in hepatocytes, this condition can often remain benign; however, when lipid accumulation is coupled with inflammatory cues, NAFLD can progress to non-alcoholic steatohepatitis (NASH), a condition associated with inflammatory cell infiltration and fibrosis. If left unchecked, NASH can lead to cirrhosis, hepatocellular carcinoma, and liver failure.

Hepatic Lipid Metabolism

With the emergence of NAFLD and NASH has come a greater interest in understanding basic aspects of hepatic lipid homeostasis and how these pathways become perturbed under conditions of excess caloric intake and the onset of insulin resistance and type 2 diabetes. There are three sources of hepatic lipid influx, which include the diet, de novo lipogenesis, and the uptake of free fatty acids released from adipocyte stores. Dietary lipids enter the small
intestine, where they are hydrolyzed by lipases, such as pancreatic lipase, and taken up by fatty acid transporters as well as by passive mechanisms at the brush border of enterocytes. In the enterocyte, long-chain fatty acids are packaged into chylomicron particles, which are then secreted into the lymphatic system. Chylomicrons enter the circulation via the thoracic duct and are then acted on by lipoprotein lipase at the surface of endothelial cells, releasing free fatty acids, which are taken up by parenchymal cells. The resulting chylomicron remnants are taken up by the liver, predominantly via low density lipoprotein receptor (LDLr) and LDLr-related protein (LRP).

In addition to dietary lipids, de novo lipogenesis also contributes to hepatic lipid formation, and is highly upregulated under conditions of insulin resistance and onset of NAFLD. De novo lipogenesis is the process by which carbohydrates are converted to free fatty acids (FFA), via the formation of pyruvate and acetyl-CoA. This process is stimulated by two major pathways, which increase the expression of lipogenic genes and provide increased substrate. The first is the upregulation of the transcription factor carbohydrate-responsive element binding protein (ChREBP). Increased glucose availability increases the activity of a specific protein phosphatase that dephosphorylates and activates ChREBP. ChREBP promotes the transcription of genes involved in FFA and triglyceride synthesis, including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). In addition, ChREBP stimulates the expression of liver-type pyruvate kinase, which catalyzes the conversion of phosphoenolpyruvate to pyruvate, thereby providing more substrate for FFA.
synthesis. Glucose also stimulates insulin secretion, which in turn induces another transcription factor, termed sterol regulatory element binding-protein 1c (SREBP-1c). As with ChREBP, stimulation of SREBP-1c activates FAS and ACC as well as other genes involved in FFA and triglyceride synthesis, such as stearoyl-CoA desaturase-1 (SCD1), elongation of very long-chain fatty acids (ELOVL), and Δ5-desaturase (DSR5)\(^6\).

The third source of hepatic lipid influx results from mobilization of TG stored in adipose depots. During fasting, insulin levels decline and epinephrine levels increase. Under these conditions, lipogenesis is downregulated and lipases, such as adipocyte triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) are activated. As a result, FFA are released into the circulation where they associate with albumin. Although many FFA are delivered to skeletal muscle, heart and other tissues for use as an energy source, many are also taken up by the liver\(^1\). Under conditions of insulin resistance, adipocyte dysfunction leads to dysregulated lipolysis, which further increases the flux of free fatty acids to the liver and becomes another major contributor to NAFLD.

Once inside the liver, there are three fates for FFA: storage, beta oxidation or secretion. FFA that enter the liver can be activated to their Co-A derivatives by acyl Co-A synthases and then esterified into complex lipids, including TG, which are then incorporated into cytosolic lipid droplets. Fatty acids are also delivered to mitochondria where they are subjected to energy-generating beta-oxidation pathways. However, one of the most important means by which the liver is able to maintain lipid homeostasis is via a lipid efflux pathway that involves the
formation and secretion of apoB-containing, TG-rich lipoproteins, termed very low density lipoprotein (VLDL).

The VLDL Assembly Pathway

The assembly of VLDL is a two-step process. In the first step, apoB undergoes co-translational translocation while associated with ribosomes located on the membrane of the rough endoplasmic reticulum (ER). During this process, a lipid transfer protein, termed microsomal triglyceride transfer protein (MTP), supplies TG to apoB and may also serve as a chaperone to promote proper apoB protein folding and maturation. During the second step of VLDL assembly, bulk core lipids are added to apoB post-translationally. It is during the second step of assembly that considerable lipoprotein size heterogeneity is generated, which is a hallmark of nascent apoB-containing lipoproteins formed both in hepatocytes and enterocytes. In hepatocytes, VLDL diameters range between 30 and 100 nm; however, under some pathophysiologic conditions, these particles can become much larger. During lipid absorption, intestinal chylomicrons can be expanded to diameters in excess of 1 µm.

Evidence for the first step of co-translational lipidation of apoB during the process of polypeptide synthesis was demonstrated when truncated forms of apoB, ranging from apoB17 (amino terminal 17%) to apoB53, or nascent forms generated by puromycin release of endogenous apoB, were analyzed in human and rat hepatoma cell lines. In all cases, a positive correlation was observed between apoB length and lipoprotein size, suggesting that lipid is likely added
during the process of translation. Upon the completion of translation, a pre-VLDL lipoprotein with a density in the HDL–LDL range is released into the ER lumen. It has been appreciated for some time that in many hepatoma cell lines, such as HepG2, these pre-VLDL particles cannot be readily converted into mature VLDL. However, a major breakthrough in this area was provided by Sven-Olof Olofsson and colleagues who demonstrated that when McA-RH7777 rat hepatoma cells were cultured in the presence of both serum and oleic acid, both apoB48 and apoB100 were converted into particles corresponding in density to native VLDL. This cell system has therefore been used extensively by many labs, including our own, to explore the mechanisms of apoB-containing lipoprotein particle assembly.

In the second step of apoB-containing lipoprotein assembly, additional TG from intracellular lipid droplets fuse with the nascent particle until a mature VLDL particle is formed. This step begins with the accumulation of large apoB-free TG-rich lipid droplets in the ER. Evidence for such droplets was first provided by direct electron microscopic visualization of osmiophilic structures within the endomembrane system of rat liver with characteristics of lipid droplets but with no immunoreactivity with antibodies to apoB. It was subsequently shown that in mice with absent apoB in the intestine, an increase in the size and abundance of such lumenal lipid droplets in the ER was observed using the same methodologies. While the trafficking of neutral lipid into the ER lumen is likely complex, it is known that MTP is essential for this process. First, apoB-replete/MTP-deficient
livers failed to reveal the presence of osmiophilic neutral lipid structures in the ER or Golgi. Subsequent studies in hepatoma cells and hepatocytes revealed reduced lumenal and membrane TG content in microsomes treated with MTP inhibitors, suggesting that MTP may be a critical cofactor required to promote the flux of neutral lipid flux from the cytosol into the ER lumen, necessary to support VLDL maturation.

Although it is clear that apoB-free lipid droplets form in the ER and perhaps the Golgi via an MTP-dependent pathway, the nature of the fusion reaction that underlies the second-step maturation of pre-VLDL is not understood in any detail. Pulse-chase studies have revealed that, while MTP is required to generate second step apoB-free precursor particles, MTP does not play a role in the actual fusion reaction \(^{14}\). In addition, the actual subcellular site where the fusion reaction occurs is also not universally agreed upon. Multiple reports, including the initial electron microscopic analyses outline above, support the hypothesis that the expansion of lipoprotein particles occurs solely in the ER. Rusinol et al. isolated apoB-containing lipoproteins from the ER and the Golgi, and found no difference in the densities of the particles, although differences in composition were observed \(^{15}\). Additionally, when lipoproteins were trapped in the ER and Golgi with transport inhibitors, such as Brefeldin A and nocodazole, only those retained in the ER were capable of expansion and not those trapped in the Golgi \(^{16}\). These studies suggest apoB-lipoprotein core expansion occurs only in the ER. However, other studies indicate that the Golgi is the sole site of lipoprotein particle expansion. For example, Nycodenz density gradient-isolated
ER and Golgi fractions from McA-RH7777 cells were examined for the occurrence of VLDL. While apoB was detected in both the ER and Golgi, VLDL was only present in the Golgi fractions. Additionally, in a cell-free system vesicle budding assay, Gusarova et al. failed to detect VLDL in ER-derived transport vesicles, but did identify VLDL in Golgi fractions. Furthermore, both large TG-rich and small dense pre-VLDL particles were present in the Golgi, suggesting a precursor-product relationship within a post-ER compartment. Thus, these latter studies suggest that lipoprotein particle core expansion may occur exclusively in the Golgi, a view that is perhaps gaining consensus.

As the main mode of hepatic lipid efflux is via VLDL assembly and secretion, it is an essential pathway for the maintenance of hepatic lipid balance. If hepatic lipid biosynthesis and storage is increased, as occurs under conditions of obesity, insulin resistance, and metabolic syndrome, compensation by enhanced TG secretion could be a factor in attenuating ectopic lipid accumulation. There are two basic mechanisms to increase hepatic TG secretion. The first is to increase the number of VLDL particles secreted; however, an increase in the number of particles secreted may result in increased levels of plasma apoB-containing lipoproteins and an increased risk for coronary heart disease. The second, and potentially more beneficial, mechanism is to increase the amount of TG packaged per particle, resulting in larger, more TG-rich particles. However, as alluded to above, the mechanisms controlling second step for particle expansion are not well understood. Nonetheless, it does appear that there are factors that may have the capacity to modulate the process of
particle expansion and could, therefore be important regulators of net lipid efflux from the hepatocyte. Further, these may represent targetable factors in the treatment of ectopic fat accumulation associated with hepatic steatosis.

Potential Modulators of Lipoprotein Particle Enlargement

To better understand lipoprotein particle expansion, key factors regulating this process need to be identified and investigated. A number of factors have been suggested to fit this role. Recent evidence reveals that the phospholipid transfer protein (PLTP) is a modulator of hepatic TG secretion acting at the level of both apoB production and particle expansion\(^3,^{23}\). In plasma, PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL; however, a number of studies have detected an important intrahepatic role of PLTP in VLDL assembly. PLTP deficient mice demonstrated reduced hepatic apoB production and atherosclerosis\(^24\). In contrast, adenoviral-mediated liver-specific expression of PLTP in PLTP-deficient mice caused an increase in both apoB and VLDL-TG secretion\(^3,^{25,26}\). These studies also revealed that PLTP overexpression enhanced the TG and phospholipid content within hepatic microsomal lumina, suggesting the PLTP may promote lipid mobilization for VLDL assembly.\(^3,^{23}\). In addition, it has been hypothesized that PLTP-mediated phospholipid transfer or exchange on the surface of pre-VLDL lipoproteins and lumenal lipid droplets might enhance their second-step fusion reaction.

In other more recent studies it was shown that the administration of the LXR agonist T0901317 in LDLr\(^/-\) mice produced a massive hypertriglyceridemia,
which was also associated with the production of very large chylomicron-sized VLDL particles. Among the genes induced by T0901317, which are responsible for this phenotype, were SREBP-1c and PLTP. While the phenotype was eliminated in SREBP-1c\(^{-/-}\) mice, administration of a PLTP adenovirus partially restored the hypertriglyceridemia with a corresponding increase in VLDL particle size. These studies suggest that T0901317-mediated activation of LXR induces lipogenesis via SREBP-1c, which in turn activates PLTP transcription. While LXR and SREBP-1c are important lipogenic activators, it was proposed the PLTP, by transferring surface lipids to nascent VLDL within the secretory pathway, created a higher capacity for TG core expansion.

**General Information on ApoA-V**

In addition to PLTP, it has been noted that several apolipoproteins, including apoE, apoC-III, A-IV and A-V can impact TG transport. Of particular relevance to studies reported here are the effects of apoA-V and -IV. ApoA-V is a 39 kDa protein expressed exclusively in the liver\(^{27}\). It is secreted into plasma and resides mainly on HDL particles. ApoA-V was discovered by comparative sequence analysis as a gene on human chromosome 11q, located 30 kb downstream from the apolipoprotein gene cluster of apoA-IV/C-III/A-I\(^{28}\). The genes found in this cluster display significant roles in lipid metabolism. Thus, due to the proximity of apoA-V to the apoA-IV/C-III/A-I gene locus, and its high structural similarity to apoA-IV\(^{28}\), a role in lipid metabolism was predicted. In an attempt to decipher a physiological role of apoA-V, the gene was knocked out in
mice and the resulting lipid phenotype revealed a 4-fold increase in plasma TG. Consistent with this finding, overexpression of apoA-V in mice led to a 65% reduction in plasma TG. Based on these initial findings, it was clear apoA-V has a potent effect on plasma TG metabolism.

**ApoA-V and Lipoprotein Clearance**

The majority of current literature suggests that apoA-V affects plasma TG turnover by stimulating LPL-mediated lipolysis of TG-rich lipoproteins. Adenoviral-overexpression of apoA-V in mice reduced hypertriglyceridemia following an intragastric fat load, and increased clearance of intravenously-injected VLDL-like TG-rich emulsions. Additionally, mice transgenically overexpressing apoA-V demonstrated increased TG turnover, increased LPL activity, and evidence of reduced VLDL particle number. However, when apoA-V transgenic mice were administered an LPL inhibitor, there was no enhancement of TG clearance.

In addition to affecting lipolysis, apoA-V may contribute to the clearance of TG-rich lipoproteins and their remnants through receptor-mediated mechanisms. Reports have indicated that apoA-V may be a ligand for LDL receptor (LDLr) family members and other potential lipoprotein receptors. For example, apoA-V binds to LDLr related protein (LRP) and SorLA, both of which are apoE-binding LDLr family members. LDLr family members are pivotal components in the internalization of apoB-containing lipoproteins.
increased clearance of TG-rich lipoproteins and their remnants could explain the inverse correlation between apoA-V and plasma TG levels.

Despite its apparent impact on TG-rich lipoprotein lipolysis and clearance, a unique characteristic of apoA-V is its very low plasma concentration. Circulating apoA-V is in the range of 100–200 µg/L, which is ~10,000-fold lower than apoA-I, and ~1,000-fold lower than apoA-IV, and corresponds to ~1 molecule of apoA-V for every 1,000 VLDL particles. This presents a conundrum as to how an apolipoprotein circulating at such low levels could exert such a potent effect on plasma TG metabolism and concentration.

**Intracellular Activities of ApoA-V**

Although it is possible that apoA-V could function in plasma at such low concentrations, relative to TG-rich lipoproteins, it has also been suggested that apoA-V might function within the hepatocyte to directly modulate hepatic TG metabolism and secretion. First, the expression of apoA-V is exclusively localized to the liver suggesting that it may have an hepatocyte-specific function. Indeed, independently of its discovery by comparative sequence analysis, the apoA-V gene was simultaneously identified based on its marked upregulation in rats following partial hepatectomy. This suggests that it could play a role in the conservation of intracellular lipids needed for liver regeneration. Secondly, the secretion kinetics of apoA-V also suggested activity in hepatocyte lipid metabolism. Very little apoA-V is secreted from hepatocytes, and within the
hepatocyte, very little apoA-V is transported to the Golgi. Therefore, the majority of apoA-V remains in the ER where it, perhaps, affects VLDL assembly.

Thus far, an effect of apoA-V on TG production has not been observed in all studies. Human apoA-V transgenic mice demonstrated equivalent hepatic TG secretion rates relative to wild type littermates. Additionally, adenoviral-mediated overexpression of human apoA-V in apoC-III transgenic mice did not demonstrate a significant reduction in hepatic TG production when compared to control adenovirus. However, Schaap et al. documented reduced hepatic TG production following adenovirus-mediated expression of human apoA-V in wild-type mouse liver. Further, as apoB secretion was unchanged, they surmised that apoA-V may reduce TG secretion by reducing lipoprotein size; however this possibility was not explored directly.

More recently, the discovery that apoA-V may reside on cytosolic lipid droplets gave further support to the concept that apoA-V responds to and potentially modulates aspects of intracellular hepatic TG metabolism. Intracellularly, apoB and apoA-V do not interact, but immunofluorescence microscopy confirmed that apoA-V colocalizes with lipid. Studies in McA-RH7777 rat hepatoma cells revealed that the colocalization was independent of the signal peptide, but did not occur if the C-terminal 200 amino acids were removed. Interaction between the C-terminus of apoA-V and lipid droplets could be indicative of a functional relationship in hepatic lipid metabolism. Indeed, studies performed in our laboratory by Blade et al. have demonstrated that when expressed in McA-RH7777 cells, the intracellular stability and
localization of apoA-V is impacted by the TG content of cells and that the expression of apoA-V reduces the secretion of VLDL-TG. The basis for this effect is explored in Chapter 2 of this thesis.

**General Information on ApoA-IV**

Intestinally-derived apoB-containing lipoproteins (chylomicrons; CM) are capable of achieving considerably larger particle diameters as compared to hepatic VLDL. To determine the factors that enable CMs to load more TG per particle, one must scrutinize the differences between the two particles. One difference is the presence of apolipoprotein A-IV (apoA-IV), which is expressed exclusively in the intestine of mammals and found on nascent chylomicrons. Therefore, it is possible that apoA-IV participates in the enlarging of secreted lipoprotein particles in the intestine.

ApoA-IV is a 46 kDa lipid binding protein, making it the largest member of the exchangeable apolipoprotein family. As mentioned above, it is located on human chromosome 11q in a gene cluster with apoA-I/C-III/A-V and is exclusively expressed in the intestine, though rodents express apoA-IV in the liver as well. In the enterocyte, it is synthesized during lipid absorption and secreted into mesenteric lymph on the surface of nascent chylomicrons. Once it reaches the bloodstream, apoA-IV rapidly dissociates from chylomicrons and is found on HDL or in lipid-free form in plasma at a concentration of ~15 mg/dL.
The rapid dissociation of apoA-IV and its occurrence in lipid-free form can be predicted from its structure. ApoA-IV, like most apolipoproteins, contains 13 tandem amphipathic α-helices; however, the radial charge distribution of these α-helices prevents deep penetration into lipid monolayers. Thus, apoA-IV has the weakest lipid affinity and the lowest interfacial exclusion pressure of any apolipoprotein allowing for rapid dissociation from chylomicrons in the blood stream.

**Proposed Functions of ApoA-IV**

Since its discovery in 1977, many roles have been suggested for apoA-IV. These include roles in satiety, cholesterol efflux via lecithin-cholesterol acyltransferase (LCAT) activation, modulation of CETP and LPL and glucose homeostasis. In addition, apoA-IV has been shown to display strong anti-oxidant and anti-atherosclerotic properties.

As apoA-IV is expressed in enterocytes, and gastrointestinal peptides can suppress food intake, a role in satiety is possible. Intravenous injection of apoA-IV in rats reduced food intake in a dose dependent manor. At the highest dose of 200 µg there was a 73% reduction in food intake. Additionally, apoA-IV injected directly into the third cerebroventricle of rats also reduces food intake in a dose dependent manor, but with 50-fold more potency. Furthermore, expression of intestinal apoA-IV is regulated by an adipokine, leptin, and the distal gut satiety hormone peptide YY (PYY). Taken together, these findings suggest a role for apoA-IV in the regulation of feeding behavior.
In plasma, apoA-IV is associated with HDL, thus roles in cholesterol efflux have been suggested. Evidence exists that apoA-IV affects the activity of integral components of the cholesterol efflux pathway. The membrane protein, ATP-binding cassette transporter A1 (ABCA1), facilitates HDL particle formation and promotes cholesterol efflux. Firstly, sera from human apoA-IV transgenic mice promote cAMP-stimulated cholesterol efflux in macrophage cells; cAMP induces cholesterol efflux by increasing ABCA1 mRNA expression and HDL binding. Second, a study examined the effects of apoA-IV expression on ABCA1 and another enzyme involved in cholesterol efflux, lecithin:cholesterol acyltransferase (LCAT). LCAT catalyzes the esterification of free cholesterol in plasma lipoproteins. Adenoviral overexpression of apoA-IV in apoA-I−/− × apoE−/− mice increased plasma lipid levels and generated α- and pre-β-like HDL subpopulations. However, in mice deficient in either ABCA1 or LCAT, spherical and α-migrating HDL particles were not detectable following gene transfer of apoA-IV. Lipid-free apoA-IV and reconstituted HDL-A-IV promoted ABCA1 and scavenger receptor BI (SR-BI)-mediated cholesterol efflux, respectively, as efficiently as apoA-I and apoE. Moreover, coexpression of apoA-IV and LCAT in apoA-I−/− mice restored the formation of apoA-IV containing HDL. Taken together, these findings suggest that apoA-IV can enhance cholesterol efflux via ABCA1 and LCAT activation.

In addition to participating in cholesterol efflux, apoA-IV has been suggested to contribute to plasma TG hydrolysis by activating lipoprotein lipase (LPL). Human plasma-derived apoA-IV increases LPL activity in the presence of
the known LPL activator, apolipoprotein C-II. This is not achieved through a
direct activation of LPL but by apoA-IV’s ability to displace apoC-II from HDL and
VLDL so that it can transfer to the surface of nascent chylomicrons.

Increasing both plasma TG hydrolysis and cholesterol efflux has the
capacity to reduce atherosclerosis. Indeed, apoA-IV has demonstrated striking
results in atheroprotection. When fed a high fat diet, mice transgenically
overexpressing murine apoA-IV (muA4-Tg) demonstrated a 30% reduction in
atherosclerotic lesions as compared to wild type littermate controls. Although
these mice had increased plasma lipids, HDL isolated from muA4-Tg mice
increased cholesterol efflux from human macrophages. When human apoA-IV
was transgenically overexpressed (huA4-Tg), atherosclerotic lesions were
reduced 90% and, when crossed onto an apoE-/- background, huA4-Tg mice had
a 64% reduction in atherosclerotic lesions.

In addition to affecting cholesterol efflux, another suggested mechanism of
anti-atherosclerosis of apoA-IV is through its anti-oxidant properties. HuA4-Tg
mice on an apoE-/- background exhibited reduced presence of anti-oxidized LDL
antibodies, oxidized proteins in tissues, and oxidation-specific epitopes in heart
sections of atherosclerotic lesions. Additionally, human apoA-IV accumulation
was detected in the atherosclerotic lesions, suggesting that apoA-IV may
decrease the progression of atherosclerosis by inhibiting oxidative damage to
local tissues.

Most recently a new function has been ascribed for apoA-IV in the area of
glucose homeostasis. Mice deficient in apoA-IV displayed reduced insulin
secretion and impaired glucose tolerance. When exogenous apoA-IV was introduced, glucose tolerance improved due to enhanced insulin secretion 62. Enhanced insulin secretion and improved glucose tolerance opens a new door to potential anti-diabetic functions of apoA-IV in addition to the anti-atherosclerotic activities discussed above.

**Functions of ApoA-IV in Lipoprotein Metabolism**

Although a broad spectrum of physiologic functions have been proposed for apoA-IV, a substantial body of evidence suggests that its core function is in lipid absorption and chylomicron assembly. First, intestinal expression of apoA-IV responds to fat infusion and high fat diet. In lymph-fistula rats, apoA-IV expression increased in the proximal jejunum with triolein dose, and apoA-IV production in lymph demonstrated a positive linear correlation with TG output 50. Additionally, the rate of synthesis of apoA-IV in rat enterocytes increased with a TG bolus and basal synthesis rates of apoA-IV were higher with high fat diet feeding 63. Thus, apoA-IV expression is regulated by both acute and chronic increases in intestinal lipid.

Although lipids regulate intestinal apoA-IV expression, this effect appears limited to long chain fatty acids. When lymph-fistula rats were administered long chain fatty acids (14:0, 18:0, 18:1, 18:2, 20:4, triolein), an increase in lymphatic apoA-IV output was observed. In contrast, short chain fatty acids (4:0, 8:0, 12:0, tributyrin, tricaprylin) did not promote apoA-IV expression 64. Additionally, jejunal apoA-IV synthesis was increased with a triolein bolus, but not with a bolus of
butric or caprylic acid. One possible explanation for these observations is that, due to their relatively high aqueous solubility, short chain fatty acids can enter the circulation directly via transcellular transport across the enterocyte. In contrast, long chain fatty acids require assembly into chylomicrons before they enter the lymphatic system. Hence, it is possible that apoA-IV is induced, not by intracellular lipid per se, but by the process of chylomicron-dependent lipid transport.

Additional evidence for the regulation of apoA-IV by chylomicron assembly is obtained in studies utilizing the surfactant, Pluronic L-81. Pluronic L-81 is a non-ionic hydrophobic detergent that inhibits chylomicron assembly. When Pluronic L-81 was infused into lymph-fistula rats, both lymph lipids and apoA-IV lymph output were inhibited. After cessation of Pluronic L-81, lipids were rapidly cleared and apoA-IV output increased. As Pluronic L-81 does not inhibit the uptake of lipid, the enterocytes remain highly lipid enriched. Despite the high intracellular concentration of lipid, the cells fail to activate the expression of apoA-IV. Again, these data validate the theory that apoA-IV synthesis and secretion is stimulated, not by the intracellular concentration of lipid, but by the packaging and secretion of lipid as chylomicrons.

Further evidence linking apoA-IV expression with chylomicron assembly and secretion lies in human conditions of lipid malabsorption. Decreased levels of plasma apoA-IV in patients with abetalipoproteinemia, hypobetalipoproteinemia, chronic pancreatitis, and malabsorption syndrome are observed. Abetalipoproteinemia is caused by a mutation in MTP, which
results in inhibition of VLDL and CM assembly and disruption of dietary fat absorption. Under these conditions, patients demonstrate a ~60% reduction in plasma apoA-IV concentration. Again, as MTP deficiency does not disrupt the uptake of dietary fat into the enterocyte, these data suggest that some other aspect of lipid absorption, most likely involving CM assembly, is involved in the regulation of apoA-IV synthesis and secretion.

Several cell-based studies have also confirmed a role for apoA-IV in lipid absorption. When swine apoA-IV was overexpressed in porcine intestinal epithelial cells (IPEC), the amount VLDL-associated TG secreted increased 5-fold as compared to control cells. In IPEC cells engineered to achieve tetracycline-inducible expression of swine apoA-IV, TG secretion was also stimulated upon addition of increasing doses of the antibiotic. This was accompanied by an increase in lipoprotein particle diameter from 53 nm in control cells to 87 nm in apoA-IV expressing cells, as well as a decrease in secreted apoB. These data establish that expression of swine apoA-IV in intestinal cells increases TG secretion by enlarging lipoprotein particles.

The mechanism in which apoA-IV may enhance lipoprotein particle expansion can be rationalized based on scrutiny of its structure. The C-terminus of apoA-IV may be key the in its lipid-binding properties; a variant allele of human apoA-IV, A-IV-2, encodes a Q360H substitution which is found in the EQQQ-rich C-terminus. A-IV-2 is associated with changes in lipid absorption, including decreased plasma TG and delayed clearance of postprandial TG-rich lipoproteins. Additionally, studies investigating manipulation of both the N- and
C-termini of apoA-IV revealed that the truncation of the C-terminal 43 amino acids resulted in increased lipid binding activity \(^1\). Taken together, these findings suggest that the C-terminal structure of apoA-IV is important to its lipid binding properties and may, therefore, also be important for apoA-IV’s capacity to enhance CM-dependent lipid transport.

To test this possibility, Lu et al. created apoA-IV C-terminal mutants to reflect either swine (pig-like) or chicken (chicken-like) apoA-IV. Pig-like apoA-IV is the result of the truncation of 13 amino acids, comprising the EQQQ-rich portion of the human apoA-IV C-terminus, as swine apoA-IV does not contain these repeats; chicken-like apoA-IV is produced by further truncation of 11 amino acids. When overexpressed in IPEC cells, human apoA-IV demonstrated a 2-fold increase and swine apoA-IV a 5-fold increase in TG secretion. However, pig-like apoA-IV demonstrated a 20-fold increase in TG secretion, and the size of the particles secreted demonstrated a 27-fold increase in average particle size to 4000 nm \(^6\). These findings suggest a TG secretion inhibitory role of the EQQQ-rich C-terminal sequence. The further truncation in the chicken-like variant of human apoA-IV resulted in no enhancement of TG secretion, suggesting disruption of the TG transport stimulatory properties of native human apoA-IV. These results supplement the argument that the C-terminus of apoA-IV regulates its lipid binding potential, and moreover, that this property is also related to its ability to enhance TG secretion.
Mechanisms of ApoA-IV-Mediated Lipoprotein Particle Expansion

Although the evidence linking apoA-IV expression and chylomicron assembly and secretion is compelling, the intracellular mechanisms by which apoA-IV might impact these processes are not well understood. For apoA-IV to facilitate the expansion of TG-rich lipoproteins, it must likely interact either directly with apoB or with the surface of apoB-containing lipoproteins within the secretory pathway. To determine if such interactions occur, human apoA-IV was modified at its C-terminus with the amino acid sequence KDEL, which is an ER-retention signal (apoA-IV-KDEL) \(^{72}\). African green monkey kidney cells (COS) were co-transfected with MTP, apoB41, and ether native human apoA-IV or apoA-IV-KDEL. First, apoA-IV-KDEL was able to retain apoB within the ER, whereas it had no effect on other secretory proteins. ApoA-IV-KDEL was able to inhibit the secretion of both lipid-associated and lipid-poor forms of apoB-25, suggesting the possibility of a direct protein-protein interaction. Expression of KDEL-modified control proteins, such as human serum albumin and apoA-I, had no effect on apoB secretion, confirming the specificity of this interaction. Immunofluorescence microscopy demonstrated colocalization of apoA-IV-KDEL and apoB in the ER, while native apoA-IV and apoB were found in both the ER and Golgi \(^{72}\). Finally, \textit{in situ} cross-linking studies were performed in COS cells transfected with apoB25 and apoA-IV. In the absence of the cross-linker, DSP, little apoB coimmunoprecipitated with apoA-IV; however, in the presence of DSP, apoB and apoA-IV appeared to be physically associated, as evidenced by extensive coimmunoprecipitation \(^{72}\). Taken together, these studies demonstrate
that apoA-IV and apoB-containing lipoproteins specifically interact with each other in the secretory pathway and that this interaction may enable apoA-IV to modulate apoB particle expansion.

**Analysis of ApoA-IV Function In Vivo**

While *in vitro* data provide a convincing argument for a role of apoA-IV in lipid transport, analogous *in vivo* studies have failed to confirm such a role. In apoA-IV knockout mice, plasma lipids were decreased, but no significant change was detected in intestinal lipid absorption, weight gain, or feeding behavior \(^73\). Likewise, when human apoA-IV was transgenically overexpressed in mice, increased plasma lipids were accompanied by delayed lipoprotein clearance, but again, no significant change was detected in intestinal lipid absorption, feeding behavior or weight gain \(^74\). However, as the intestine possesses excess absorptive capacity and numerous adaptive mechanisms, these properties may well mask the more subtle effects of apoA-IV on lipid trafficking \(^75\).

In contrast to the intestine, the liver is more sensitive to perturbations in TG metabolism, as is evidenced by the many genetic and metabolic perturbations that can lead to hepatic steatosis \(^1\). Thus, in the following studies we utilized the unique ability of rodents to endogenously express apoA-IV in the liver to examine the effects of apoA-IV on lipid packaging and secretion *in vivo*. Specifically, we measured the response of hepatic apoA-IV expression to liver TG accumulation, explored the impact of apoA-IV deficiency on VLDL-mediated hepatic lipid efflux, and also created an apoA-IV adenovirus, which allowed us to
examine the effects of hepatic apoA-IV overexpression on TG transport. These studies have provided the first evidence that apoA-IV can directly stimulate lipid transport pathways, in vivo, via the promotion of apoB lipoprotein particle expansion and without increasing the number of apoB lipoprotein particles produced by the liver. Further, apoA-IV-mediated enhancement of hepatic lipid efflux was accompanied by a dramatic decrease in hepatic lipid content. Hence, in addition to establishing a fundamental role of apoA-IV in modulating hepatic lipid efflux, these studies also suggest that agonism of apoA-IV expression may provide a potential therapeutic modality to decrease hepatic lipid burden, associated with hepatic steatosis, without increasing hepatic production of atherogenic apoB-containing lipoproteins.
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CHAPTER II

APOLIPOPROTEINS A-IV AND A-V MODULATE VERY LOW DENSITY LIPOPROTEIN PARTICLE EXPANSION IN MCA-RH7777 CELLS

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ABSTRACT

Several apolipoproteins (apo) can impact triglyceride (TG) transport by means of VLDL assembly and secretion. Here we explore the impact of apoA-IV and apoA-V expression on VLDL particle expansion. ApoA-V is a potent regulator of intravascular TG metabolism, yet its plasma concentration is very low compared to other apolipoproteins. Thus, in addition to its widely explored role in increasing lipoprotein catabolism, we hypothesized a function for apoA-V in suppressing hepatic TG secretion. In a stably transfected doxycycline-inducible McA-RH7777 rat hepatoma cell line, apoA-V expression inhibited TG secretion by ~50%, increased cellular TG, and reduced Z-average VLDL₁ particle diameter from 81 to 67 nm; however, no impact on apoB secretion was observed. These data demonstrate that apoA-V can modulate VLDL TG mobilization and can thereby impact the core lipid content of VLDL. In contrast to apoA-V, previous studies demonstrated that apoA-IV expression promotes apoB lipoprotein-mediated TG secretion in transfected enterocytes and hepatoma cells. We therefore examined the impact of apoA-IV expression on VLDL particle dynamics in stably transfected McA-RH7777 cells. Expression of apoA-IV caused an increase in TG secretion that was attributed to 10.1 nm increase in peak VLDL₁ particle diameter. ApoA-IV expression had no significant impact on expression of MTP or apoB, suggesting that apoA-IV can directly impact VLDL particle expansion, perhaps by modulating the trafficking kinetics of apoB. In conclusion, in stably transfected McA-RH7777 cells, inducible expression of apoA-V reduced TG secretion and VLDL₁ diameter, while apoA-IV expression increased TG secretion.
and VLDL, diameter. Hence, apoA-IV and apoA-V may be important modulators of hepatic VLDL particle expansion and TG secretion.

INTRODUCTION

The growth, reproduction, and survival of all multicellular organisms are dependent upon the efficient absorption, transport, and storage of exogenous lipids. The assembly and secretion of triglyceride (TG)-rich lipoproteins by the intestine and liver is central to these critical metabolic processes. TG-rich lipoprotein assembly is thought to occur in two sequential steps, in which the endoplasmic reticulum (ER)-localized cofactor, microsomal triglyceride transfer protein (MTP), plays an essential role. In the first step, MTP directs the cotranslational lipidation of apolipoprotein (apo)B in the ER with phospholipid and TG to form precursor particles with diameters of ~10–20 nm. In the second step, MTP promotes the movement of lipid from the cytosol into the ER to form luminal lipid droplets, which then fuse with apoB precursor particles in the ER and/or post-ER compartments. It is during the second, lipid loading step that the size of the particle is determined. However, the mechanisms of particle expansion, including the regulation of these processes, are not well understood. It has been noted that several apolipoproteins, including apoE, apoC-III, apoA-IV and apoA-V can impact TG transport. Of particular relevance to studies reported here are the effects of apoA-V and -IV.
ApoA-V is a member of the exchangeable apolipoprotein family synthesized predominantly in the liver and is a potent regulator of intravascular triglyceride (TG) metabolism. When it is overexpressed in transgenic mice, apoA-V reduces plasma TG levels by 65%, whereas inactivation of the apoA-V gene increases plasma TG by 4-fold. The preponderance of current literature suggests that apoA-V affects plasma TG turnover by stimulating LPL-mediated lipolysis of TG-rich lipoproteins, either directly or indirectly. ApoA-V has also been found to serve as a ligand for LDL receptor family members and other potential lipoprotein receptors and may thus contribute to the clearance of TG-rich lipoproteins and their remnants. However, recent studies have also revealed that the effects of apoA-V on plasma TG concentration are complex and variable. In humans, several loss-of-function and null apoA-V alleles are associated with both reduced plasma apoA-V levels and elevated plasma TG, yet other studies have found both positive and negative associations between plasma apoA-V and TG concentrations. Moreover, recent studies in mice have found a positive correlation between plasma apoA-V and TG concentrations. Despite its apparent impact on intravascular TG-rich lipoprotein lipolysis and clearance, a peculiar characteristic of apoA-V is that its plasma concentration is in the range of 100–200 µg/l, which is ~10,000-fold lower than apoA-I and ~1,000-fold lower than apoA-IV and corresponds to ~1 molecule of apoA-V for every 1,000 VLDL particles. This presents a conundrum as to how an
apolipoprotein circulating at such low levels could exert such a potent effect on plasma TG metabolism and concentration. Although it is certainly possible that apoA-V could function in plasma at extreme substoichiometric concentrations relative to that of TG-rich lipoproteins, it has also been suggested thatapoA-V might function within the hepatocyte to directly modulate hepatic TG metabolism and secretion. Indeed, the apoA-V gene was first identified based on its marked upregulation in rats following partial hepatectomy, suggesting that it could play a role in the conservation of intracellular lipids needed for liver regeneration. While an effect of apoA-V on TG production has not been observed in all studies, Schaap et al. documented reduced hepatic TG production following adenovirus-mediated expression of human apoA-V in mouse liver. More recently, the discovery that apoA-V may reside on cytosolic lipid droplets further supported the concept that it may respond to and perhaps modulate aspects of intracellular hepatic TG metabolism. Recent studies from our lab have indicated that in McA-RH7777 cells stably transfected with apoA-V, oleate-induced TG synthesis reduces apoA-V secretion efficiency, while promoting the localization of apoA-V on cytosolic lipid droplets. Thus, movement of apoA-V onto lipid droplets may directly compete with the exocytic trafficking of apoA-V and could also modulate the process of lipid mobilization for VLDL assembly and secretion. Hence, in the experiments described here, the impact of apoA-V expression on apoB particle characteristics in McA-RH7777 cells were explored using dynamic laser light scattering. These studies revealed a negative impact of
apoA-V on nascent lipoprotein particle diameter, leading to reduced TG secretion.

Another protein that may impact second-step particle expansion is apoA-IV. A unique aspect of intestinal apoB-containing lipoproteins (chylomicrons; CM), is their capacity for enhanced particle expansion, relative to hepatic VLDL. As in most mammals, apoA-IV is restricted to the intestine, it has been hypothesized that apoA-IV may, in part, be an important factor responsible for chylomicron expansion. Although considerable evidence linking apoA-IV expression and chylomicron assembly and secretion is compelling, the intracellular mechanisms by which apoA-IV might impact these processes remain poorly understood. For apoA-IV to facilitate the expansion of TG-rich lipoproteins, it may interact either directly with apoB or with the surface of apoB-containing lipoproteins within the secretory pathway. In previous studies, we cotransfected apoA-IV, apoB, and MTP into COS cells and found that apoA-IV modified with the C-terminal ER retention signal KDEL (apoA-IV-KDEL) inhibited the secretion of apoB constructs larger than apoB25, suggesting the existence of a protein-protein interaction between apoA-IV and specific sequences in the N-terminal region of the apoB molecule in the early stages of TG-rich lipoprotein assembly. Furthermore, we reported that stably transfected apoA-IV in McA-RH7777 cells not only increased TG secretion, but also reduced the rate of secretion of endogenous apoB. It was hypothesized based on these findings that apoA-IV’s interaction with apoB, increased nascent apoB particle residence time in the
ER/Golgi, thus allowing for increased lipid loading and core lipoprotein particle expansion.

In the current study, we investigated the roles of both apoA-V and apoA-IV in VLDL-TG secretion in stably transfected, inducible cell lines. First, we revealed that apoA-V gene expression reduces TG secretion and decreases particle size, suggesting an intrahepatic mechanism by which apoA-V could modulate TG metabolism and plasma TG levels. We also used similar techniques to examine the physical properties of secreted apoB-containing TG-rich lipoproteins produced during apoA-IV overexpression. Our data suggest that, in contrast to apoA-V, apoA-IV has the capacity to promote particle expansion and, ultimately, TG secretion.
METHODS

Cell culture
McA-RH7777 cells were grown in DMEM containing 4.5 g/l glucose and 10% FBS, supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C in an atmosphere containing 5% CO₂.

Plasmids
Construction of a human apoA-IV cDNA plasmid was described previously. Plasmids containing the tetracycline regulator (pTet-On) and the tetracycline response element (pTRE2hyg) were obtained from Clontech Laboratories, Inc. (Mountain View, CA). Human apoA-IV cDNA was generated by polymerase chain reaction using linearized apoA-IV plasmid DNA as template and 5’ and 3’ flanking primers containing engineered BglII and MluI restriction enzyme sites, respectively. Following BglII and MluI digestion, the cDNA was ligated to BamHI and MluI-digested pTRE2hyg. The integrity of the plasmid was verified by sequence analysis.

Selection of stable clones
Either apoA-IV or apoA-V cloned into pTRE2hyg was cotransfected into McA-RH7777 cells at a 1:1 ratio (20 µg of total DNA) with plasmid pTet-On. Twenty-four hours post-transfection, cells were subjected to selection with DMEM-10% FBS (Media Tech, Manassas, VA) supplemented with 750 µg/ml G418 and 200 µg/ml hygromycin. Selection medium was replaced every 48 h for ~14 days.
Individual clones were selected and maintained in media containing 250 µg/ml G418 and 100 µg/ml hygromycin. To induce expression, cells were incubated for the indicated times with 1 µg/ml doxycycline (Dox; BD Biosciences). Individual clones were analyzed for inducible expression by immunoblot analysis.

**Metabolic radiolabeling and analysis from transfected McA-RH7777 cells**

Unless otherwise indicated, McA-RH7777 cells were grown in 100 mm dishes containing DMEM with 10% FBS (growth medium). One µg/ml doxycycline (Dox) was added to the media and left on the cells for 48 h. Two h prior to experiments, media was replaced with growth medium containing 1 µg/ml Dox supplemented with 0.8 mM oleic acid complexed to 1.5% fatty acid–free BSA (both from Sigma-Aldridge, St. Louis, MO). Transfected cells were then metabolically radiolabeled by incubation for the indicated times with 100 µCi/ml [³⁵S]Met and Cys (EasyTag Express Protein Labeling Mix; Perkin Elmer Life Sciences, Waltham, MA) in Met-and Cys-deficient DMEM (Gibco-Life Technologies), also supplemented with 10% FBS, 0.8 mM oleic acid, and 1 µg/ml of Dox. Pulse-chase studies were performed in 60 mm dishes. After a 10 min pulse with [³⁵S]Met/Cys as described above, cells were washed and incubated with oleate-containing growth medium containing an additional 2.5 mM Met and 1 mM Cys for the indicated times. Cell lysate and media samples were prepared as described previously [43, 45] and subjected to immunoprecipitation with rabbit anti-apoA-IV, rabbit anti-human apoA-V serum or goat anti-human apoB (Academy Biomedical, Houston, TX), as indicated. Immune complexes were fractionated by SDS-PAGE, and dried gels
were exposed to BioMax MS film backed with a BioMax TransScreen-LE intensifying screen (Eastman Kodak, Rochester, NY) at -70°C. Band intensities were quantified using a Molecular Dynamics 445 SI phosphorimager or a Fujifilm BAS5000 phosphorimager. The phosphorimager values were used to calculate apoB secretion efficiency, intracellular retention, and degradation.

**Quantification of intracellular and secreted lipids from transfected McA-RH7777 cells**

Stably transfected, inducible cells were incubated in medium containing 20% FBS and 0.4 mM oleate complexed to 0.75% BSA, with or without 1 µg/ml Dox, for 24 hours. Cells were then radiolabeled with 10 µCi [³H]oleate, in the same media for 24 hours. Media was then removed and subjected to Bligh-Dyer extraction. Extracted lipids were fractioned by thin layer chromatography using heptane-ether-acetic acid [90:30:1]; bands were visualized by incubation in iodine vapor, and TG- and phospholipid (PL)-containing fractions were cut from the plate and quantified by liquid scintillation counting. Protein concentration of cell extracts was determined by the bicinchoninic acid method using the Pierce BCA Protein Assay Reagent kit (Thermo Scientific, Rockford, IL).

**Immunofluorescence microscopy**

ApoA-V stably transfected McA-RH7777 cells were plated in 35 mm tissue culture dishes and incubated for 24 h. Cells were fixed and permeabilized by incubation with 3.7% formaldehyde in PBS for 20 min followed by incubation for
1 hour in PBS containing 10 mM glycine, 0.1% saponin, and 3% BSA. Cells were then incubated for 1 hour with rabbit anti-human apoA-V primary serum diluted 1:300 in PBS, 0.1% saponin, 1% BSA, followed by rhodamine-conjugated goat anti-rabbit IgG (1:20; Jackson ImmunoResearch) in the same buffer. Live cells were incubated with anti-apoA-V serum diluted 1:300 in PBS containing 1% BSA for 1 hour at 4° C, followed by 1 hour incubation with rhodamine-conjugated goat anti-rabbit IgG in the same buffer. All cells were post-fixed with 3.7% formaldehyde in PBS and mounted under cover slips with 90% glycerol, 10% PBS. Cells were viewed using a Zeiss Xioplan 2 microscope with a 63x oil objective.

**VLDL particle diameter distribution**

The hydrodynamic diameters of VLDL particles produced by transfected McA-RH7777 cells were measured using a Zetasizer Nano-S® model ZEN1600 dynamic laser light-scattering instrument (Malvern Instruments) at 633 nm. Following density gradient ultracentrifugation, as described previously 12, 48, gradients were fractionated into twelve 1 ml fractions from the top, using an Auto Densi-Flow gradient fractionator. Fractions of VLDL₁ (Svedberg units of flotation [Sf] >100) and VLDL₂ (Sf 20–100) were transferred to a quartz cuvette, and light scatter readings were performed at 20°C. Gradient samples were subsequently subjected to immunoprecipitation with anti-apoB antibodies and analyzed by 12.5% SDS-PAGE and phosphorimager analysis.
Gene expression in inducible McA-RH7777 cells

Inducible apoA-IV expressing McA-RH7777 cells were grown in 100 mm dishes containing DMEM with 10% FBS with and without 1 μg/ml Dox. After 48 h, cellular RNA was extracted using TRIzol (Invitrogen). Total RNA (1 μg) was converted into cDNA using Omniscript RT kits (Qiagen, Valencia, CA). Quantitative PCR (qPCR) was performed on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). A typical PCR reaction (20 μl) contained 10 μl 2 X Fast SYBR Green Master Mix (Applied Biosystems), 1 μl each of 5 μM forward and reverse primers, and 2 μl cDNA. Copy numbers were normalized to GAPDH. The following primers were used. ApoA-IV forward: TTC CTG AAG GCT GCG GTG CTG; apoA-IV reverse: CTG CTG AGT GAC ATC CGT CTT CTG. MTP forward: CCT ACC AGG CCC AAC AAG AC; MTP reverse: CGC TCA ATT TTG CAT GTA TCC. Immunoblot analyses were performed with anti-mouse MTP monoclonal antibody (BD Biosciences) and rabbit anti-human apoA-IV, as described previously.

Production and verification of mouse apoA-IV antibody

Full length, mature apoA-IV was fused via cDNA cloning to maltose-binding protein (MBP) in plasmid pMALc2x (New England Biolabs). The apoA-IV-MBP fusion protein was expressed in E. coli and purified by amylose affinity chromatography. The purified protein was sent to Lampire Biological Products, where it was injected into two rabbits for antibody production. To test for efficacy in recognizing mouse apoA-IV, 100 mm dishes of COS cells were transfected
with 6 µg of either human serum albumin (HSA), mouse apoA-IV (mA4), or C-terminal FLAG-tagged mouse apoA-IV (mA4-F). Cells were harvested in lysis buffer (25 mM Tris HCl pH 7.4, 300 mM NaCl and 1% Triton X-100) containing protease inhibitors (1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 10 mM EDTA). Protein was separated by 12.5% SDS-PAGE and then transferred to a PVDF membrane. ApoA-IV protein was detected using rabbit anti-serum (1:500) from rabbit #13477, 28 days post-injection, or mouse anti-FLAG-M2 antibody at a concentration of 2.5 µg/ml (Sigma-Aldrich #F3165).

For detection of apoA-IV in mouse plasma and liver, either C57BL/6 mice or apoA-IV knock out (A4-KO) mice were fed a high fat diet (48% fat from calories, 0.2% cholesterol) for 16 weeks. Following a 4-hour fast, mice were euthanized using a ketamine/xylazine cocktail. Blood was collected via heart puncture, and organs were flushed with saline. Livers were harvested and snap frozen in liquid N₂. For immunoblot analysis ~500 mg of frozen tissue was homogenized with a Polytron homogenizer in lysis buffer containing protease inhibitors, as indicated above. Protein was separated by 12.5% SDS-PAGE and then transferred to a PVDF membrane. ApoA-IV protein was detected using the rabbit anti-serum (1:1000) described above.
RESULTS

ApoA-V does not bind to cell surface after secretion

Previous studies have documented inefficient secretion of apoA-V from transiently transfected COS cells undergoing continuous metabolic radiolabeling with \[^{35}\text{S}]\text{Met-Cys}\ 37. Reports from our lab using McA-RH7777 cells stably transfected with apoA-V, indicated that only 20% of newly synthesized apoA-V underwent secretion with the remainder undergoing degradation or cell association 42. As apoA-V can associate with heparin sulfate proteoglycans 23, 50, the limited recovery of apoA-V from medium fractions could result from binding of newly secreted apoA-V to the cell surface. To determine if newly secreted apoA-V associated with heparin-competable binding sites in McA-RH7777 cells, the relative distribution of apoA-V in cell and medium fractions was explored in the absence and presence of heparin at concentrations up to 100 U/ml; however, no significant change in cell-associated apoA-V was observed 42. These results suggest that the low apoA-V secretion efficiency was not due to cell surface proteoglycan-mediated sequestration. To explore whether apoA-V bound to the surface of McA-RH7777 cells via some other mechanism, staining of live cells with anti-apoA-V antibody was performed. However, no cell staining was observed unless cells were first permeabilized with saponin (Fig. 1). Taken together with previous studies, these data suggest the low plasma concentration of apoA-V is a result of inefficient hepatic secretion.
Figure 1. Live and permeabilized cell staining of apoA-V transfected McA-RH7777 cells. ApoA-V stably transfected McA-RH7777 cells were plated in 35 mm tissue culture dishes and incubated for 24 h. A and B: Cells were fixed and permeabilized by incubation with 3.7% formaldehyde in PBS for 20 min followed by incubation for 1 h in PBS containing 10 mM glycine, 0.1% saponin, and 3% BSA. Cells were then incubated for 1 h with rabbit anti-human apoA-V primary serum diluted 1:300 in PBS, 0.1% saponin, 1% BSA, followed by rhodamine-conjugated goat anti-rabbit IgG (1:20; Jackson ImmunoResearch) in the same buffer. C and D: Live cells were incubated with anti-apoA-V serum diluted 1:300 in PBS, 1% BSA for 1 h at 4° C, followed by 1 h incubation with rhodamine-conjugated goat anti-rabbit IgG in the same buffer. E and F: Live cells were incubated with PBS, 1% BSA, followed by secondary antibody. All cells were post-fixed with 3.7% formaldehyde in PBS and mounted under cover slips with 90% glycerol, 10% PBS. Cells were viewed using a Zeiss Xioplan 2 microscope with a 63x oil objective. A, C, and E, rhodamine fluorescence; B, D, and F, phase contrast.
Effect of apoA-V expression on apoB and triglyceride secretion

Based on its low concentration in plasma, the possibility that apoA-V modulates intracellular hepatic TG metabolism has been proposed \(^{20, 36, 37}\). Hence, we explored whether the expression of apoA-V could affect TG secretion in McA-RH7777 hepatoma cells. To rule out possible phenotypic variability associated with clonally selected cell lines, we generated McA-RH7777 cell lines that expressed apoA-V under the control of a Dox-inducible promoter. As shown in Fig. 2A, these cells displayed undetectable basal apoA-V expression and a robust induction when cells were incubated with Dox. To examine the consequences of apoA-V expression on TG secretion, cells were labeled with \(^{3}H\)oleate in the absence and presence of Dox for 24 h. Induction of apoA-V expression resulted in a \(~50\%\) increase in cellular TG content (Fig. 2B) and a roughly corresponding decrease in TG secretion (Fig. 2C). To assess the impact of apoA-V on apoB, control and induced cells were subjected to radiolabel pulse-chase analysis with \(^{35}S\)Met-Cys. As shown in Fig. 3A and B, apoA-V expression appeared to have no impact on the secretion or the intracellular stability of apoB.

The finding that apoA-V impacts TG but not apoB secretion suggests an effect on particle size but not particle number. To assess apoB particle characteristics in the presence and absence of apoA-V expression, stably transfected McA-RH7777 cells treated with and without Dox were metabolically radiolabeled with \(^{35}S\)Met-Cys for 4 h, and medium samples were subjected to cumulative rate density gradient ultracentrifugation \(^{12, 48}\). No apparent change in apoB density
Figure 2. Effect of apoA-V on apoB secretion. A: McA-RH7777 cells, which express human apoA-V under the control of a Dox-inducible promoter, were incubated without (−) or with (+) 1 µg/ml Dox for 48 h. Cells were labeled with [35S]Met-Cys for 4 h, also without and with Dox, and cell lysates were subjected to immunoprecipitation with anti-apoA-V serum, SDS-PAGE, and phosphorimaging analysis. B, C: Cells were incubated in medium containing 20% FBS and 0.4 mM oleate complexed to 0.75% BSA, without (−) or with (+) 1 µg/ml Dox for 24 h, followed by radiolabeling with [3H]oleate (10 µCi/ml) in the same medium for 24 h. Cells (B) and medium (C) fractions were extracted with chloroform-methanol, and the lipid extracts were fractionated by TLC. TG bands were quantified by liquid scintillation counting (values shown are means ± SEM; N = 3). All data were analyzed using a paired Student t-test (*P < 0.05).
Figure 3. Effect of apoA-V on apoB secretion. McA-RH7777 cells, which express human apoA-V under the control of a Dox-inducible promoter, were incubated in medium containing 20% FBS and 0.4 mM oleate complexed to 0.75% BSA, without (−) or with (+) 1 µg/ml Dox for 24 h, followed by pulse radiolabeling with [35S]Met-Cys for 30 min and chase with unlabeled medium for 0 or 2 h, as indicated. A: ApoB in cells and medium samples was immunoprecipitated and subjected to SDS-PAGE followed by phosphorimaging analysis. B: Percentage of newly synthesized apoB (0 h chase) recovered from medium after a 2 h chase. Control (without Dox [−]) efficiency was set to 100% (values shown are means ± SEM; N = 3). All data were analyzed using a paired Student’s t-test (*, P < 0.05).
distribution was observed in response to apoA-V expression (Fig. 4A); however, when the VLDL₁ fraction (Sf,>100) was analyzed by dynamic laser light scattering, the Dox-treated cells displayed a 24 nm reduction in peak VLDL₁ particle size diameter, from 66 to 42 nm, and a 14 nm reduction in Z-average diameter, from 81 to 67 nm (Fig. 4B); apoA-V had no impact on the VLDL₂ (Sf, 20–100) peak diameter of ~31 nm, as expected. These data indicate that the reduced TG secretion observed upon induction of apoA-V expression is due primarily to attenuation of second-step particle maturation, essential for the formation of TG-rich VLDL.

Dox-induced apoA-IV expression in McA-RH7777 cells increases VLDL particle size

As our previous results demonstrated that in contrast to apoA-V, apoA-IV expression increased TG secretion in association with an increased percentage of apoB that forms VLDL (44). Hence, we directly measured the effect of apoA-IV expression on VLDL particle diameter. When an inducible apoA-IV cell line was incubated with Dox for 48 h, intracellular apoA-IV increased dramatically (Fig. 5A). Media from these Dox-treated cells were then subjected to density gradient centrifugation to obtain the VLDL₁ fraction, and its particle size distribution was determined by dynamic light scattering (51). Addition of Dox caused a rightward shift in VLDL₁ particle distribution (Fig. 5B), caused by an increase in peak diameter from 59.4 to 69.5 nm (Fig. 5C), and an increase in the intensity-weighted mean ("Z-averaged") diameter from 49.35 to 59.18 nm. Together these
Figure 4. ApoA-V expression impacts lipoprotein particle size distribution. Inducible McA-RH7777 cells were incubated in the absence (−) or presence (+) of Dox for 48 h and radiolabeled as described in the legend to Fig 1A. A: Medium samples were harvested and subjected to cumulative rate flotation ultracentrifugation, as described previously. Size distributions of particles contained in the VLDL₁ (B) and VLDL₂ (C) fractions were determined using dynamic laser light scattering analysis.
Figure 5. Effect of apoA-IV expression on VLDL size distribution. Tet-inducible apoA-IV McA-RH7777 cells were incubated without (−) or with (+) 1 µg/ml (Dox) for 48 h and then incubated with DMEM containing 20% FBS and 0.4 mM oleate complexed to 0.75% BSA, also with and without Dox, for 4 h. A: Cell lysates (50 µg) were analyzed by 12.5% SDS-PAGE, followed by immunoblot analysis with anti-apoA-IV antibody. B: Media were harvested and subjected to cumulative rate flotation ultracentrifugation, as described in Methods. The size distributions in the VLDL₁ fractions were determined using dynamic laser light scattering. C: Peak VLDL₁ particle diameters (mean ± SE; n = 3; *P < 0.001 by unpaired t-test).
data suggest that Dox-induced apoA-IV expression in McA-RH7777 cells facilitates second-step particle lipidation, thereby resulting in the secretion of larger, more TG-enriched lipoproteins.

**Dox-induced apoA-IV expression in McA-RH7777 cells does not alter MTP gene expression**

As it was previously reported that the effects of apoA-IV on TG transport in IPEC-1 cells was associated with an increase in endogenous MTP expression\(^5\), we assessed whether the changes in lipid and lipoprotein secretion and particle characteristics observed in our Dox-inducible McA-RH7777 cells could also be attributable in whole or part to a similar upregulation of MTP. To assess the effect of apoA-IV expression on MTP, cells were incubated for 48 h either without or with Dox, and apoA-IV and MTP gene expression was measured by qPCR. As shown in Fig. 6A, as expected, incubation with Dox increased apoA-IV gene expression ~5-fold; however, in the same cells, MTP gene expression displayed a nonsignificant increase of 25% (Fig. 6B). To confirm this outcome, extracts from both −Dox- and +Dox-treated cells were subjected to immunoblot analysis. While apoA-IV was upregulated by Dox, no detectable change in MTP protein abundance was observed (Fig. 6C).

**Production of mouse-specific apoA-IV antibody.**

While our findings in the above *in vitro* studies of apoA-IV are compelling, to fully explore the physiological capabilities of apoA-IV, it is important to explore its role
Figure 6. Impact of Dox-induced expression of apoA-IV on MTP mRNA and protein abundance. Tet-inducible apoA-IV McA-RH7777 cells were incubated in the absence (−) or presence (+) of 1 µg/ml doxycycline (Dox) for 48 h and apoA-IV (A) and MTP (B) mRNA abundance was measured by qPCR, normalized to GAPDH, and shown as the fold increase in the presence of Dox. Data are mean ± SD; N=3; *P < 0.001 by unpaired t-test. C: Immunoblot analysis of transfected human apoA-IV and endogenous rat MTP.
*in vivo*. For this purpose, a murine-specific anti-apoA-IV antibody was required, and our attempts to identify an effective commercial antibody were unsuccessful. Hence, full length, mature mouse apoA-IV was fused to maltose-binding protein (apoA-IV-MBP) via cDNA cloning in the vector pMALc2x. ApoA-IV-MBP was expressed, purified, and injected into rabbits, as described under Methods. To test for efficacy in recognizing mouse apoA-IV, 100 mm dishes of COS cells were transfected with 6 µg of either human serum albumin (HSA), mouse apoA-IV (mA4), or C-terminal FLAG-tagged mouse apoA-IV (mA4-F). Cells were harvested in lysis buffer, as described in methods, fractionated by 12.5% SDS-PAGE and immunoblotted against rabbit anti-serum from a 28 days post-injection test bleed. A strong signal was detected in cells transfected with both mA4 and mA4-F, but not in those transfected with HSA (Fig. 7A). To confirm the identity of the band detected by the anti-apoA-IV serum, anti-FLAG antibody M2 was also used to detect the FLAG tag of mA4-F (Fig. 7B).

To verify that our antibody was capable of detecting endogenous apoA-IV in mice, 1 µl of plasma from wild type or apoA-IV knock-out mice was fractionated by SDS-PAGE followed by immunoblot analysis. Our antibody was capable of detecting a strong signal, running in conjunction with the positive control from mA4-transfected cells (Fig. 7C). While detection of mouse apoA-IV protein has been previously demonstrated in plasma, detection of protein in the liver is lacking. Thus, we wanted to demonstrate the ability of our antibody to detect hepatic apoA-IV. We were able to detect apoA-IV in whole liver protein lysates.
Figure 7. Production of antiserum against full-length murine apoA-IV. An apoA-IV-maltose binding protein fusion was constructed and expressed in E. coli. The fusion protein contained in bacterial extract was purified by amylose affinity chromatography. Two rabbits were immunized with 0.5 mg each of purified fusion protein. Twenty-eight days after immunization, test bleeds were obtained and verified. A and B: COS cells were transfected with either a control protein (human serum albumin; HSA), mouse apoA-IV (mA4), or C-terminal flag-tagged mouse apoA-IV (mA4-F), as indicated. Cell lysates (50 µg) were analyzed by 12.5% SDS-PAGE, followed by immunoblot analysis with post-immune serum from rabbit #13477 (A) or anti-M2-FLAG antibody (B). C: Alongside mA4-transfected COS cell lysate as a positive control, 1 µL plasma from wild-type or apoA-IV knock out (A4-KO) mice were analyzed by 12.5% SDS-PAGE, followed by immunoblot analysis with post-immune serum from rabbit #13477. D: Along with 1 µL mouse plasma as a positive control, 50 µg of whole liver protein lysates from high fat diet fed wild-type or A4-KO mice were analyzed by 12.5% SDS-PAGE, followed by immunoblot analysis with post-immune serum from rabbit #13477. The arrow indicates the position of mouse apoA-IV, which appears to have a slightly different size than plasma apoA-IV, perhaps due to differences in O-linked glycosylation.
from wild type mice fed a high fate diet but not apoA-IV knockout mice (Fig. 7D). These results indicate that we have successfully created an antibody against murine apoA-IV that can detect protein levels in the liver as well as plasma.

Although we had developed and used a rabbit anti-human apoA-IV antibody for previous studies, a systematic characterization of its use *in vitro* and *in vivo* had not been published. Hence, either human apoA-IV (hA4) or a control protein (human serum albumin; HSA) was transfected into McA-RH7777 cells. Cell lysates were fractionated by SDS-PAGE followed by transfer to a PVDF membrane. When membranes were probed with post-immune serum from rabbit #5344 the appropriate sized band was detected abundantly in the hA4- but not the HSA-transfected cells (Fig. 8A). To test the antibody for *in vivo* applications, plasma and liver extracts from human apoA-IV and LacZ control adenovirus-infected mice (see Chapter 3) were also subjected to SDS-PAGE and immunoblot analysis with rabbit 5344 serum. In both plasma (Fig 8B) and liver samples (Fig 8C), apoA-IV protein was detected only in samples from apoA-IV adenovirus (Ad-huA4)-infected mice and not in the Ad-LacZ samples.
Figure 8. Production of antiserum against full-length human apoA-IV. A full length mature apoA-IV-maltose binding protein fusion was constructed and expressed in E. coli. The fusion protein contained in the bacterial extract was purified by amylose affinity chromatography. Two rabbits were immunized with 0.5 mg each of purified fusion protein. Fifty-two days after immunization, production bleeds were obtained and tested. A: McA-RH7777 cells were transfect with either a control protein (human serum albumin; HSA) or human apoA-IV (hA4), as indicated. Cell lysates (50 µg) were fractionated by 12.5% SDS-PAGE, followed by immunoblot analysis with post-immune serum from rabbit #5344. B and C: Wild-type mice were administered 1.5x10⁹ pfu of either apoA-IV-adenovirus (huA4) or the control LacZ-adenovirus (LacZ). One µL of plasma (B) or 50 µg of whole protein lysate (C) were analyzed by 12.5% SDS-PAGE, followed by immunoblot analysis with post-immune serum from rabbit #5344.
Discussion

Several apolipoproteins can impact TG transport by means of VLDL assembly and secretion. For example, overexpression of apoE enhances VLDL-TG production, while apoE deficiency is associated with increased liver lipid accumulation and reduced VLDL-TG production. A complementary decrease in VLDL particle diameter was also observed in apoE deficient mice. Similar to apoE, overexpression of apoC-III in either hepatoma cells or in mice increased hepatic TG secretion with an increase in VLDL1 production. Thus, expression of either apoE or apoC-III can impact hepatic VLDL-TG by augmenting particle expansion. In the current study, we expose additional apolipoproteins, apoA-IV and apoA-V, as modulators of hepatic TG transport via particle expansion.

Recent data provide evidence of a linkage between intracellular apoA-V trafficking and TG metabolism. Thus in the current study, the consequence of apoA-V expression and localization was explored using a regulatable apoA-V expression system. When transfected apoA-V expression was induced with Dox, neither the secretion nor the density distribution of apoB in McA-RH7777 cells was altered dramatically. This finding agrees with observations of Shu et al., who also noted no change in apoB secretion or density gradient distribution in Hep3B hepatoma cells stably transfected with human apoA-V. However, in the current study, apoA-V expression was associated with a ~50% reduction in TG secretion and a corresponding increase.
in cellular TG content. To explore the basis for this observation, particle size analysis was performed. The VLDL<sub>1</sub> peak particle diameter was reduced, which was calculated to correspond to a ~40% reduction in VLDL volume. Hence, the reduction in TG content in medium from apoA-V-expressing McA-RH7777 cells appears to result from a reduction in VLDL particle size distribution but not in particle number. These data are consistent with findings of Schaap at al. 20, who found that adenovirus-mediated expression of human apoA-V inhibited VLDL-TG secretion, also without affecting particle number. While other studies have failed to establish a link between apoA-V expression and VLDL secretion in transgenic mice 22, it is worth noting that upon induction of apoA-V in vivo during liver regeneration 38, hepatic TG synthesis and accumulation are upregulated without an accompanying increase in VLDL secretion 61.

Observations from animal and human studies provide further evidence of the linkage between apoA-V gene expression and hepatic TG synthesis, storage, and secretion. Shu et al. 50 observed that both the hepatic TG content and the apoA-V lipid droplet association were increased in human apoA-V transgenic mice, although inactivation of the mouse apoA-V gene had little effect on liver TG. Although the impact on plasma TG was not examined in that study, Pamir et al. 62 found that when human apoA-V transgenic mice were fed a high-fat and high-sucrose diet, fasting plasma TG levels fell instead of increasing, suggesting that apoA-V gene expression had inhibited diet-induced hepatic VLDL-TG secretion. Werner et al. 63 observed that hepatic steatosis induced by essential
fatty acid deficiency is accompanied by increased apoA-V gene expression; yet, Huang et al. 64 found that plasma apoA-V levels were 45% lower in obese, insulin-resistant, dyslipidemic subjects, suggesting that hepatic steatosis, which is a concomitant condition of the metabolic syndrome, reduced hepatic apoA-V secretion. Presently, the mechanism of linkage between apoA-V and TG metabolism is not well understood, but the current data suggests it could be a result of reduced hepatic TG secretion caused by inhibition of TG mobilization and particle expansion.

While apoA-V modulates hepatic lipid secretion by decreasing lipoprotein particle size, apoA-IV may have the opposite effect 65. To examine this possibility, we used McA-RH7777 cells stably transfected with regulatable apoA-IV expression to show that apoA-IV increased the diameter of VLDL1 particles. Although these data corroborate the previous studies of Lu et al., who found that constitutive and Dox-induced overexpression of apoA-IV in IPEC-1 cells facilitates transcellular TG transport by increasing lipoprotein size 65,66, they are seemingly at odds with work of Yao et al. 67. These investigators observed that constitutive expression of rat apoA-IV under control of the constitutive CMV promoter in McA-RH7777 cells had no obvious effect on the size of d<1.006 g/ml lipoprotein particles as assessed by electron microscopy 67. However, these studies used serum-free media and a much lower concentration of oleate (0.1 mM), which may have resulted in suboptimal stimulation of apoB and VLDL secretion, thereby obscuring an effect of apoA-IV. Another important caveat in comparing these
studies is that rat apoA-IV is much more hydrophobic than human apoA-IV and it lacks one of four EQQQ repeats that are a unique feature of the human apoA-IV C terminus and may mediate the interaction between apoA-IV and apoB.

Previous studies from our lab found evidence for a direct presecretory protein-protein interaction between apoA-IV and apoB that decreased the trafficking kinetics of apoB and increased TG secretion. Considered together with these previous findings, our current data suggest that apoA-IV may act as a chaperone that modulates the trafficking of apoB through the secretory pathway, thereby prolonging the residence time of nascent apoB-containing particles in cellular compartments where lipidation occurs, enhancing TG loading and, ultimately, increasing TG secretion via secretion of larger lipoprotein particles.

Given the critical role of MTP in the assembly and secretion of TG-rich lipoproteins it is also pertinent to consider whether the impact of apoA-IV on TG secretion observed in the present studies was mediated by an effect on MTP gene expression. Using the IPEC-1 intestinal cell model, Yao et al. observed that Dox-induced expression of swine apoA-IV increased MTP gene expression by ~80%; in a previous study, this was associated with a 2-fold increase in TG secretion. However, Dox-induced expression of a truncated “pig-like” human apoA-IV (lacking the distinctive C-terminal repeated EQQQ motif) increased MTP gene expression by only ~50%. As previous studies noted a 25-fold increase in TG secretion associated with the “pig-like” construct it would appear the MTP
induction by apoA-IV cannot account for of its impact of TG secretion. In the
present study, we observed that Dox-induced expression of human apoA-IV
cau a nonsignificant 25% increase in MTP mRNA abundance and no
detectable change in protein levels (Fig. 6). Hence, it is unlikely that MTP is
responsible for apoA-IV’s impact on TG secretion in our current studies.

While these data elucidate how apoA-IV could modulate lipoprotein assembly
and lipid absorption at the cellular level, the puzzling issue has remained that no
obvious phenotypes were observed in either apoA-IV knockout ⁷² or human
apoA-IV transgenic mice ⁷³. One possibility is that in these studies lipid
absorption was determined by plasma TG appearance curves after a single lipid
bolus. Because the intestine possesses a large reserve capacity for lipid
absorption ⁷⁴, ⁷⁵, it is possible that these animals were not given a big enough
lipid challenge to discern an effect of apoA-IV deletion or overexpression on
dietary fat absorption efficiency.

With the current disconnect between in vitro findings and the observations from
mouse models, additional studies are necessary to discern the in vivo
consequences of apoA-IV expression. An essential tool in examining the role of
apoA-IV in vivo is an effective antibody. As there are no reliable commercially
available antibodies that detect mouse apoA-IV, we developed rabbit antiserum
that recognizes the full length, mature protein. Thus, we are now well positioned
to explore the role of endogenous apoA-IV on mouse lipid metabolism, in vivo.
In summary, our data from a hepatic cell line model establishes that upregulation of gene expression of apoA-V and apoA-IV affects VLDL-TG secretion by altering lipoprotein particle expansion. These data suggest that in addition to its well established function in regulating plasma TG levels by catalyzing the peripheral lipolysis and clearance of TG-rich lipoproteins, apoA-V may also play a critical role in modulating hepatic lipoprotein secretion and TG storage. ApoA-V may thus stand at the crossroads between hepatic lipid storage and export and may be an important factor in determining human susceptibility to hepatic steatosis, lipotoxicity, and insulin sensitivity.

Additionally, our apoA-IV data established that expression of human apoA-IV in McA-RH7777 rat hepatoma cells facilitates TG transport by increasing the size and core TG content of lipoproteins. When considered in the light of previous work on the structure and function of apoA-IV and apoB, we propose that by functioning as a secretory chaperone for apoB, apoA-IV increases the residence time of nascent apoB-containing lipoproteins in intracellular compartments where second-step lipidation occurs, thereby allowing more time for them to undergo core expansion prior to secretion. In the liver, increased apoA-IV expression induced by hepatic fat accumulation could play a role in mediating more efficient TG export and hence have beneficial effect on resisting steatosis induced by diet and other metabolic perturbations\textsuperscript{76}.
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CHAPTER III

APOLIPOPROTEIN A-IV EXPRESSION IN MOUSE LIVER ENHANCES TRIGLYCERIDE SECRETION AND REDUCES HEPATIC LIPID CONTENT BY FACILITATING VLDL PARTICLE EXPANSION

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Abstract

Objective: Previous studies have demonstrated that apolipoprotein A-IV (apoA-IV) expression promotes apoB lipoprotein-mediated triglyceride (TG) secretion in transfected enterocytes and hepatoma cells; however, evidence for a role in lipid transport in vivo is lacking. Using mouse models we explored the role of apoA-IV in hepatic VLDL-mediated lipid efflux under conditions which promote hepatic steatosis.

Approach and Results: Hepatic steatosis induced by either a high fat diet or enhanced de novo lipogenesis caused by transgenic overexpression of SREBP-1a (SREBP-1aTg) was associated with a robust induction (up to 43-fold) of hepatic apoA-IV mRNA and protein levels. In both models, a positive linear correlation between hepatic TG content and apoA-IV mRNA abundance was observed ($r^2 = 0.8965$). To examine whether induction of apoA-IV affected hepatic TG secretion, SREBP-1aTg mice were crossed with apoA-IV knock out mice (A4KO). With Triton blockade of peripheral lipolysis, SREBP1aTg/A4KO mice demonstrated a 24% reduction in hepatic TG secretion rate, relative to SREBP1aTg controls, but no change in apoB production. Negative stain electron microscopy revealed a 33% decrease in the abundance of secreted large VLDL particles with diameters >120 nm. Conversely, mice infected with a recombinant human apoA-IV adenovirus demonstrated a 38% increase in hepatic TG secretion rate and a 39% reduction in liver TG content relative to LacZ controls, associated with a 43% increase in large diameter VLDL particles and no change in apoB secretion.
Conclusions: Hepatic steatosis in mice induces hepatic apoA-IV expression, which, in turn, promotes lipoprotein particle expansion and reduces hepatic lipid burden without increasing the number of secreted atherogenic apoB-containing lipoprotein particles.
Introduction

The epidemic of obesity and associated metabolic syndrome has caused a rapid increase in the incidence of nonalcoholic fatty liver disease (NAFLD), which now affects approximately one-third of adults in developed countries \(^1\), \(^2\). A predominant means by which the liver protects itself from excess lipid accumulation is the assembly and secretion of very low density lipoproteins (VLDL) \(^1\). Bulk triglyceride (TG) export from the liver can be increased by two non-exclusive mechanisms: assembly and secretion of a greater number of VLDL particles or secretion of larger VLDL particles which contain an increased amount of core lipid. Although a broad understanding of the VLDL assembly pathway has emerged, the question of how the liver integrates particle number with particle size to achieve a given rate of hepatic lipid efflux is poorly understood \(^3\), \(^4\), \(^5\).

One factor which may play a role modulating apoB lipoprotein assembly and particle expansion is apolipoprotein (apo) A-IV. ApoA-IV is a 46 kDa lipid binding protein which is expressed in the mammalian intestine, but also in rodent liver. Since its discovery in 1977, apoA-IV has been ascribed a wide variety of functions in lipid metabolism and metabolic regulation \(^6\)-\(^9\). Perhaps the most notable characteristic of apoA-IV is the close association between active intestinal lipid absorption and induction of intestinal apoA-IV gene expression \(^10\). The first direct functional connection between apoA-IV expression and bulk lipid transport was observed in a cultured pig intestinal epithelial cell model (IPEC-1),
in which transfection of apoA-IV constructs strongly enhanced transcellular TG transport, primarily by promoting lipoprotein particle expansion \textsuperscript{11, 12}. Similar results were observed in transfected rat hepatoma cells, where the impact of apoA-IV on apoB lipoprotein assembly was attributed to its ability to interact with apoB within the secretory pathway and alter the trafficking kinetics of nascent apoB-containing lipoproteins \textsuperscript{13, 14}. However, despite the considerable physiological and \textit{in vitro} evidence linking apoA-IV and intestinal TG transport, a longstanding conundrum has been that no significant impact of genetic apoA-IV deficiency or transgenic overexpression on intestinal lipid absorption and growth was observed in mouse models \textit{in vivo} \textsuperscript{15, 16}.

In contrast to the intestine - an organ which possesses both excess absorptive capacity and robust adaptive mechanisms which can mask subtle defects in lipid transport \textsuperscript{17} - TG transport in the liver is highly sensitive to signals which regulate lipid metabolic pathways, as evidenced by the many genetic, hormonal, and dietary factors which can lead to NAFLD \textsuperscript{1, 18}. We therefore have explored the function of apoA-IV in the liver by assessing its impact on TG secretion in two models which promote hepatic steatosis, i.e., unregulated \textit{de novo} lipogenesis caused by expression of the constitutively active form of SREBP-1a \textsuperscript{19, 20} and feeding a high fat/high cholesterol diet. We found that, unlike in the intestine, apoA-IV deficiency and overexpression exert a powerful impact on hepatic TG secretion rate and lipid content, due to its ability to promote nascent VLDL particle expansion. These data provide the first evidence for a direct role of
apoA-IV in modulating lipid transport in vivo, and suggest that its expression can dramatically enhance VLDL-mediated hepatic TG efflux by promoting particle expansion without increasing the number of atherogenic apoB lipoprotein particles secreted by the liver.
Materials and Methods

Animals

All animal procedures were approved by the Wake Forest School of Medicine Animal Care and Use Committee. C57BL/6 mice (Harlan) were fed either a chow diet or a high fat/high cholesterol diet (HFHC, 48% of calories from fat, 0.2% cholesterol) for 16 weeks. SREBP-1a<sup>Tg</sup> mice (The Jackson Laboratory, stock #002840) were crossed with apoA-IV knock out mice<sup>17</sup> to yield SREBP-1a<sup>Tg</sup>:A4-KO and SREBP-1a<sup>Tg</sup>:A4-WT littermates. Transgenic mice were maintained on chow diet throughout the study or at 6–8 weeks of age were switched to a low carbohydrate, high protein diet (Purina Test Diet #5789) for 2-3 weeks. ASO-treated mice were obtained from the lab of J. Mark Brown and described previously<sup>21</sup>. Briefly, C57BL/6N of 8 weeks of age were either maintained on standard rodent chow diet or switched to a HFD (~45% of energy as lard and ~0.015% (w/w) cholesterol). In conjunction with diet feeding, mice received biweekly intraperitoneal injections for 8 weeks of either saline, a nontargeting control ASO (Control), or an ASO targeting CGI-58 obtained from ISIS Pharmaceuticals (Carlsbad, CA). For liver-specific knock out of MTP, mice with a floxed MTP allele (MTP<sup>floxfloxflox</sup>), obtained from Dr. Lawrence Chan (Baylor College of Medicine) were crossed with albumin Cre mice to generate liver-specific deficiency of MTP. Eight week-old MTP<sup>flox/flox</sup> or MTP-LKO mice were maintained on chow diet or HFHC (described above) for 4 weeks. For all models, mice were euthanized with ketamine and xylazine. Blood was collected via heart
puncture, and organs were perfused with saline. Livers were harvested and snap frozen in liquid N\textsubscript{2}.

**Recombinant Adenoviruses**

Recombinant adenoviruses expressing genes for either human apoA-IV or LacZ, under control of the human cytomegalovirus promoter, were constructed using the Adeno-X system (Clontech #631513). Recombinant adenoviruses were expanded in HEK293 cells, purified by cesium chloride gradient ultracentrifugation, and stored in 10% (v/v) glycerol in phosphate-buffered saline at −80°C, as recommended by the supplier. Retro-orbital injection of 1.5 \times 10^9 plaque forming units (pfu) of adenovirus per mouse was administered under isofluorane sedation. Three days after injection, the mice were euthanized, and plasma and liver tissue were harvested as described above.

**mRNA and Protein Quantification**

RNA was extracted from frozen liver samples using TRIzol (Invitrogen). Total RNA was reverse transcribed into cDNA with random primers using the Omniscript RT kit (Qiagen, Valencia, CA) or qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR (qPCR) was performed using a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). A typical PCR reaction (20 µl) contained 10 µl 2X Fast SYBR Green Master Mix (Applied Biosystems), 1 µl each of 5 µM forward and reverse primers, and 25 ng of cDNA. Copy numbers were normalized to GAPDH. The following mouse primers were used: forward
apoA-IV, TTC CTG AAG GCT GCG GTG CTG; reverse apoA-IV, CTG CTG AGT GAC ATC CGT CTT CTG; forward GAPDH, TGT GTC CGT GGA TCT GA; reverse GAPDH, CCT GCT TCA CCA CCT TCT TGA T; forward CREB-H, GGC CAT TGA CCT GGA CAT GT; reverse CREB-H, TTC ACA GTG AGG TTG AAG CGG; forward HNF-4α, ATG CGA CTC TCT AAA ACC CTT G; reverse HNF-4α, ACC TTC AGA TGG GGA CGT GT; forward PPARα, TCT GTC GGG ATG TCA CAC AA; reverse PPARα, TGT TCA CAG GTA AGA ATT TCT GCT; forward SREBP-1c, CAG CCA CAC TTC ATC AAG G; reverse SREBP-1c, ACT GTG GCC AAG ATG GTC CCT.

For immunoblot analysis ~500 mg of frozen tissue was homogenized with a Polytron homogenizer in lysis buffer (25 mM Tris HCl pH 7.4, 300 mM NaCl and 1% Triton X-100 containing 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin). Protein was separated by SDS-PAGE and then transferred to a PVDF membrane. ApoA-IV protein mass was detected using rabbit anti-serum raised against purified mouse apoA-IV protein (1:1000 dilution) or rabbit anti-serum raised against purified human apoA-IV protein (1:2000 dilution). LacZ was detected with an anti-β-galactosidase monoclonal antibody.

**Analysis of Plasma and Liver Lipids**

Following a 4 hour fast, blood was collected by heart puncture and was placed into a tube containing a protease inhibitor cocktail (Sigma #P2714) dissolved in 0.05% EDTA, 0.05% NaN₃. Blood was centrifuged at 12,000 x g for 10 min at
4°C, and the plasma was analyzed for TG concentration using an enzymatic colorimetric assay (Triglycerides/GB kit, Wako). For analysis of liver lipids, ~100 mg of liver was thawed, minced, and weighed in a glass tube. Lipids were extracted in 2:1 CHCl₃/methanol and dried down under a stream of nitrogen; 1% Triton X-100 in CHCl₃ was then added and the solvent was evaporated. Deionized water was then added to each tube and vortexed until the solution was clear. Lipids were then quantified with an enzymatic colorimetric assay (Triglycerides/GB kit, Wako).

**Hepatic TG and ApoB Secretion Rates**

After a 4 hour fast, mice were anesthetized by isofluorane inhalation and then given 500 mg/kg Triton WR1339 (Triton) (Sigma #T0307-5G) and in some studies, 200 µCi [³⁵S]Met/Cys (PerkinElmer) by retro-orbital injection. Blood was collected in heparanized capillaries by retro-orbital bleeding at 0 (before injection), 30, 60, 120, and 180 min. TG concentration in plasma samples was measured by enzymatic assay, as described above. TG secretion rates were calculated as the mean slope of linear regressions time versus plasma TG for each individual animal, using GraphPad Prism 5. To measure secretion of newly synthesized apoB, 15 µl of the plasma from terminal bleeds was diluted into 1 ml of lysis buffer containing protease inhibitors (above), and 0.2% bovine serum albumin. Samples were immunoprecipitated by addition of 5 µl of rabbit anti-mouse apoB antibody (Biodesign). After 18 h of incubation with rotation at 4 °C, 20 µl of protein G-Sepharose (50:50 slurry; Amersham Biosciences) was added
to the samples followed by an additional 90 min incubation. Beads were collected by centrifugation at 10,000 rpm for 10 s and washed three times with lysis buffer. Proteins were eluted from the beads by heating (100°C for 5 min) in SDS-PAGE sample buffer and fractionated by 12.5% SDS-PAGE. Gels were then dried and visualized with a Fuji BAS5000 PhosphorImager.

**Electron Microscopy of Plasma VLDL**

For plasma lipoprotein fractionation, 30 μl of post-Triton block plasma was diluted in 2 mL of saline containing 1mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 mM EDTA and centrifuged at 100,000 rpm for 4 hours at 4°C using a TLA 100.2 rotor (Beckman) in a TL-100 centrifuge. The d<1.006 g/mL (VLDL) fraction was collected from the top 0.5 ml of the tube by tube slicing. For negative staining, VLDL were absorbed onto Formvar substrate 200 mesh copper grids stabilized with carbon (Pelco #01801) for 30 seconds. The grid was then stained with 2% phosphotungstic acid (pH 6.6) for 1 minute, and excess stain was removed using filter paper. Grids were then examined using a FEI Technai BioTwin 120 keV transmission electron microscope.

**Statistics**

Results are presented as means ± S.E. Data were analyzed using GraphPad Prizm by unpaired Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons, as indicated.
Results

Hepatic ApoA-IV Expression is Increased in Steatosis Caused by a High Fat Diet and Increased de novo Lipogenesis

Two models of hepatic steatosis were employed to assess the impact of cellular TG content on hepatic apoA-IV expression. In the first model, transgenic expression of a constitutively active form of SREBP-1a (SREBP-1aTg) promotes the transcription of genes responsible for de novo hepatic lipogenesis. As the transgene is under the control of the PEPCK promoter, feeding a low carbohydrate diet further induces SREBP-1a, which upregulates lipogenic gene expression and causes massive TG accumulation. As shown in Figure 1A, SREBP-1a transgenic mice (Tg) displayed a 15-fold increase in liver TG content relative to wild type littermates (WT) on the chow diet and a 35-fold increase on the low carbohydrate diet. Under these conditions, apoA-IV mRNA abundance increased 18- and 43-fold, respectively (Figure 1B); immunoblot analysis confirmed that this was accompanied by a corresponding increase in hepatic apoA-IV protein levels (Figure 1C). In the second model, hepatic steatosis was induced by feeding a HFCD diet. After 16 weeks on diet, liver TG content had increased 15-fold relative to chow-fed controls (Figure 1D) and apoA-IV mRNA abundance increased 27-fold (Figure 1E), with a corresponding increase in apoA-IV protein mass (Figure 1F). Analysis of all data from both models revealed a strong positive linear correlation between apoA-IV mRNA abundance and hepatic TG content (Figure 1G). These data indicate that hepatic apoA-IV mRNA and protein levels are dramatically increased by conditions which promote
Figure 1. Hepatic apoA-IV expression and TG content in two models of steatosis. SREBP-1a^{Tg} (SB-Tg) and wild-type littermates were fed either a chow or low carbohydrate (Low Carb) diet for 2 weeks. Mice were sacrificed after a four hour fast and liver and blood were collected. A, Hepatic TG content measured by enzymatic assay. B, ApoA-IV mRNA levels measured by quantitative PCR. C, Hepatic apoA-IV protein mass detected by western blot. C57BL/6J mice were fed either chow or a high fat diet (48% calories from fat, 0.2% cholesterol) for 16 weeks. D, Hepatic TG content measured by enzymatic assay. E, ApoA-IV mRNA levels measured by quantitative PCR. F, Hepatic apoA-IV protein mass detected by western blot. G, Correlation between apoA-IV mRNA abundance and hepatic TG content. Linear regression and Pearson correlation coefficients are given. Data are mean ± SE (n = 5). Bars labeled with different letters are P<0.05 by ANOVA. *P<0.0001, #P<0.005 by unpaired Student’s t test.
hepatic TG accumulation, and suggest that apoA-IV gene expression may be regulated either directly or indirectly by intracellular TG content.

**ApoA-IV Deficiency in SREBP-1a\(^{Tg}\) Mice Reduces Hepatic TG secretion**

To determine if the induction of apoA-IV expression that accompanies hepatic TG accumulation impacts TG secretion, we examined the consequences of apoA-IV gene inactivation in SREBP-1a\(^{Tg}\) mice. For this purpose, SREBP-1a\(^{Tg}\) mice were crossed with apoA-IV knockout mice (A4-KO) to yield SREBP-1a\(^{Tg}\)/A4-KO mice and SREBP-1a\(^{Tg}\)/wild type littermates (SREBP-1a\(^{Tg}\)/A4-WT). Mice were maintained on the low carbohydrate diet for 3 weeks, after which hepatic TG secretion rates were measured as described above. The absence of apoA-IV expression in the SREBP-1a\(^{Tg}\)/A4-KO mice significantly reduced plasma TG accumulation at times greater than 30 minutes compared to the SREBP-1a\(^{Tg}\)/A4-WT control littermates (Figure 2A), and reduced the overall hepatic TG secretion rate by 24% (Figure 2B). Although there was a trend towards increased hepatic TG content in the SREBP-1a\(^{Tg}\)/A4-KO mice, this difference was not significant (Figure 2C). These data indicate that in the absence of apoA-IV, SREBP-1a\(^{Tg}\) mice were less efficient in secreting TG from the liver.

To explore the basis for the lower TG secretion rate in SREBP-1a\(^{Tg}\)/A4-KO mice, we first considered whether apoB secretion had been affected. SDS-PAGE and phosphorimager analysis of radiolabeled apoB in plasma VLDL revealed no differences in the amount of total secreted apoB protein (Figure 2D and E),
Figure 2. ApoA-IV deficiency inhibits TG secretion in SREBP-1a\textsuperscript{Tg} mice. SREBP-1a\textsuperscript{Tg} and apoA-IV knockout mice were cross bred and TG secretion was measured in SB-Tg/A4WT mice and their SB-Tg/A4KO littermates. Following a 4 hour fast, mice were injected with 500 mg/kg Triton WR1339 to inhibit lipolysis and 200 µCi of \[^{35}\text{S}\]Met and Cys to measure apoB production. Blood samples were collected at 0, 30, 60, 120, and 180 minutes post Triton injection. A, Plasma TG concentration. B, Mean secretion rate calculated from the slopes of individual plots of TG versus time. C: Hepatic TG content. D, Accumulation of newly synthesized and secreted plasma apoB in terminal plasma. E: Relative phosphorimager intensity of total apoB (apoB100 + B48), normalized to SB-Tg/A4-WT mice sample. Data expressed as mean ±SE (for A-C, n = 8; for D and E, n = 5). *P<0.05, **P<0.01 by unpaired Student’s t test.
indicating that apoA-IV deletion did not change the number of secreted apoB-containing VLDL particles. We next analyzed the size distribution of VLDL isolated from the Triton block experiments using negative stain electron microscopy (EM) (Figure 3A). Visual inspection of representative images suggested that plasma VLDL from the SREBP-1a\textsuperscript{Tg}/A4-WT mice contained a greater proportion of larger lipoprotein particles compared to the SREBP-1a\textsuperscript{Tg}/A4-KO mice (Figure 3A). Indeed, systematic analysis of the particle size distribution revealed that the percentage of VLDL particles with diameters $\geq 120$ nm was lower in SREBP-1a\textsuperscript{Tg}/A4-KO plasma (Figure 3B and C). This finding suggests that VLDL particle expansion was impaired in the absence of apoA-IV expression.

**Overexpression of Human ApoA-IV Increases Hepatic TG Secretion in SREBP-1a\textsuperscript{Tg} Mice.**

As apoA-IV deficiency appeared to decrease the rate of hepatic TG secretion by reducing VLDL particle size, we next explored whether overexpression of human apoA-IV in SREBP-1a\textsuperscript{Tg} mouse liver would have the converse effect. For this purpose we generated a recombinant adenovirus expressing human apoA-IV (ad-huA4) and a control adenovirus expressing bacterial $\beta$-galactosidase (ad-LacZ). Infection of SREBP-1a\textsuperscript{Tg} mice with the ad-huA4 and ad-LacZ adenoviral constructs caused abundant hepatic expression of the respective proteins 3 days post-injection (Figure 4A). Mice receiving ad-huA4 also demonstrated an increase in plasma TG levels to $\sim 175$ mg/dL, presumably due to increased VLDL
Figure 3. ApoA-IV deficiency in SREBP-1a<sup>Tg</sup> mice causes reduced VLDL particle diameter. Pooled blood samples were collected 180 minutes after Triton injection and VLDL was isolated from plasma by ultracentrifugation. **A**, VLDL visualized by negative staining EM (x 49,000). **B**, Size distribution of VLDL particles (more than 500 particles were measured). **C**, Percentage of total VLDL particles ≥120 nm.
Figure 4. Adenoviral overexpression of human apoA-IV increases the rate of TG secretion in SREBP-1aTg mice. SREBP-1aTg (SB-Tg) mice maintained on chow diet were administered 1.5x10⁹ pfu of either apoA-IV-adenovirus (huA4) or the control LacZ-adenovirus (LacZ). After 3 days, animals were fasted for 4 h and liver and blood were collected. A, LacZ and huA4 protein in liver as detected by western blot. B, Plasma TG levels were measured by enzymatic assay. C, Following a 4 hour fast, mice were injected with 500 mg/kg Triton WR1339 and blood samples were collected at 0, 30, 60, 120, and 180 min post injection. Plasma TG content was measured by enzymatic assay and the TG concentration at t=0 was subtracted from each time point. D, Mean secretion rate calculated from the slopes of individual plots of TG versus time. E, Hepatic TG content measured by enzymatic assay. Data are expressed are mean ± SE. For B-D, n = 8 for LacZ and 9 for huA4. For E, n = 6, *P<0.01 by unpaired Student’s t-test.
secretion and/or inhibition of VLDL clearance by displacement of the lipoprotein lipase activator apoC-II from their surface by apoA-IV\textsuperscript{15}.

To explore the impact of apoA-IV overexpression on the hepatic TG secretion rate, mice treated with ad-huA4 and ad-LacZ mice were given Triton to block lipolysis, and plasma TG accumulation was measured as a function of time. In SREBP-1\textsuperscript{a}\textsuperscript{Tg} mice treated with ad-huA4, a 50% increase in the rate of TG secretion was observed relative to LacZ controls (Figure 4C and D). As the expression of apoA-IV produced a dramatic increase in the rate and amount of TG secreted, we examined if there was a corresponding decrease in hepatic lipid burden. For this purpose, mice were separately injected with ad-huA4 or ad-LacZ and three days post-injection, animals were sacrificed and liver lipids were measured. These data revealed a 39% decrease in the hepatic TG content of mice overexpressing huA4 compared to mice administered ad-LacZ (Figure 4E). A similar reduction in hepatic TG content was also observed in the animals that had been subjected to Triton block analysis used in Figure 4C (data not shown).

To explore the basis of the enhanced TG secretion in the mice treated with ad-huA4, radiolabeled apoB in VLDL was isolated from terminal bleeds by immunoprecipitation and quantitated by SDS-PAGE and phosphorimager analysis. As with the apoA-IV knockout experiments, no difference in the secretion of radiolabeled apoB was observed, indicating that overexpression of apoA-IV did not increase the number of VLDL particles secreted by the liver.
(Figure 5A and B). However, negative stain EM analysis of VLDL particles found that apoA-IV overexpression caused a 43% increase in the percentage of VLDL particles with diameters ≥ 120 nm (Figure 5C-5E). These data suggest that the increase in TG secretion and concomitant decrease in hepatic TG observed in mice overexpressing apoA-IV is due to secretion of larger, more TG-enriched apoB-containing VLDL, rather than a greater number of lipoprotein particles.

**Hepatic steatosis associated with impaired VLDL-TG assembly does not induce apoA-IV expression.**

As shown in Figure 1, hepatic apoA-IV expression is enhanced in two models of hepatic steatosis. We next asked if apoA-IV responds similarly in additional models of hepatic steatosis caused by disruption in different processes regulating hepatic lipid homeostasis. First, as a model of defective lipolysis and lipid signaling, anti-sense oligonucleotides (ASO) were employed to inhibit expression of CGI-58, which is a co-activator of adipose triglyceride lipase 21, 24. CGI-58 inhibition impairs VLDL production 21 and increases liver TG levels by 4-fold when fed either chow or HFD, as compared to mice treated with the control ASO (Figure 6A). Steatosis caused by CGI-58 ASO treatment increased hepatic TG to levels, reaching 150 mg/g wet weight on HFD, but actually reduced apoA-IV abundance (Figure 6B, and C).

Next, we employed the steatotic mouse model caused by ablation of hepatic microsomal triglyceride transfer protein (MTP) expression. MTP is the essential
Figure 5. ApoA-IV deficiency reduces particle diameter in SREBP-1a\textsuperscript{Tg} mice. After a 4-hour fast, mice were injected with 500 mg/kg Triton and 200 µCi \([35\text{S}]\text{Met and Cys to measure apoB production. Blood samples were collected 180 minutes post detergent injection. VLDL was isolated by ultracentrifugation, followed by immunoprecipitation with anti-apoB antibody, SDS-PAGE, and phosphorimager analysis. A, Accumulation of }35\text{S-labeled newly synthesized and secreted apoB in the terminal plasma. B, Relative phosphorimager units of total apoB (apoB100 + B48), normalized to LacZ mouse sample (n=4 per group). C: Negative staining electron microscopy (x 49,000) of pooled VLDL D: Size distribution of VLDL particles. The diameters of more than 500 particles were measured. E, Percentage of total VLDL particles ≥120 nm.}
Figure 6. Knockdown of CGI-58 induces TG accumulation but not apoA-IV expression. C57BL/6 mice were maintained on a high fat, low cholesterol diet (HFD; 45% of energy as fat and 0.015% (w/w) cholesterol) for 12 weeks while receiving injections of a control (Ctl) ASO or an ASO directed against CGI-58 (CGI). A: liver TG; B: apoA-IV mRNA; C: apoA-IV protein levels (pooled samples in duplicate) were analyzed by immunoblot analysis. Data are mean ± SE (n = 4). Bars labeled with different letters are \( P<0.05 \) by ANOVA with post hoc analysis.
cofactor for apoB lipoprotein biogenesis; thus, the liver-specific MTP knockout mouse is an ideal model of hepatic steatosis caused by defective VLDL secretion. Mice with a floxed MTP allele (MTP$^{\text{floox/floox}}$), were crossed with albumin Cre mice to generate liver-specific deficiency of MTP (MTP$^{\text{LKO}}$). We show in Figure 7 that feeding a HFHC diet to control MTP$^{\text{floox/floox}}$ mice for just 4 weeks begins to induce apoA-IV mRNA (Fig. 7B), even without a significant increase in liver TG (Fig 7A); however, in MTP$^{\text{LKO}}$ mice, the same short-term dietary regimen caused a 10-fold increase in TG accumulation, yet the apoA-IV mRNA remained at the baseline levels observed in chow fed control (MTP$^{\text{floox/floox}}$) mice. These data suggest that hepatic apoA-IV expression is not induced in these two models of hepatic steatosis caused by impaired lipolysis, lipid signaling, and VLDL secretion.
Figure 7. Analysis of liver TG and apoA-IV mRNA levels in chow and HFCD fed control and liver-specific MTP knockout mice. MTPflox/flox (f/f) and albumin Cre mice were crossed to generate liver specific MTP knockout mice (MTP-LKO; abbreviated here as LKO). A: Two individual mouse liver extracts from each genotype were fractionated by SDS-PAGE, followed by immunoblot analysis with anti-mouse MTP antibody to confirm absence of MTP protein in MTP-LKO livers. B & C: Eight week-old MTPflox/flox or MTP-LKO mice were maintained on chow diet or HFCD for 4 weeks. Liver TG (panel B) and apoA-IV mRNA levels (expressed as fold-increase above value in chow fed MTPflox/flox mice (panel C) were plotted. Data in B and C are means ± SE (n=5-6). Bars labeled with different letters are $P<0.05$ by ANOVA with post hoc analysis.
Discussion

Although a broad spectrum of physiologic functions has been proposed for apoA-IV, a preponderance of evidence suggests that it plays a role in intestinal lipid absorption and chylomicron assembly. Indeed, recent studies with cultured intestinal cells have established that apoA-IV expression increases bulk TG transport by enabling secretion of larger TG-rich lipoproteins. Nonetheless, elucidation of the specific role of apoA-IV in lipid transport in vivo has remained elusive, for studies with apoA-IV knockout and transgenic mice found no gross abnormalities in dietary lipid absorption. In part, this may be due to the fact that the intestine possesses a large absorptive reserve capacity and compensatory cellular mechanisms which can mask the impact of perturbed apoA-IV expression on lipid transport. However, in rodents, apoA-IV is also expressed in the liver. In this organ, TG uptake, synthesis, and secretion is controlled by complex metabolic pathways and regulated by multiple dietary and hormonal factors, such that even small changes in the relative rates of TG import/synthesis versus oxidation/secretion can rapidly lead to intracellular TG accumulation (steatosis) and inflammation (steatohepatitis). We thus reasoned that examining the impact of altered apoA-IV expression on hepatic VLDL secretion and TG content would reveal an unequivocal metabolic phenotype with direct clinical relevance.

We therefore examined the impact of altering hepatic apoA-IV expression by genetic deletion and adenoviral-induced over-expression on VLDL-TG secretion.
and cellular TG content in two models of hepatic steatosis: unregulated de novo lipogenesis caused by transgenic expression of the constitutively active form of SREBP-1a\textsuperscript{19,20}, and feeding a high fat/high cholesterol diet. Using this approach, we not only observed that hepatic apoA-IV gene expression and protein levels are increased by conditions that promote hepatic TG accumulation, but, for the first time, have demonstrated an unequivocal metabolic phenotype directly related to apoA-IV expression: i.e., genetic absence of apoA-IV reduces hepatic TG secretion and VLDL particle size, whereas adenoviral overexpression of human apoA-IV enhances VLDL-TG secretion, increases VLDL particle size, raises plasma TG levels, and reduces hepatic TG content. These data reveal that apoA-IV expression can exert a powerful impact on hepatic TG export, attributable to its ability to promote VLDL particle expansion in the secretory pathway.

The parallel increases in hepatic apoA-IV mRNA and TG content which we observed in steatosis induced by increased lipogenesis and a HFHC diet have been reported in other mouse models of steatosis. A 100-fold induction of hepatic apoA-IV mRNA was seen in suckling fatty liver dystrophy (fld) mice, which returned to baseline when liver TG content rapidly fell upon weaning. Williams et al. also observed that a high fat diet induces hepatic apoA-IV expression, although in their studies the effect was strain-specific\textsuperscript{36}. The strong correlation between apoA-IV mRNA, protein abundance, and hepatic TG content in all these
models of steatosis implies that cellular TG or fatty acid accumulation may provide a specific signal for upregulating apoA-IV gene expression.

In this regard, the mechanisms by which lipids regulate apoA-IV gene transcription are not well understood \(^{37-39}\). Two hormone response elements in the 5' proximal promoter of the apoA-IV gene bind HNF4\(\alpha\) and HNF4\(\gamma\), and are both necessary to generate the physiological pattern of intestinal apoA-IV gene expression \(^{38}\). ApoA-IV expression is also induced by PPAR\(\alpha\) in human and murine hepatoma cells \(^{40}\). Interestingly, free fatty acids serve as ligands for these nuclear receptors, but the functional significance of this interaction is unclear \(^{41}\).

Recently, it was discovered that the endoplasmic ER-tethered, liver-specific transcription factor, cAMP response element-binding protein H (CREB-H) is required for hepatic apoA-IV synthesis \(^{42}\). Activation of CREB-H requires translocation from ER to Golgi, proteolytic processing, and release of the active form back into the nucleus. Although the exact metabolic cues which mediate this activation sequence are unknown \(^{43}\), CREB-H expression is enhanced by a number of metabolic conditions - including fasting, insulin resistance \(^{44}\) and ER stress \(^{45}\) - which also promote VLDL secretion \(^{46, 47}\).

Nonetheless, several observations suggest that it is processes which direct the assembly of apoB-containing lipoproteins, not intracellular signaling by fatty acids or TG, which drive apoA-IV gene expression. Intestinal apoA-IV RNA and protein synthesis increase up to 5-fold during absorption of long-chain fatty acids, which
require chylomicron assembly for secretion into lymph; yet absorption of short chain fatty acids — which do not require chylomicron assembly for transport into the portal circulation 48 — does not increase apoA-IV gene expression or protein synthesis 49. The hydrophobic surfactant Pluronic L-81 simultaneously blocks intestinal apoA-IV synthesis, chylomicron assembly, and TG secretion, but has no effect on fatty acid uptake or enterocyte TG accumulation 33, 34. Thus, it is intriguing to consider that the MTP mediated movement of lipid across the ER membrane 50-52 or incorporation into nascent primordial particles in the ER 53 integrates apoA-IV gene expression with VLDL assembly, perhaps via CREB-H production and/or processing, which then, in turn, serves as the specific signal which regulates apoA-IV transcription.

This consideration then raises the key question as to whether apoA-IV gene expression is upregulated in models of steatosis in which chylomicron assembly is either decreased or completely inhibited. For example, knockdown of CGI-58 inhibits lipolysis of TG stored in cytosolic fat storage droplets, thereby impeding their entry into the rough ER, which reduces VLDL production and causes a 4-fold increase in liver TG content 51, 52; however CGI-58 knockdown does not enhance hepatic apoA-IV expression. In fact, on a HFD, inhibition of CGI-58 reduced apoA-IV expression. Likewise, apoA-IV expression was not stimulated by hepatic steatosis in liver-specific MTP knockout mice (MTP-LKO). In MTP-LKO mice, TG-rich lipoprotein assembly is completely inhibited, and the liver accumulates massive amounts of lipid, which cannot be secreted into the
circulation. In both of these models with impaired VLDL-TG assembly, we observed a dissociation of hepatic TG content and apoA-IV levels. Hence, while TG accumulation in the liver may be necessary for apoA-IV induction, it may not be sufficient. In fact, the demands for VLDL assembly could underlie the integration of VLDL assembly with the regulation of apoA-IV and perhaps other genes involved in TG mobilization. To confirm this hypothesis, additional models could be employed, such as the murine model of NASH induced by a methionine-choline deficient diet. In these mice intracellular TG accumulate secondary to reduced VLDL secretion, increased hepatic fatty acid uptake, and impaired fatty acid oxidation. Examination of the behavior of apoA-IV in numerous models could not only be very informative in elucidating the cellular mechanisms which regulate apoA-IV gene expression, but could also lead to new approaches to treating NAFLD and nonalcoholic steatohepatitis.

Two mechanisms have been proposed to explain how apoA-IV facilitates the assembly of larger lipoprotein particles. As only a single molecule of apoB is incorporated into each nascent lipid particle in the first step of TG-rich lipoprotein assembly, the expansion of the TG core in the second stage of assembly exposes surface lipids to the aqueous milieu, which decreases their free energy of stabilization. As the interfacial properties of apoA-IV are ideally suited to stabilizing expanding lipid interfaces, we have proposed that adsorption of apoA-IV molecules to the expanding particle surface renders core expansion more thermodynamically favorable, which thus facilitates particle growth.
However, we have also presented evidence in cultured rat hepatoma cells that a protein-protein interaction between apoA-IV and a domain in the amino terminus of apoB delays the secretory trafficking of nascent TG-rich lipoproteins in a manner which enables their cores to become more fully lipidated prior to final secretion\textsuperscript{14}. These mechanisms are not mutually exclusive.

In summary, we have demonstrated in two models which cause liver steatosis that hepatic TG accumulation and apoA-IV RNA levels increase in parallel, and that adenoviral-mediated hepatic apoA-IV expression increases the rate of hepatic VLDL-TG transport and reduces liver TG content by enabling the assembly of larger apoB-containing lipoprotein particles, rather than by increasing the number of particles that are secreted. As efficient TG export is an important means by which the liver protects itself from toxic accumulation of intracellular lipids, these data suggest that increasing hepatic apoA-IV expression by dietary, pharmacological, or biological approaches could constitute an novel strategy for treating non-alcoholic fatty liver disease without raising the long term risk of atherosclerotic cardiovascular heart disease due to increased secretion of increased numbers of atherogenic apoB-containing lipoproteins.
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CHAPTER IV
SUMMARY AND CONCLUSIONS

Introduction

Triglyceride (TG), which is composed of three fatty acids esterified to a glycerol backbone, is the most concentrated biological fuel available and is utilized by both higher and lower organisms as an energy source via oxidative mechanisms. However, TG is extremely hydrophobic, which presents unique challenges for intestinal absorption and transport through the circulation. In order to move through the aqueous environment of the small intestine, TG is emulsified by bile acids and then hydrolyzed by pancreatic lipase to yield two free fatty acids (FFA) and a single molecule of monoacylglycerol (MAG) $^1$. Both FFA and MAG are taken up by enterocytes at the brush border membrane by both active and passive mechanisms $^2$. As bile acid micelles cannot at high concentrations be used in the blood to solubilize TG, as this would perturb cellular membranes $^3$, and transport of FFA in monomolecular form is inefficient, animal evolution gave rise to a distinct transport vehicle for moving TG and other neutral lipids and hydrophobic compounds through the blood—the lipoprotein.

The evolution of lipoproteins

The evolution of lipoproteins arose from a need to enhance the efficiency of diet-derived fatty acid transport, storage and utilization, while conserving the advantage of TG as a concentrated energy source. In lower organisms, such as
nematodes, the intestine is the site of both lipid adsorption and synthesis and there exists a relatively straight-forward, unidirectional flux of lipid transport from intestine to peripheral tissues\(^4\). However, even in these systems, lipids are transported on so-call primitive lipoproteins composed of neutral lipids, surface lipids and lipid binding proteins, such as vitellogenin\(^5\).

The evolution of insects brought a more complex lipid transport system\(^6\). In insects, dietary and endogenously synthesized lipid is transferred from the mid-gut to the fat body, where it is esterified to form TG for storage as lipid droplets. However, reusable shuttles were necessary to transport lipids stored in fat body for use by flight muscle and for other metabolic needs. Hence, high density lipophorins (HDLp) are produced within the fat body secretory pathway\(^7\). Upon their secretion into the hemeolymph, they function as efflux acceptors for TG lipolytic products generated in fat body cells and which are translocated across the plasma membrane by the extracellular lipid transfer particle. As these lipids, primarily in the form of diacylglycerol, are incorporated into the HDLp, the particle is enlarged to become a low density lipophorin. These lipid-rich particles then shuttle their lipid to flight muscle, where they discharge their cargo via a process of selective lipid uptake. These lipid-depleted lipophorins then return to the fat body to complete another round of transport. The ability of the fat body to both store and mobilize lipid and engage in lipoprotein formation and secretion suggests that this cell type may be a hybrid progenitor of the vertebrate adipocyte and hepatocyte\(^4\).
The evolution of vertebrates resulted in large-bodied, highly active animals with increased energy requirements. Thus, there was increased survival advantage for the efficient absorption, biosynthesis, transport and storage of TG. This resulted in the anatomical separation of the lipid storage and lipoprotein assembly functions. Adipose tissue evolved as a dedicated fat storage depot, responding to hormonal cues for both lipid accretion and mobilization. In contrast, the liver played a less critical role in energy storage but provided complex integration of overall lipid and glucose homeostasis, in part by the ability to engage in de novo lipogenesis and to efflux large amounts of both endogenous and diet-derived lipids via the assembly and secretion of apolipoprotein (apo)B-containing lipoproteins.

Mammalian lipoprotein particles

To facilitate lipid transport, mammals rely on two different apoB-containing, TG-rich lipoproteins for TG transport in circulation: chylomicrons derived from the gut and very low density lipoproteins (VLDL) derived from the liver. Chylomicrons and VLDL consist of a TG-rich core also containing cholesteryl esters, which is encapsulated by a surface monolayer composed of phospholipids, non-esterified cholesterol, and apolipoproteins. Chylomicrons are produced from dietary TG during absorption. VLDL-TG can be also derived from the diet by the uptake of remnant chylomicron particles; however the predominant source of VLDL lipids arise from conversion of carbohydrate to free fatty acids via de novo lipogenesis or from hepatic accumulation of albumin-
bound fatty acids derived from adipocyte lipolysis. While hepatic free fatty acids can be oxidized by mitochondria or stored as lipid droplets, abundant lipid is transported out of the hepatocyte by the VLDL assembly and secretion pathway. While VLDL functions to transport hepatic TG to extra-hepatic tissues for both storage and utilization, VLDL is also responsible for protecting the liver from excess lipid accumulation. Disruptions in the balance between lipid accretion and efflux results in hepatic lipid accumulation, or hepatic steatosis. While often a benign state, termed nonalcoholic fatty liver disease (NAFLD), steatosis can progress to the pathological inflammatory condition of nonalcoholic steatohepatitis (NASH), which in turn can progress to cirrhosis, and ultimately hepatocellular carcinoma.

Hepatic steatosis, VLDL assembly and secretion, and cardiovascular disease

Although hepatic steatosis can be prevented by increasing VLDL production, this efflux pathway has a finite capacity. Furthermore, increasing the export of VLDL results in increased circulating plasma lipids, which is associated with increased risk of heart disease. More specifically, VLDL is exported as a TG-rich lipoprotein, but following intravascular lipolysis of TG and modification of particles by cholesterol ester transfer protein and other processes, VLDL is ultimately converted to a cholesterol ester-rich LDL particle. Increased concentrations of LDL are strongly linked with increased risk of cardiovascular disease, as at elevated concentrations these particle can enter the artery.
wall and initiate the formation of atherosclerosis \textsuperscript{14}. Thus, a delicate balance exists between the need to protect the liver from ectopic fat accumulation by increasing VLDL-TG secretion and the over-production of proatherogenic lipoprotein particles.

While, it is important to acknowledge the increased risk of cardiovascular disease associated with increased VLDL-TG production, it is also of interest that apolipoprotein (apo)B-containing lipoprotein particle number, rather than total cholesterol or LDL-cholesterol levels, may best predict susceptibility to premature cardiovascular disease \textsuperscript{11}. Therefore, enhancing TG secretion by increasing the number of VLDL particles secreted may be more proatherogenic than secreting the same amount of TG on fewer but larger particles. However, the mechanisms that integrate particle number with particle size during the lipoprotein assembly process are not well understood. Since chylomicrons are capable of achieving considerably larger particle diameters, as compared to hepatic VLDL, we chose to scrutinize the differences between these two systems. One difference that we chose to focus on is the exclusive presence of apoA-IV in the intestine, but not the liver of most mammalian species. Hence, we hypothesized that apoA-IV might be one factor responsible for promoting particle expansion during the lipoprotein assembly process.

\textbf{Regulation of hepatic apoA-IV expression by lipid}

While a broad spectrum of physiologic functions has been proposed for apoA-IV \textsuperscript{15}, its primary role may be in lipid absorption. One important piece of
evidence that supports this notion is that intestinal apoA-IV expression is acutely induced during lipid absorption in the gut. As apoA-IV is also endogenously expressed in livers of mice, it is possible that hepatic apoA-IV may also respond to lipid accumulation associated with the onset of hepatic steatosis. Indeed, previous studies in a mouse model with an autosomal recessive mutation in the lipin-1 gene, termed fatty liver dystrophy (fld), revealed neonatal mice with enlarged and fatty livers. Under these conditions a 100-fold induction of hepatic apoA-IV mRNA was seen in the suckling fld mice, which returned to baseline when liver TG content rapidly fell upon weaning. Additionally, C57BL/6 mice fed an atherogenic diet containing 1.25% cholesterol and 15% fat demonstrated a 10-fold increase in apoA-IV mRNA levels. However, a response of apoA-IV expression to the same diet was not observed in BALB/c or C3H mice. In the current studies we sought to determine if hepatic apoA-IV responds to hepatic TG accumulation in other mouse models of steatosis.

Indeed, an increase in apoA-IV expression in response to hepatic lipid accumulation was observed in two mouse models of steatosis, one caused by feeding a high fat and cholesterol diet and the other caused by increased de novo lipogenesis caused by transgenic overexpression of sterol regulatory element binding protein 1a (SREBP-1a). Both of these models demonstrated a robust increase in hepatic apoA-IV mRNA and protein mass and both models displayed a robust positive linear association between TG and apoA-IV mRNA content. These data suggest that the upregulation of apoA-IV may be signaled exclusively by the TG content within the hepatocyte. Surprisingly, however, in
two additional mouse models of hepatic steatosis, the relationship between TG and apoA-IV mRNA was not observed. In the first model, inhibition of expression of CGI-58, a co-activator of adipose triglyceride lipase, increased hepatic TG content by reducing intracellular lipolysis, thus reducing lipolytic turnover of TG and also reducing the efflux of lipid on VLDL\textsuperscript{19}. In another model of hepatic steatosis, caused exclusively by inhibition of the VLDL assembly and secretion pathway via genetic disruption of the microsomal triglyceride transport protein (MTP) gene\textsuperscript{20, 21}, it was also observed that increased liver TG failed to induce apoA-IV expression. Thus, in mouse models with reduced hepatic TG secretion, apoA-IV expression is not induced, despite the massive accumulation of hepatic TG.

In sum, these findings suggest the enhancement of apoA-IV expression in steatotic liver depends on the mechanism of lipid accretion. Specifically, it appears that only conditions of TG accumulation that simultaneously promote VLDL secretion are capable of inducing apoA-IV expression. This is similar to the regulation of apoA-IV in the intestine, where conditions that promote fat absorption via chylomicron assembly also induce apoA-IV expression, whereas conditions that promote the uptake, but not the transcellular transport, of lipid via chylomicron assembly, fail to induce apoA-IV\textsuperscript{16, 22, 23}.

Further studies in additional mouse models may be beneficial to confirm our hypothesis that the regulation of hepatic apoA-IV expression responds only to conditions of hepatic lipid accumulation accompanied by increased TG secretion. One possibility is the hyperphagic ob/ob mouse model, which has
defects in leptin signaling \(^{24}\). Young ob/ob mice develop NAFLD without accompanying NASH, unless subjected to a low dose endotoxin challenge \(^{25}\). While ob/ob mice demonstrate a 10-fold increase in \textit{de novo} hepatic lipogenesis, surprisingly no increase in VLDL-TG secretion was observed relative to lean controls \(^{26}\). Therefore, the regulation of apoA-IV expression in ob/ob mice may be of particular interest.

Another model murine hepatic steatosis is induced by the feeding of a methionine-choline deficient diet. This model promotes microvesicular steatosis secondary to reduced VLDL secretion, increased hepatic fatty acid uptake, and impaired fatty acid oxidation \(^{27,28}\). As this model progresses to NASH it provides another distinct mode of lipid dysregulation. Examination of the behavior of apoA-IV in these models would not only be informative in terms of elucidating the cellular mechanisms that regulate apoA-IV gene expression, but may also lead to new approaches for treating NAFLD and nonalcoholic steatohepatitis.

**Transcriptional regulation of hepatic apoA-IV expression**

While our current findings have unveiled intriguing results related to the hepatic expression of apoA-IV \textit{in vivo}, further scrutiny of the signaling pathways responsible for transcriptional regulation of apoA-IV are required. Currently, the transcriptional regulation of apoA-IV is not fully understood \(^{29-31}\), although several transcription factors have been implicated. It is appreciated that two hormone response elements within the 5’ proximal promoter region bind to hepatocyte nuclear factor 4\(\alpha\) (HNF-4\(\alpha\)) and \(\gamma\) and both are necessary to reproduce the
physiological pattern of apoA-IV gene expression, *in vivo* \(^{30}\). Binding of as yet unidentified transcriptional repressors also appears important for the regulation of apoA-IV by lipid absorption \(^{32}\), \(^{33}\). Interestingly, both HNF isoforms bind to fatty acid ligands, although the functional significance of this interaction in the transcriptional regulation of apoA-IV and other targets of these hormone receptors is unclear \(^{34}\).

Most recent is the discovery that the endoplasmic reticulum (ER)-tethered, hepatocyte-specific transcription factor, cAMP response element-binding protein H (CREB-H; also termed, CREB3L3) is essential for hepatic expression of apoA-IV as well as other proteins involved in TG metabolism \(^{35}\). CREB-H expression is regulated by a number of stimuli including fasting, insulin resistance \(^{36}\) and ER stress \(^{37}\). However, the metabolic cues that signal the translocation of CREB-H from ER to Golgi for proteolytic processing, and release of the active form into the nucleus, are unknown \(^{38}\). As both fasting and insulin resistance increase fatty acid and TG content of the liver and promote VLDL secretion \(^{39}\), \(^{40}\) (conditions that we believe may also promote apoA-IV expression), it is possible that enhanced lipid mobilization for VLDL assembly signals CREB-H production and/or processing leading to upregulation of apoA-IV transcription. Specifically, it is intriguing to consider that the enhanced movement of lipid across the ER membrane, mediated by MTP \(^{41}\)-\(^{43}\) and the demands for VLDL assembly, could underlie the integration of VLDL assembly with the regulation of apoA-IV and perhaps other genes involved in TG mobilization.
In addition to the factors discussed above, evidence for other transcriptional regulators of apoA-IV expression have emerged. Estrogen-related receptor alpha (ERR-α) knockout mice display decreased intestinal apoA-IV levels and impaired fat absorption in pups but not in adults. ApoA-IV is also induced in human hepatoma cells by peroxisome proliferator-activated receptor α (PPARα) and in mice and human hepatoma cells by liver X receptor (LXR). To better define how these various transcription factors coordinate the highly regulated pattern of apoA-IV expression during lipid accumulation, further scrutiny is needed. In addition to measuring mRNA expression in the various methods employed in the current studies, the DNA binding and transactivation activities of these and other transcription factors should be examined, as it is the activity of transcription factors, such as SREBP-1c and CREB-H, that determine gene regulation as opposed to the mere abundance. Thus, further studies on the transcriptional regulation of apoA-IV will be required to understand how TG content and/or the process of mobilizing TG for VLDL secretion is able to signal apoA-IV expression.

**ApoA-IV expression modulates apoB-containing lipoprotein particle expansion**

While further investigation of the regulation of apoA-IV expression is still needed, we have established in the current studies that hepatic apoA-IV expression is, in fact, increased under steatotic conditions in SREBP-1aTg mice. Thus, it was valuable to determine the consequence of this expression. In the
current study, SREBP-1a\textsuperscript{Tg} mice demonstrated a 24% reduction in hepatic TG secretion under conditions of apoA-IV deficiency. To explore the basis for this observation, particle size analysis was performed. The total average VLDL diameter was reduced 18 nm from 110 to 92 in apoA-IV deficient mice. This change corresponds to an \(~42\%\) reduction in total lipoprotein particle volume; however, only a subset of VLDL particles present in the terminal bleeds were produced after administration of Triton to inhibit lipolysis. Thus, an ill-defined proportion of VLDL in our samples is not reflective of the nascent state, as they had been undergoing lipolysis prior to Triton administration. Hence, the disproportionate decrease in total particle volume could be attributable to lipolytic modification of existing particles, which may be enhanced under conditions of apoA-IV deficiency.

As expected from the apoA-IV knockout data, overexpression of human apoA-IV in SREBP-1a\textsuperscript{Tg} mice was associated with a 38% increase in hepatic TG secretion and a corresponding 39% decrease in hepatic TG content. Again, when mean particle diameters were calculated, a 13 nm diameter increase was observed in the Ad-A4 mice, relative to the LacZ controls. This corresponds to a \(~39\%\) increase in mean volume, a value consistent with the 38% increase in TG secretion. The close agreement between the increase in TG secretion and the increase in lipoprotein particle volume is perhaps attributable to the fact that apoA-IV overexpression both increases nascent VLDL size and may also increase circulating VLDL size by virtue of its ability to inhibit lipolysis, via displacement of apoC-II \textsuperscript{48}. 
Irrespective of assumptions used to calculate changes in TG secretion attributable to changes in particle size, it is clear that the Triton block method gives primarily qualitative data in this regard. Other methodologies to more quantitatively capture nascent VLDL could include use of recirculating liver perfusion systems, where preexisting particles are not present and lipolytic modification of newly secreted particles in negligible. Another method to assess quantitatively only new secreted particles would be to employ primary hepatocytes. However, one caveat associated with this approach is that hepatocytes can change their phenotype rapidly in culture, so there is no guarantee that the nature of particles secreted under these conditions would mirror those produced in vivo or ex vivo.

Regardless of the quantitative assessment of particle volume, one of the most significant findings that emerged from our current studies established that overexpression of human apoA-IV reduced hepatic TG content and that this resulted from an increase in VLDL particle size but not in particle number, as apoB production was unaffected. These data are consistent with our in vitro studies in McA-RH7777 cells and the findings of Lu et al., who found that constitutive and Dox-induced overexpression of apoA-IV in IPEC-1 cells facilitates transcellular TG transport by increasing lipoprotein size. However, they are seemingly at odds with work done previously in mice transgenically overexpressing or deficient in apoA-IV expression. These in vivo studies focused on examining intestinal consequences of apoA-IV expression, and were not under conditions of continuous lipid challenge. Thus, any effects on hepatic
VLDL-TG production did not achieve statistical significance, although there was a trend for reduced hepatic TG VLDL-TG production in the apoA-IV knockout mice\(^{52}\).

In terms of the impact of apoA-IV deficiency and overexpression, our current studies focused on only the SREBP-1a\(^{Tg}\) mouse; however, further studies are required to discern if the effects of human apoA-IV overexpression can attenuate lipid deposition in other models of steatosis. Two models relevant to obesity and the metabolic syndrome are mice fed a high fat, high cholesterol diet and the above mentioned, hyperphagic ob/ob mice. These models could prove useful in investigating not only whether apoA-IV overexpression can attenuate hepatic lipid deposition, but also if it could delay or prevent the onset of NASH.

**Mechanism of apoA-IV particle expansion**

Conclusions from the present studies, both *in vitro* and *in vivo*, have demonstrated an augmentation of the size of apoB-containing lipoproteins in response to apoA-IV expression. However, the mechanism of particle expansion by apoA-IV is not fully understood. Previous studies from our lab found evidence for a direct presecretory protein-protein interaction between apoA-IV and apoB\(^{53}\) that decreased the trafficking kinetics of apoB and increased TG secretion\(^{54}\). Considered together with these previous findings, our current data suggest that apoA-IV may act as a chaperone that modulates the trafficking of apoB through the secretory pathway, thereby prolonging the residence time of nascent apoB-containing particles in cellular compartments where lipidation occurs\(^{55-58}\).
enhancing TG loading and, ultimately, increasing TG secretion via secretion of larger lipoprotein particles.

It was previously noted that the inhibitory effect of apoA-IV-KDEL on apoB secretion in COS cells appears between apoB21–25 and is independent of apoB lipidation. Furthermore, IPEC-1 cell transfection studies found that deletion of residues 345 to 357 in the apoA-IV C-terminus increases transcellular TG transport 25-fold but that additional truncation by only 11 residues completely prevents the effect. These data are consistent with a protein-protein interaction between specific domains in apoA-IV and apoB, and suggest that apoA-IV may act as a secretory pathway chaperone for apoB. In this regard, apoA-IV displays slower secretion kinetics than apoB and thus, its association with apoB could retard export of apoB-containing TG-rich particles from the ER and/or slow their passage through ER or post-ER lipidation compartments.

ApoA-IV could also serve as a bridge between apoB and other ER chaperone proteins that modulate apoB secretion, such as ERp72, GRP94, calreticulin, and BiP. Molecular chaperones bind to nascent polypeptides and mediate their folding into mature native forms. The folding of apoB is potentially more complex than most other secretory proteins, so molecular chaperones remain associated with apoB following its translocation into the lumen as a lipidated intermediate. The same chaperones are associated with apoB in ER and Golgi, with at least GRP94 remaining associated in the trans Golgi network. Thus, apoA-IV, by bridging apoB with one or more chaperone
proteins, could also increase residence time of apoB in ER and/or Golgi, allowing for increased lipidation.

**Therapeutic consequences of hepatic apoA-IV expression**

While the mechanism of apoA-IV’s involvement in particle expansion remains elusive, there still exists a potential therapeutic role for apoA-IV in alleviating hepatic steatosis without increasing atherogenic lipoproteins. However, a concern with increasing apoA-IV is increased plasma TG levels. Our current data reveal an increase in plasma TG concentration when human apoA-IV is adenovirally overexpressed, as did previous studies transgenically overexpressing human apoA-IV. Aalto-Setälä et al. attributed their observations to reduced TG clearance, which they hypothesized was caused by displacement of lipoprotein-catalytic apolipoproteins, such as apoC-II, by apoA-IV. While we hypothesize increased plasma TG concentration is also due to enhanced hepatic VLDL-TG production, a concern lies in an increased risk of heart disease associated with elevated levels of plasma TG. For example, elevated levels of circulating TG are seen in patients with metabolic syndrome, cardiovascular disease, and type 2 diabetes and in type 2 diabetes, overproduction of VLDL₁ is associated with insulin resistance. It is also suggested that TG-rich VLDL₁ underlies the formation of small dense LDL, which have been considered an independent predictor of cardiovascular disease risk. However, this pathway exists mainly in the setting of VLDL overproduction and dysregulated intravascular catabolism of TG-rich lipoproteins. Hence, while VLDL₁ may give
rise to small dense LDL under certain metabolic circumstances, it is unlikely that the formation of VLDL$_1$, *per se*, is the driver of this process. However, one consistent theme is that apoB particle number best predicts the risk of cardiovascular disease, superior to even LDL cholesterol levels$^{11,67-69}$. Also, the rapid catabolism of intestinally derived lipoproteins suggests a metabolic advantage associated with particle expansion, at the expense of particle number$^{70}$. Thus, under most conditions of hepatic and whole body lipid balance, the production of larger, more TG-rich VLDL at the expense of increased particle number might be advantageous by protecting the liver from abnormal lipid accumulation, while simultaneously producing a less atherogenic lipoprotein profile.

Despite the potential TG-raising impact of apoA-IV, other studies in mice have revealed apoA-IV is anti-atherosclerotic. Under conditions of feeding an atherogenic diet and in mice crossed onto an apoE/- background, transgenic overexpression of human apoA-IV produced a 41-90% reduction in atherosclerotic plaque size$^{71,72}$. ApoA-IV possesses a plethora of ancillary roles, including anti-oxidant and anti-inflammatory activities and a capacity to enhance cholesterol efflux$^{15}$. Thus, despite elevation in plasma TG levels, apoA-IV has the potential to alleviate hepatic lipid burden and decrease the risk of developing cardiovascular disease.

In summary, our current findings reveal that apoA-IV expression increases the rate of hepatic VLDL-TG transport and reduces liver TG content by enabling the assembly of larger apoB-containing lipoprotein particles, rather than by
increasing the number of particles that are secreted. As efficient TG export is an important means by which the liver protects itself from toxic accumulation of intracellular lipids, these data suggest that increasing hepatic apoA-IV expression by dietary, pharmacological, or biological approaches could constitute a novel strategy for treating non-alcoholic fatty liver disease, without raising the long term risk of atherosclerotic cardiovascular heart disease due to secretion of increased numbers of atherogenic apoB-containing lipoproteins.
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