Apolipoprotein A-IV Expression in Mouse Liver Enhances Triglyceride Secretion and Reduces Hepatic Lipid Content by Promoting Very Low Density Lipoprotein Particle Expansion

Melissa A. VerHague, Dongmei Cheng, Richard B. Weinberg, Gregory S. Shelness

Objective—Previous studies demonstrated that apolipoprotein A-IV (apoA-IV) 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 very low density lipoprotein–mediated lipid efflux under conditions that promote hepatic steatosis.

Approach and Results—Hepatic steatosis, induced by either high-fat diet or enhanced de novo lipogenesis caused by transgenic overexpression of SREBP-1a (SREBP-1aTg), was associated with up to a 43-fold induction 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 Apoa4 knockout mice. With Triton blockade of peripheral lipolysis, SREBP-1aTg/Apoa4 knockout mice demonstrated a 24% reduction in hepatic TG secretion rate, relative to SREBP-1aTg controls, but no change in apoB production. Negative stain electron microscopy revealed a 33% decrease in the abundance of secreted very low density lipoprotein particles with diameters $\geq 120$ nm. Conversely, mice infected with a recombinant human apoA-IV adenovirus demonstrated a 52% increase in the hepatic TG secretion rate, relative to controls, a 38% reduction in liver TG content, and a 43% increase in large diameter ($\geq 120$ nm) very low density lipoprotein particles, with 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. (Arterioscler Thromb Vasc Biol. 2013;33:2501-2508.)

Key Words: apolipoprotein B ■ metabolic syndrome ■ nonalcoholic fatty liver disease ■ triglyceride ■ VLDL

The epidemic of obesity and associated metabolic syndrome has caused a rapid increase in the incidence of nonalcoholic fatty liver disease, which now affects approximately one third of adults in developed countries. A predominant means by which the liver protects itself from excess lipid accumulation is the assembly and secretion of very low density lipoproteins (VLDLs). Bulk triglyceride (TG) export from the liver can be increased by 2 nonexclusive mechanisms: assembly and secretion of a greater number of VLDL particles or secretion of larger VLDL particles containing 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.

One factor that may play a role in modulating apoB lipoprotein assembly and particle expansion is apolipoprotein A-IV (apoA-IV). ApoA-IV is a 46-kDa lipid-binding protein, which is expressed not only 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. Perhaps the most notable characteristic of apoA-IV is the close association between active intestinal lipid absorption and the induction of intestinal apoA-IV gene expression. The first direct functional connection between apoA-IV expression and bulk lipid transport was observed in a cultured pig intestinal epithelial cell model, in which transfection of apoA-IV constructs strongly enhanced transcellular TG transport, primarily by promoting lipoprotein particle expansion. Similar results were observed in transfected rat hepatoma cells, in which the impact of apoA-IV on apoB lipoprotein assembly was attributed to its ability to alter the trafficking kinetics of nascent apoB-containing lipoproteins by interacting with apoB in the secretory pathway. However, despite the considerable physiological and in vitro evidence linking apoA-IV and intestinal TG transport, a
long-standing conundrum has been that no significant impact of genetic apoA-IV deficiency or transgenic (Tg) overexpression on intestinal lipid absorption and growth was observed in mouse models in vivo.\(^{15,16}\)

In contrast to the intestine—an organ that possesses both excess absorptive capacity and robust adaptive mechanisms, which may mask subtle defects in lipid transport\(^{17}\)—the liver is highly sensitive to signals that alter lipid metabolic pathways, as evidenced by the many genetic, hormonal, and dietary factors that contribute to nonalcoholic fatty liver disease.\(^{1,18}\) We, therefore, chose to explore the function of apoA-IV in the liver by assessing its impact on TG secretion under conditions of hepatic steatosis, induced by either feeding a high-fat and high-cholesterol diet or by unregulated de novo lipogenesis, caused by Tg expression of a constitutively active form of SREBP-1a.\(^{19,20}\) We found that, unlike in the intestine, apoA-IV deficiency and overexpression exert a powerful impact on hepatic TG secretion rate and lipid content, attributable to its ability to promote nascent VLDL particle expansion. These data provide the first evidence for a direct role of hepatic apoA-IV mRNA and protein levels are dramatically increased by conditions that promote hepatic TG accumulation and suggest that apoA-IV gene expression may be regulated either directly or indirectly by intracellular TG content.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Hepatic ApoA-IV Expression Is Increased in Steatosis

Two models of hepatic steatosis were used to assess the impact of cellular TG content on hepatic apoA-IV expression. In the first model, Tg expression of a constitutively active form of SREBP-1a (SREBP-1a\(^{Tg}\)) promotes the transcription of genes responsible for hepatic de novo lipogenesis.\(^{19,20}\)

Because the transgene is under the control of the PEPCK promoter, feeding a low-carbohydrate diet further induces SREBP-1a and lipogenic gene expression, causing massive TG accumulation.\(^{19}\) As shown in Figure 1A, SREBP-1a Tg mice displayed a 15-fold increase in liver TG content relative to wild-type (WT) littermates on the chow diet and a 35-fold relative 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 high-fat and high-cholesterol diet to WT C57BL/6 mice. 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 (\(r^2=0.8965\)) between hepatic TG content and apoA-IV mRNA abundance (Figure 1G). These data indicate that hepatic apoA-IV mRNA and protein levels are dramatically increased by conditions that promote 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 whether the induction of apoA-IV expression that accompanies hepatic TG accumulation affects TG secretion, we examined the consequences of Apoa4 gene inactivation in SREBP-1a\(^{Tg}\) mice. For this purpose, SREBP-1a\(^{Tg}\)

![Figure 1.](image-url)
mice were crossed with Apoa4 knockout (A4-KO) mice to yield SREBP-1aTg/A4-KO mice and SREBP-1aTg/WT (SREBP-1aTg/A4-WT) littermates. As shown in Figure 2, neither apoA-IV mRNA (Figure 2A) nor protein (Figure 2B) was detected in the livers of SREBP-1aTg/A4-KO mice. ApoA-IV deficiency produced a small reduction in plasma TG levels, consistent with previous reports, although in this study the reduction did not reach statistical significance (Figure 2C). Mice were maintained on the low-carbohydrate diet for 3 weeks, and then hepatic TG secretion rates were measured after administration of Triton WR1339 (Triton) to block peripheral lipolysis. The absence of apoA-IV expression in the SREBP-1aTg/A4-KO mice significantly reduced plasma TG accumulation at times >30 minutes compared with the SREBP-1aTg/A4-WT control littermates (Figure 2D) and reduced the overall hepatic TG secretion rate by 24% (Figure 2E). Although there was a trend toward increased hepatic TG content in the SREBP-1aTg/A4-KO mice, this difference was not significant (Figure 2F). These data indicate that in the absence of apoA-IV, SREBP-1aTg mice were less efficient in secreting TG from the liver.

To explore the basis for the lower TG secretion rate in SREBP-1aTg/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 3A and 3B), indicating that apoA-IV gene 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. Although the presence of Triton in the plasma samples might be perturbing, others have assessed particle characteristics in postdetergent plasma by size exclusion chromatography and negative stain electron microscopy. Visual inspection of representative images suggested that plasma VLDL from the SREBP-1aTg/A4-WT mice contained a greater proportion of larger lipoprotein particles compared with the SREBP-1aTg/A4-KO mice (Figure 3C). Indeed, systematic analysis of the particle size distribution revealed that the percentage of VLDL particles with diameters ≥120 nm was lower in SREBP-1aTg/A4-KO plasma (Figure 3D and 3E). 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-1aTg Mice**

Because 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-1aTg mouse liver would have the converse effect.

**Figure 2. Apolipoprotein A-IV (apoA-IV) deficiency inhibits triglyceride (TG) secretion in SREBP-1aTg mice. SREBP-1aTg/SB-Tg and Apoa4 knockout mice (A4-KO) were crossed to produce SB-Tg/A4-KO mice and wild-type littermates (SB-Tg/A4-WT). A. Relative apoA-IV mRNA abundance normalized to SB-Tg/A4-WT. B. Pooled liver samples were subjected to immunoblot analysis in triplicate to quantify relative apoA-IV abundance. C. Fast- ing plasma TG levels. D and E. After a 4-hour fast, mice were injected with 500 mg/kg Triton WR1339 to inhibit peripheral lipolysis and 200 μCi of [35S]Met and Cys to measure apoB production. Blood samples were collected at 0, 30, 60, 120, and 180 minutes post-Triton injection. D. Plasma TG concentrations after Triton block. E. Mean secretion rate calculated from the slopes of individual plots of TG vs time. F. Hepatic TG content. Data are expressed as means±SE, n=8 for A to G and F, n=5 for D and E. *P<0.05; **P<0.01 by unpaired Student t test. Tg indicates transgenic.

**Figure 3. Apolipoprotein A-IV (apoA-IV) deficiency in SREBP-1aTg mice reduces nascent very low density lipoprotein (VLDL) particle diameter. Pooled blood samples were collected 180 minutes after Triton injection, and VLDL was isolated from plasma by ultracentrifugation, as described under Materials and Methods. A. Accumulation of [35S]-labeled newly synthesized and secreted apoB in terminal plasma. B. Relative phosphorimager units of total apoB (apoaB100+B48) normalized to SB-Tg/A4-WT. C. VLDL visualized by negative stain electron microscopy (×49000). D. Size distribution of VLDL particles; >500 particles were measured. E. Percentage of total VLDL particles ≥120 nm. Data in B were analyzed by unpaired Student t test (n=5), A4-KO indicates Apoa4 knockout; Tg, transgenic; and WT, wild type.
For this purpose, we generated a recombinant adenovirus expressing human apoA-IV (ad-huA4) and a control adenovirus expressing bacterial β-galactose (ad-LacZ). Infection of SREBP-1αTg mice with either ad-huA4 or ad-LacZ caused abundant hepatic expression of the respective proteins 3 days postinjection (Figure 4A). Mice receiving ad-huA4 also demonstrated an increase in plasma TG levels to ∼175 mg/dL, presumably attributable to increased VLDL secretion and/or inhibition of VLDL clearance. Studies with apoA-IV Tg mice, with overexpression predominantly in the intestine, also noted decreased lipolysis, possibly because of displacement of the lipoprotein lipase coactivator apoC-II from the TG-rich lipoprotein surface by apoA-IV.15,25,26

To explore the impact of apoA-IV overexpression on the hepatic TG secretion rate, mice treated with ad-huA4 and ad-LacZ were administered Triton to block peripheral lipolysis and plasma TG accumulation was measured as a function of time. In SREBP-1αTg mice treated with ad-huA4, a 52% increase in the rate of TG secretion was observed relative to LacZ controls (Figure 4C and 4D). Because the expression of apoA-IV produced a dramatic increase in the rate and amount of TG secreted, we examined whether there was a corresponding decrease in hepatic lipid burden. For this purpose, mice were separately injected with ad-huA4 or ad-LacZ and 3 days postinjection, animals were euthanized and liver lipids measured. These data revealed a 38% decrease in the hepatic TG content of mice overexpressing huA4 compared with mice administered ad-LacZ (Figure 4E). A similar reduction in hepatic TG content was also observed in the animals that had been subjected to the 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 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 5B). However, negative stain electron microscopy analysis of VLDL particles revealed 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 because of secretion of larger, more TG-enriched apoB-containing VLDL, rather than a greater number of lipoprotein particles. It should be noted that the lipoprotein diameters observed in the control LacZ mice were smaller than those in the SB-Tg/A4-WT control mice in Figure 3. This is likely attributable to the fact that the secretion of very large chyomicron-sized VLDL from SREBP-1αTg mice is dependent on dysregulated de novo lipogenesis and hepatic lipid accretion.20 Because the animals used for the adenovirus experiments were maintained on a chow diet

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Adenoviral overexpression of human apolipoprotein A-IV (apoA-IV) increases the rate of hepatic triglyceride (TG) secretion and reduces hepatic lipid content in SREBP-1αTg mice. SREBP-1αTg (SB-Tg) mice maintained on chow diet were administered 1.5×10⁹ pfu of either apoA-IV adenovirus (huA4) or the control LacZ adenovirus (LacZ), and experiments were performed 3 days later. A, LacZ and huA4 protein in liver from 3 separate animals as detected by immunoblot blot analysis. B, Fasting plasma TG levels. C, Mice were injected with 500 mg/kg Triton WR1339, and TG concentrations were measured at 0, 30, 60, 120, and 180 minutes postinjection. The basal TG concentration (t=0) for each time point was subtracted. 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 as means±SE. For B to D, n=8 for LacZ and 9 for huA4. For E, n=6, *P<0.01 by unpaired Student t test. Tg indicates transgenic.

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Adenoviral overexpression of human apolipoprotein A-IV (apoA-IV) increases nascent very low density lipoprotein (VLDL) particle diameter in SREBP-1αTg mice. Blood samples from the Triton block experiment in Figure 4 were collected at the 180-minute time point. A, VLDL was isolated by ultracentrifugation, followed by immunoprecipitation with anti–apoB antibody, SDS-PAGE, and phosphorimager analysis. B, Relative phosphoimager units of total apoB (apoB100+B48) normalized to LacZ (n=4). C, Negative stain electron microscopy (×49000) of pooled VLDL. D, Size distribution of VLDL particles; >500 particles were measured. E, Percentage of total VLDL particles ≥120 nm. Tg indicates transgenic.
(ie, intermediate steatosis), their lipoprotein particles were likely smaller than those that were maintained on the low-carbohydrate diet, which induces maximal steatosis (Figure 1A).

Recent studies have demonstrated that apoA-IV might impact apoB lipoprotein particle dynamics by modulating the expression of the microsomal triglyceride transfer protein (MTP).27,28 Hence, we specifically examined whether MTP mRNA or protein mass was affected by apoA-IV. As seen in Figure 6, neither MTP mRNA nor protein abundance was affected by apoA-IV deficiency (Figure 6A and 6C) or overexpression (Figure 6B and 6D). We next explored whether other genes with roles in hepatic lipid metabolism could account for the altered TG secretion observed in these studies. No changes in expression of genes responsible for de novo lipogenesis, fatty acid esterification, intracellular lipolysis, β-oxidation, or VLDL assembly were observed (Figure III in the online-only Data Supplement), with the exception of DGAT1, where in both the A4-KO and in the ad-huA4 livers we observed a small (≈1.5- and 2-fold, respectively) but significant increase in mRNA abundance, relative to controls. Because this pattern of expression could not explain the observed changes in VLDL-TG secretion, particularly in the knockout situation, we suggest that this modest change in one lipogenic gene is not likely to be biologically relevant to the observed effects of apoA-IV on hepatic TG secretion and instead confirm the core conclusion that apoA-IV may directly impact the extent of VLDL lipidation.

**Discussion**

Although a broad spectrum of physiological functions has been proposed for apoA-IV,29 a preponderance of evidence suggests that it plays a role in intestinal lipid absorption and chylomicron assembly.30–33 Indeed, recent studies with cultured intestinal and hepatoma cells have established that apoA-IV expression increases bulk TG transport by enabling secretion of larger TG-rich lipoproteins.12,13 Nonetheless, elucidation of the specific role of apoA-IV in lipid transport in vivo has remained elusive, for studies with apoA-IV knockout and Tg mice found no gross abnormalities in dietary lipid absorption.13,16 In part, this may be attributable to the fact that the intestine possesses a large absorptive reserve capacity and robust 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.34 In this organ, TG uptake, synthesis, and secretion is controlled by complex metabolic pathways and is regulated by multiple dietary and hormonal factors, such that even small changes in the relative rates of TG import and synthesis versus oxidation and secretion can rapidly lead to intracellular TG accumulation (steatosis) and inflammation (steatohepatitis).1

We thus reasoned that examining the impact of altered apoA-IV expression on hepatic VLDL secretion and TG content could 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 overexpression on VLDL-TG secretion and cellular TG content under conditions of hepatic steatosis caused by Tg expression of the constitutively active form of SREBP-1a.19,20 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 also, for the first time, have demonstrated an unequivocal metabolic phenotype directly related to apoA-IV expression: that is, 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, which is attributable to its ability to promote VLDL particle expansion within the secretory pathway.

The parallel increases in hepatic TG and apoA-IV mRNA content, which we observed in steatosis induced by increased lipogenesis and a high-fat and high-cholesterol 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.35 Williams et al16 also observed that a high-fat diet induces hepatic apoA-IV expression, although in their studies the effect was strain specific. The strong positive correlation between apoA-IV mRNA abundance (and in the current study, protein concentration) 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 hepatic apoA-IV gene transcription are not well understood.37–39 Recently, it was discovered that the endoplasmic reticulum (ER)-tethered,
liver-specific transcription factor cAMP response element-binding protein H is required for hepatic apoA-IV synthesis.40,41 Activation of cAMP response element-binding protein H requires translocation from ER to Golgi, proteolytic processing, and release of the active form into the nucleus. Although the exact metabolic cues that mediate this activation sequence are unknown,42 cAMP response element-binding protein H expression is enhanced by a number of metabolic conditions, including fasting, insulin resistance,42 and ER stress,43 all of which also promote VLDL secretion.44,45 Thus, it is intriguing to consider that the MTP-mediated movement of lipid across the ER membrane46–48 or incorporation into nascent primordial particles in the ER49 integrates apoA-IV gene expression with VLDL assembly, perhaps via cAMP response element-binding protein H production and/or processing, which then, in turn, serves as the specific signal that regulates apoA-IV transcription.

A possible issue compromising the interpretation of results using the A4-KO mouse is the reduction in expression of the neighboring gene, Apoc3.16 Because Apoc-III is a lipase inhibitor, its lower abundance affects TG-rich lipoprotein clearance, as was observed in previous studies16 and in Figure 2D, in which a nonsignificant decrease in plasma TG was observed in SREBP-1aTg/A4-KO mice. Apoc-III overexpression, both in hepatoma cells and in vivo, is also associated with enhanced VLDL-TG production.50,51 In contrast, studies in Apoc3 knockout mice failed to observe reductions in hepatic TG secretion, even after feeding a high-fat diet.52,53 Hence, it is unlikely that the decreased hepatic VLDL-TG secretion associated with apoA-IV deficiency observed in these studies is the result of decreased apoC-III expression. This conclusion was supported by the 52% increase in TG secretion observed when apoA-IV was selectively overexpressed using a recombinant adenovirus (Figure 4D).

Although the apoA-IV/C-III effects on lipolysis may also complicate interpretation of the impact of production versus clearance on steady-state plasma TG levels, the use of the Triton block method clearly demonstrates an impact of apoA-IV on hepatic VLDL-TG production. Because these changes were not accompanied by altered apoB secretion, the implication is that modification of particle size and not particle number was the basis for the observed effects. This conclusion was supported by direct analysis of particle diameters from post-Triton block plasma in which, based on the changes in TG values between the 0- and 180-minute time points, >90% to 95% of the TG-rich particles present were produced under conditions of lipase inhibition.

Two mechanisms have been proposed to explain how apoA-IV facilitates the assembly of larger lipoprotein particles. Because 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.54,55 Because the interfacial properties of apoA-IV are ideally suited to stabilizing expanding lipid interfaces,56,57 we have proposed that adsorption of apoA-IV molecules to the expanding particle surface renders core lipid incorporation more thermodynamically favorable, which thus facilitates particle growth.58 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 that enables their cores to become more fully lipidated before final secretion.59 These mechanisms are not mutually exclusive.

Another possible explanation for the impact of apoA-IV on VLDL-TG secretion centers on its ability to modulate MTP expression or the expression of other genes involved in hepatic lipid metabolism. Previous studies demonstrated that apoA-IV stimulates MTP expression in both intestine and Huh7 hepatoma cells via the regulation of the transcription factors FoxA2 and FoxO1.27,28 However, in the current study, we failed to observe a similar relationship between apoA-IV and MTP expression (Figure 6). A further exploration of genes involved in lipid metabolism also failed to identify a pattern of gene expression that could account for the enhanced TG secretion associated with apoA-IV expression (Figure III in the online-only Data Supplement). Again, these data suggest that apoA-IV may play a direct role in the modulation of apoB lipoprotein particle expansion and TG transport.

In summary, we have demonstrated in 2 models of 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. Because 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 attributable to secretion of increased numbers of atherogenic apoB-containing lipoproteins.

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Disclosures
None

References

### Significance

Plasma apolipoprotein B (apoB) lipoprotein particle number may be the best predictor of susceptibility to atherosclerotic cardiovascular disease. Because nascent very low density lipoprotein particles are heterogeneous, an important issue is how the hepatocyte integrates particle number with particle size to achieve a given rate of hepatic lipid efflux. The current studies revealed that in mouse liver, apoA-IV is acutely regulated by hepatic triglyceride content and modulates hepatic lipid efflux by promoting nascent very low density lipoprotein particle expansion. Furthermore, human apoA-IV overexpression in steatotic mouse liver both stimulated very low density lipoprotein-triglyceride secretion and dramatically reduced hepatic lipid content. Defining this previously unexplored role of apoA-IV in hepatic lipid transport, in vivo, has important translational potential, for if very low density lipoprotein-mediated hepatic lipid efflux could be achieved by a process of particle expansion, at the expense of particle number, this would simultaneously protect the liver from ectopic lipid accumulation while potentially generating a less atherogenic lipoprotein profile.
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Apolipoprotein A-IV Expression in Mouse Liver Enhances Triglyceride Secretion and Reduces Hepatic Lipid Content by Promoting VLDL Particle Expansion

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Supplementary Figure I. Production and characterization of rabbit polyclonal antiserum against murine apoA-IV. Full-length mature murine apoA-IV cDNA was cloned to form a fusion protein with bacterial maltose binding protein in plasmid pMAL-c2X (New England Biolabs). Fusion protein in bacterial extract was purified by amylose affinity chromatography (New England Biolabs), as recommended by the supplier. Rabbits were immunized with 0.5 mg of purified fusion protein on day 1 and 21 and on day 28, serum was obtained and tested. 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 fractionated by 12.5% SDS-PAGE, followed by immunoblot analysis with 28 day post-immune serum from rabbit #13477 (Rα-mA4-MBP) (A) or anti-FLAG monoclonal antibody M2 (Sigma) (B). C, 1 µL of plasma from wild-type or apoA-IV knock out (A4-KO) mice and 50 µg COS cell extract, containing transfected mouse apoA-IV (mA4 in COS), were fractionated by 12.5% SDS-PAGE. D, 50 µg of whole liver protein extract from high fat/cholesterol diet (HFCD) fed wild-type (WT1 and WT2), A4-KO mice, and 1 µL wild type mouse plasma, were fractionated by 12.5% SDS-PAGE. For both C and D, immunoblot analyses were performed with Rα-mA4-MBP. In D, the arrow indicates the position of mouse apoA-IV, which is absent in the A4-KO livers but appears to have a slightly different gel mobility relative to plasma apoA-IV, perhaps due to differences in O-linked glycosylation.

A | Rα-mA4-MBP
---|---
HSA | mA4 | mA4-F

B | anti-FLAG M2
---|---
HSA | mA4 | mA4-F

C | Plasma
---|---
WT | A4-KO | mA4 in COS

D | HFCD-Liver
---|---
WT 1 | WT 2 | A4-KO | mouse plasma

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Supplementary Figure II. Production and characterization of rabbit polyclonal antiserum against human apoA-IV. Full-length mature human apoA-IV cDNA was cloned to form a fusion protein with bacterial maltose binding protein in plasmid pMAL-c2X (New England Biolabs). Fusion protein in bacterial extracts was purified by amylose affinity chromatography (New England Biolabs), as recommended by the supplier. Rabbits were immunized with 0.5 mg each of purified fusion protein on days 1, 21 and 28 and on day 52 production bleeds were obtained and tested. A, McA-RH7777 cells were transfected with plasmids expressing 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 $1.5 \times 10^9$ pfu of either apoA-IV-adenovirus (huA4) or the control LacZ-adenovirus (LacZ). One µL of plasma (B) or 50 µg of liver protein lysate (C) were fractionated by 12.5% SDS-PAGE, followed by immunoblot analysis with the same anti-serum used in A.
Supplementary Figure III. Quantitative PCR of hepatic lipid metabolism genes. **A**, Liver RNA samples from SREBP-1α<sup>Tg</sup>/A4-WT (A4-WT) and SREBP-1α<sup>Tg</sup>/A4-KO (A4-KO) mice, maintained on a low carbohydrate diet, were used for quantitative PCR; data are normalized to A4-WT. **B**, Liver RNA samples from SREBP-1α mice maintained on chow diet and infected with Ad-LacZ (LacZ) or Ad-huA4 (huA4) were used for quantitative PCR; data are normalized to LacZ. Data are expressed as mean ± SE. For A, n=6 for A4-WT and 8 for A4-KO; For B, n=6 per group. *, *P*<0.05 by Student’s unpaired T-test; all other differences were not significant. Quantitative PCR for LDLr and LRP were performed using pooled RNA samples. ACC-1 and -2, Acetyl-CoA carboxylase 1 and 2; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase 1; DGAT 1 and 2, diacylglycerol O-acyltransferase 1 and 2; CPT-1, Carnitine palmitoyltransferase 1; LCAD, Long-chain acyl-coenzyme A dehydrogenase; MCAD, Medium-chain acyl-CoA dehydrogenase; HADH, hydroxyacyl-CoA dehydrogenase; ACOX, Peroxisomal acyl-coenzyme A oxidase 1; ATGL, adipocyte triglyceride lipase; CGI-58, comparative gene identification-58; HSL, hormone sensitive lipase; TGH, triacylglycerol hydrolase, PLTP, phospholipid transfer protein; ApoB, apolipoprotein B; LDLr, LDL receptor; LRP, LDL receptor related protein 1.
Apolipoprotein A-IV Expression in Mouse Liver Enhances Triglyceride Secretion and Reduces Hepatic Lipid Content by Promoting VLDL Particle Expansion

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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 and cholesterol diet (HFCD) containing (as percent of calories) 48% fat, 36% carbohydrate, 16% protein and 0.2% w/w cholesterol for 16 weeks. The diet composition per 100 grams was as follows: 23.0 g lard, 2.5 g soybean oil, 22.0 g sucrose, 11.3 g maltodextrin, 8.0 g dextrin, 19.3 g casein, 5.0 g Hegsted salt mixture, 0.35 g L-cystine, 2.5 g vitamin mix, 5.85 g cellulose, and 0.2 g crystalline cholesterol. SREBP-1a Tg mice (The Jackson Laboratory, stock #002840) were crossed with Apoa4 knock out mice to generate SREBP-1a Tg/A4-KO and SREBP-1a Tg/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. 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 N2.

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 x 10⁹ plaque forming units (pfu) of adenovirus per mouse was administered under isofluorane sedation. Experiments were performed three days after injection. The extent of hepatic expression of human apoA-IV is documented in Figure 4 by immunoblot analysis. Although, the absolute level of overexpression cannot be directly ascertained, because our anti-human and anti-mouse apoA-IV antisera do not cross-react, it should be noted that the relative band intensities of mouse apoA-IV in low carbohydrate diet-fed SREBP-1a transgenic mouse liver (~40-fold induction; Figure 1C) and human apoA-IV adenovirus-infected livers (Figure 4A) is nearly identical. Although these antisera may not have identical sensitivities, both were raised in rabbits against full-length mature apoA-IV fused to maltose binding protein. Assuming a similar degree of sensitivity, this comparison would imply that Ad-A4 achieves a similar ~40-fold level of overexpression of human apoA-IV as is observed for the TG-mediated induction of the endogenous gene in mouse.
mRNA and Protein Quantification
RNA was extracted from frozen liver samples using TRIzol (Invitrogen). Total RNA (2 μg; 20 µl reaction volume) was reverse transcribed into cDNA with random primers using the Omniscript RT kit (Qiagen) 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 5 µl of cDNA reaction mixture that was previously diluted 1:20 into water. Copy numbers were normalized to GAPDH. The following primers were used for mouse apoA-IV: forward, TTC CTG AAG GCT GCG GTG CTG; reverse, CTG CTG AGT GAC ATC CGT CTT CTG. 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, 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 was detected using rabbit anti-serum against mouse apoA-IV (1:1000 dilution. Supplementary Figure I) or human apoA-IV (1:2000 dilution) (Supplementary Figure II). LacZ was detected with a mouse anti-β-galactosidase monoclonal antibody (Sigma #G-4644). Mouse MTP was detected with a mouse monoclonal antibody (BD Biosciences #612022).

Analysis of Plasma and Liver Lipids
Following a 4 hour fast, blood was collected by heart puncture and placed into a tube containing a protease inhibitor cocktail (Sigma #P2714) dissolved in 0.05% EDTA, 0.05% NaN₃. Samples were 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 kit (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 CHCl₃:methanol (2:1)² and dried down under a stream of nitrogen. Triton X-100 (1%) in CHCl₃ was then added and the solvent was again evaporated under nitrogen³. Deionized water was added and each tube was vortexed until the solution was clear. Lipids were then quantified by enzymatic colorimetric assay, as described above for plasma.

Hepatic TG and ApoB Secretion Rates
Following a 4 hour fast, mice were sedated by isofluorane inhalation and then administered 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 heparinized capillary tubes by retro-orbital bleeding at 0 (before injection), 30, 60, 120, and 180 min. TG concentration in plasma samples was measured by enzymatic assay. TG secretion rates were calculated as the mean of the slopes of linear regressions of TG concentration versus time for each individual animal, using GraphPad Prism 5.

To measure secretion of newly synthesized apoB, 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 in a TL-100 centrifuge for 4 hours at 4°C using a TLA 100.2 rotor (Beckman). The d<1.006 g/mL (VLDL) fraction was collected
from the top 0.5 ml of the tube by tube slicing. The sample was then adjusted to lysis buffer conditions (above) and 0.2% bovine serum albumin using concentrated stocks. Samples were immunoprecipitated by addition of 5 µl of rabbit anti-mouse apoB antibody (Biodesign International [Meridian Life Science] #K23300R). 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 in SDS-PAGE sample buffer at 100°C for 5 min, and then fractionated by 12.5% SDS-PAGE. Gels were then dried and visualized with a Fuji BAS5000 PhosphorImager.

Electron Microscopy of Plasma VLDL
Negative stain electron microscopy of VLDL from post-Triton terminal bleeds was performed as described4. The d<1.006 g/mL (VLDL) fraction obtained as above, was collected from the top 0.5 ml of the tube by tube slicing. 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 with filter paper. Grids were then examined using a FEI Technai BioTwin 120 keV transmission electron microscope.

Statistics
Results are presented as means ± SE. Data were analyzed using GraphPad Prism by unpaired Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons, as indicated. Significant differences are indicated with symbols defined within the legend of each figure; where no symbol is indicated, differences of mean values did not reach statistical significance (P>0.05).

References


