Hepatic apolipoprotein A-IV (apoA-IV) expression is correlated with hepatic triglyceride (TG) content in mouse models of chronic hepatosteatosis, and steatosis-induced hepatic apoA-IV gene expression is regulated by nuclear transcription factor cAMP-responsive element-binding protein H (CREBH) processing. To define what aspects of TG homeostasis regulate CREBH processing and apoA-IV gene expression, several mouse models of attenuated VLDL particle assembly were subjected to acute hepatosteatosis induced by an overnight fast or short term ketogenic diet feeding. Compared with chow-fed C57BL/6 mice, fasted or ketogenic diet-fed mice displayed increased hepatic TG content, which was highly correlated ($r^2 = 0.95$) with apoA-IV gene expression, and secretion of larger, TG-enriched VLDL, despite a lower rate of TG secretion and a similar or reduced rate of apoB100 secretion. When VLDL particle assembly and secretion was inhibited by hepatic shRNA-induced apoB silencing or genetic or pharmacologic reduction in microsomal triglyceride transfer protein (MTP) activity, hepatic TG content increased dramatically; however, CREBH processing and apoA-IV gene expression were attenuated compared with controls. Adenovirus-mediated reconstitution of MTP expression proportionately restored CREBH processing and apoA-IV expression in liver-specific MTP knock-out mice. These results reveal that hepatic TG content, per se, does not regulate CREBH processing. Instead, TG mobilization into the endoplasmic reticulum for nascent VLDL particle assembly activates CREBH processing and enhances apoA-IV gene expression in the setting of acute steatosis. We conclude that VLDL assembly and CREBH activation play key roles in the response to hepatic steatosis by up-regulating apoA-IV and promoting assembly and secretion of larger, more TG-enriched VLDL particles.

Over the past decade, the incidence of nonalcoholic fatty liver disease (NAFLD)\(^2\) has reached epidemic levels in developed countries, and it has emerged as a major preventable cause of cirrhosis (1, 2). The central pathophysiology underlying NAFLD is the accumulation of abnormal amounts of triglyceride (TG) within hepatocytes (steatosis), which can initiate inflammation (steatohepatitis) and fibrosis (3). Hepatic TG content is controlled by multiple dietary, hormonal, and genetic factors that regulate the balance among fatty acid uptake, synthesis, oxidation, and export via secretion of TG-rich VLDLs (3, 4). In NAFLD, both TG synthesis and secretion are increased, but TG export is inadequate to prevent steatosis (5, 6). The efficiency of hepatic TG export via the VLDL secretion pathway can be increased by assembly of a greater number of VLDL particles and/or larger VLDL particles that contain more core TG (7, 8). Understanding the mechanisms that integrate VLDL particle number and size to maintain a rate of hepatic TG efflux sufficient to prevent steatosis could provide new insights into the pathogenesis of NAFLD and suggest novel strategies for its prevention and treatment.

One factor that may play an important role in modulating VLDL particle size during TG-rich lipoprotein assembly is apolipoprotein A-IV (apoA-IV). ApoA-IV is a 46-kDa lipid-binding protein, which is expressed in the mammalian intestine and liver (9–12). Although a broad spectrum of functions in lipid metabolism and metabolic regulation has been ascribed to apoA-IV since its discovery in 1977 (9), the largest body of literature has focused on the powerful impact of TG absorption on the induction of intestinal apoA-IV gene expression (13, 14). More recently, Lu et al. (15) reported the direct impact of apoA-IV expression on intestinal TG transport efficiency by demonstrating in a transfected cultured pig intestinal epithelial cell line that apoA-IV expression enhances transcellular TG transport, primarily by promoting lipoprotein particle expansion (16). We subsequently observed similar effects in transfected rat hepatoma cells and proposed that apoA-IV alters the
Hepatic ApoA-IV Expression Is Increased in Models of Acute Steatosis—We previously demonstrated that hepatic apoA-IV expression is increased in several mouse models of chronic and acute steatosis (19, 20). To further explore the basis for the relationship between hepatic TG homeostasis and apoA-IV expression, we examined hepatic apoA-IV gene expression in mice after an overnight fast (20, 21), in mice after short term (6 days) keto diet exposure (20), or in animals provided free access to chow (i.e. fed). Previous studies showed that overnight fasting or keto diet increases CREBH processing (20, 22). Plasma TG concentrations were unchanged after an overnight fast compared with fed mice (fasted 48 ± 15 mg/dl versus fed 64 ± 4 mg/dl, mean ± S.D., n = 3); however, hepatic TG content increased 9.6-fold for fasted mice relative to fed controls (Fig. 1A). In comparison, the keto diet significantly raised plasma TG content 26- and 2.7-fold versus chow-fed and fasted mice, respectively (Fig. 1A). Quantitative real-time PCR revealed that relative to chow-fed controls, hepatic apoA-IV mRNA abundance increased 8-fold with overnight fasting and 27-fold with keto diet exposure (Fig. 1B). Although apoA-IV protein abundance was below the limits of detection for fed and fasted mice, the keto diet-fed mice displayed dramatically increased levels of hepatic apoA-IV (Fig. 1C). Plasma apoA-IV was also increased in keto diet-fed mice compared with fed and fasted mice (Fig. 1D). Of note, hepatic apoB protein was also increased with fasting and keto diet feeding, relative to fed mice; microsomal triglyceride transfer protein (MTP) abundance was similar for all three groups of mice (Fig. 1C). Analysis of individual animal data from both acute steatosis models revealed a strong association between hepatic TG content and apoA-IV mRNA expression (Fig. 1E; ρ² = 0.95). In vivo TG secretion after detergent block of TG lipolysis demonstrated significant (p < 0.001) decreases in the TG secretion rate of fasted and keto diet-fed mice compared with chow-fed controls (Fig. 1F), probably due to increased shunting of hepatic fatty acids into fatty acid oxidation (23, 24). After the 3-h detergent block experiment, plasma VLDLs were isolated, and particle sizes were measured by dynamic laser light scattering analysis. Despite a lower in vivo TG secretion rate compared with fed mice, fasted and keto diet-fed mice had a distribution of VLDL particles that was skewed toward larger sizes, which was particularly exaggerated for keto diet-fed mice (Fig. 1G). Aggregating the VLDL particle size data into three broad size ranges revealed that fasted and keto diet-fed mice had significantly fewer VLDL particles in the smallest (<100 nm) size range and more in the 100–200 nm range compared with chow-fed mice (Fig. 1H). Moreover, the keto diet-fed mice had significantly more VLDL particles in the largest (>200 nm) size range compared with the other two groups. Secretion of newly synthesized plasma [35S]apoB100 during the Triton block experiment was similar for fed and fasted mice but was significantly (p < 0.05) reduced for keto diet-fed mice (Fig. 1I).

ApoB Expression Is Essential for Promoting CREBH Processing and Hepatic ApoA-IV Expression—Early studies reported that hepatic apoA-IV expression is regulated by multiple factors, including nutritional and metabolic stress (21), insulin (25), liver X receptors (26), and transcription factors such as LUMAN (27), HNF-4α, and PGC-1α (21). However, recently we demonstrated that CREBH is a major regulator of apoA-IV transcription (20, 22) and is responsible for increasing apoA-IV expression in steatotic conditions via tandem binding sites on the apoA-IV promoter.

To explore whether VLDL assembly and secretion are required for CREBH processing and apoA-IV expression, adenoviruses harboring apoB shRNA (shRNA-apoB) or non-targeting control shRNA (shRNA-NT) were given by retro-orbital injection to keto diet-fed C57BL6 mice. Immunoblot analysis 3 days after injection showed that both hepatic and plasma apoB protein were greatly reduced in the apoB shRNA-treated mice compared with the non-targeting shRNA controls (Fig. 2, A and B). Quantitative PCR confirmed that this was the consequence of a >95% decrease in hepatic apoB mRNA abundance (data not shown). As expected, the keto diet induced steatosis in the control mice, and apoB silencing resulted in a further doubling of hepatic TG content (Fig. 2C). Despite the dramatic increase in hepatic TG accumulation caused by apoB silencing, dramatic reductions in hepatic apoA-IV protein (Fig. 2D), mRNA (76% reduction; data not shown), and CREBH processing were observed in mice treated with the higher adenoviral dose of shRNA-apoB (Fig. 2E, mice 135 and 136). Two additional mice treated with the lower dose of shRNA-apoB (Fig. 2E, mice 133 and 134) had a less striking reduction in hepatic apoB protein expression and CREBH processing. Neither microsomal CREBH (i.e. CREBH full-length (CREBH(F)); Fig. 2E) nor
mRNA abundance (data not shown) was affected by apoB silencing. These data suggest that VLDL particle assembly is necessary for CREBH processing and subsequent up-regulation of apoA-IV gene expression under steatotic conditions.

**MTP Is Essential for Promoting CREBH Processing and Hepatic ApoA-IV Expression**—VLDL assembly and secretion require MTP (28). To examine the role of MTP protein and activity on steatosis-induced CREBH processing and up-regu-
lation of hepatic apoA-IV expression, we generated MTP liver-specific knock-out (MTPLKO) mice to eliminate hepatocyte MTP expression and treated C57BL6 WT mice with an MTP inhibitor (BMS-212122) to reduce MTP activity (29). MTPLKO mice and WT were fasted overnight for 16 h to induce acute hepatosteatosis. Immunoblot analysis confirmed that there was no detectable MTP expression in MTPLKO mouse liver (Fig. 3A). The absence of hepatic MTP caused both plasma and hepatic apoB100 (Fig. 3B) to fall to undetectable levels and decreased plasma TG levels in both fed and fasted states compared with WT mice (Fig. 3C), indicating that hepatic MTP deficiency had blocked VLDL particle assembly and secretion, as anticipated (28, 30). Fasting induced hepatic TG accumulation in both WT and MTPLKO mice, although the magnitude of steatosis was 4-fold greater in MTPLKO mice (Fig. 3C). Fasting-induced steatosis increased apoA-IV mRNA expression 16-fold in WT mice relative to fed mice but did not increase apoA-IV gene expression in MTPLKO mice (Fig. 3D), despite more severe steatosis (Fig. 3C). Inhibition of MTP activity with BMS-212122 (MTPi) also reduced plasma TG concentrations (Fig. 3E), increased hepatic TG content (Fig. 3F), and decreased apoA-IV gene expression (Fig. 3G), relative to vehicle-treated mice, in a manner similar to the responses seen in fasted MTPLKO versus WT mice. MTP protein (Fig. 3H) and mRNA abundance (data not shown) were not affected by MTP inhibition; however, as compared with vehicle-treated control mice, CREBH protein processing was suppressed to levels similar to that observed in vehicle-treated MTPLKO mice (Fig. 3H). These data strongly support the conclusion that MTP and VLDL particle assembly plays an important role in activating CREBH processing and up-regulation of hepatic apoA-IV expression in steatosis.

Reconstitution of VLDL Assembly/Secretion with MTP Restores CREBH Processing and Hepatic apoA-IV Expression in MTPLKO Mice—To further explore the importance of VLDL assembly for CREBH processing and apoA-IV expression, MTPLKO mice were administered adenovirus expressing human MTP (Ad-hMTP) or LacZ (Ad-LacZ). Three days after infection, mice were fasted overnight to induce steatosis before liver and plasma were harvested. Immunoblot analysis con-
firmed efficient hepatic hMTP expression in mice injected with Ad-hMTP as well as increased plasma apoB levels, particularly apoB100 (Fig. 4A). Plasma TG concentration was significantly increased (Fig. 4B), whereas hepatic TG content was significantly reduced (Fig. 4C) for Ad-hMTP- versus Ad-LacZ-treated mice. As anticipated, quantitative real-time PCR revealed that reconstitution of VLDL assembly with Ad-hMTP did not affect CREBH mRNA abundance (Fig. 4D) but restored steatosis-induced CREBH processing (Fig. 4E) and apoA-IV gene expression (Fig. 4F). Quantitative real-time PCR revealed that the genes encoding other apolipoproteins involved in VLDL synthesis and secretion (i.e. apoC-III, apoB, and apoA-V) were unchanged by hMTP reconstitution (Fig. 4F), indicating that apoB/MTP-directed VLDL assembly and secretion play a direct and specific role in CREBH processing and apoA-IV expression.

To determine whether hepatic CREBH processing and apoA-IV expression were quantitatively regulated by the level of MTP expression, increasing doses of Ad-hMTP were given to keto diet-fed MTPLKO mice. Quantitative real time PCR and immunoblot analysis demonstrated that hepatic abundance of hMTP mRNA (Fig. 5A) and protein (Fig. 5B) were
induced in a dose-dependent manner by Ad-hMTP; control mice (MTPLKO or Mtpflox/flox mice) given the highest dose of Ad-LacZ had no increase in human MTP expression (Fig. 5A).

Increased hepatic hMTP expression resulted in a trend toward decreased hepatic TG content (Fig. 5C) and a proportional increase in plasma apoB100 (Fig. 5D), CREBH processing (Fig. 5E), and apoA-IV mRNA (Fig. 5F) and protein (Fig. 5G) abundance. These data suggest that restoration of hepatic MTP in MTPLKO mice proportionately stimulates CREBH processing, apoA-IV expression, and VLDL assembly and secretion.

**Discussion**

Past studies have shown that hepatic steatosis induces CREBH processing and induction of apoA-IV gene expression (20) and that hepatic apoA-IV overexpression reduces hepatic TG content and increases VLDL TG secretion by promoting an increase in VLDL particle size in a mouse model of chronic steatosis driven by transgenic overexpression of sterol regulatory element-binding protein 1a (SREBP1a) (19). In the current study, we explored more fully the basis for the promotion of CREBH processing and apoA-IV expression and, specifically, whether hepatic TG accumulation, per se, promotes CREBH activation or whether processes associated with TG mobilization for nascent VLDL assembly are instead responsible. To distinguish between these possibilities, we investigated hepatic TG content, CREBH processing, and apoA-IV gene expression in mouse models of acute steatosis with intact or disrupted VLDL particle assembly.

Our studies reveal several novel findings. First, using two additional mouse models of acute steatosis, caused by either fasting or feeding a keto diet, we observed a similar relationship between liver TG content and apoA-IV expression as was observed previously using SREBP1a transgenic and high fat diet-fed mice (19). Hence, whether hepatic TG accumulation is induced by enhanced de novo lipogenesis, increased dietary fat, or mobilization of adipocyte TG via activated lipolysis, apoA-IV expression is increased via activation of CREBH proteolytic processing. As was observed for SREBP1a transgenic mice (20), increased apoA-IV synthesis was associated with secretion of larger, TG-enriched VLDL particles. Second, attenuation of VLDL particle assembly by apoB silencing or genetic or pharmacologic reduction in MTP activity severely attenuated CREBH processing and apoA-IV expression, despite a dramatic increase in liver TG content. Finally, a direct role of MTP-mediated VLDL assembly in CREBH processing and apoA-IV expression was demonstrated by the fact that MTP reconstitution in MTP-deficient mouse liver with adenoviral expression of human MTP restored both CREBH processing and apoA-IV expression. These studies demonstrate that whereas increasing hepatic TG content is necessary for CREBH-dependent apoA-IV activation, it is not sufficient. Instead, some aspect of the VLDL assembly and secretion pathway is essential for CREBH activation.

Although our current results do not point to which aspect of the VLDL assembly pathway activates CREBH processing, it is
intriguing to note that ER cholesterol content regulates proteolytic activation of SREBP2, another ER-tethered basic helix-loop-helix leucine zipper transcription factor that controls the cellular biosynthesis and uptake of cholesterol (31, 32). Hepatocytes are unique relative to non-lipoprotein-producing cells in that TG synthesis and storage must be coupled to its translocation across the ER membrane to form lumenal lipid droplets, which then serve as a substrate for TG acquisition by apoB (8, 33). Hence, it is intriguing to consider that lipid flux across the ER membrane is required to regulate CREBH cleavage. Previous efforts to dissect the roles of apoB and MTP in VLDL assembly have suggested that MTP is predominantly responsible, perhaps independently of apoB, for the formation of lumenal TG-enriched droplets (34, 33). Hence, it is intriguing to consider that lipid flux across the ER membrane is required to regulate CREBH cleavage. Previous efforts to dissect the roles of apoB and MTP in VLDL assembly have suggested that MTP is predominantly responsible, perhaps independently of apoB, for the formation of lumenal TG-enriched droplets (34, 33). However, clearly apoB, as the sole TG acceptor in the ER, is also required to maintain ongoing flux of lipid across the ER membrane. Our finding that both apoB and MTP deficiency block CREBH processing suggests that either lipid movement into the ER or some other related function of these proteins initiates the vesicular trafficking of CREBH to the Golgi and processing by site 1 and site 2 proteases to release the active form.

Irrespective of the exact mechanisms, our results reveal a novel pathway in which processes associated with VLDL particle assembly activate CREBH processing, resulting in up-regulation of apoA-IV expression. Since its discovery in 1977 (9), apoA-IV has been reported to have many and varied physiological and metabolic functions (36). Most studies have examined the role of apoA-IV in small intestine physiology, where its expression is robustly up-regulated during lipid absorption (13). We recently explored the role of apoA-IV in hepatic lipid mobilization and demonstrated that steatosis-induced apoA-IV expression enhanced TG secretion and reduced

![Figure 5](image_url)
hepatic lipid content by promoting VLDL particle expansion without increasing the number of VLDL particles (19). This pathway probably evolved to increase hepatic TG flux from steatotic liver into the plasma compartment through VLDL particle expansion, thereby protecting the liver from lipid toxicity. Because LDL particle number is more predictive of coronary heart disease than LDL cholesterol (37, 38), the net result of increased hepatic apoA-IV expression would be more efficient hepatic lipid mobilization without increasing secretion of VLDL particles, the precursors of atherogenic plasma LDL particles.

We propose two mechanisms by which apoA-IV may facilitate VLDL particle expansion. The first relates to the interfacial activity and elasticity of apoA-IV, allowing apoA-IV to bind and stabilize the surface monolayer of expanding VLDL particles (39). Second, a direct interaction between apoA-IV and the amino terminus of apoB slows the secretory trafficking of VLDL particles, allowing more lipid addition to the expanding VLDL particle before secretion (18). In the present study, we show that acute steatosis also stimulates apoA-IV expression and secretion of larger VLDL particles, even under metabolic conditions (i.e. overnight fasting and keto diet feeding) that lead to an overall reduction in hepatic TG secretion (Fig. 6). This is particularly evident in keto diet-fed mice in which both VLDL TG and apoB100 secretion were reduced compared with chow-fed mice, but plasma VLDL particles were considerably larger and enriched in apoA-IV (data not shown). These results suggest that apoA-IV plays a major role in VLDL particle expansion and in mobilizing TG for secretion to protect the liver from steatosis without increased demand for apoB synthesis.

In addition to increasing hepatic apoA-IV to facilitate VLDL particle expansion, CREBH processing also increases hepatic expression and secretion of apoC-II and apoA-V, both of which activate lipoprotein lipase, increasing lipolysis and clearance of plasma VLDL TG (22). The net result of this CREBH-coordinated physiological response is to shunt TG (i.e. fatty acids) out of the liver, through the circulation, and into peripheral tissues. To our knowledge, this is the first evidence for an integrated pathway to protect against hepatosteatosis by exporting excess TG in larger VLDL particles that enter the plasma to undergo stimulated lipolysis with increased fatty acid delivery to peripheral tissues.

ApoA-IV is predominantly expressed in human enterocytes and is highly up-regulated after a fatty meal to facilitate intestinal chylomicron assembly and TG secretion (13, 14). Although human liver apoA-IV mRNA abundance is <5% of intestinal levels (40, 41), hepatic apoA-IV expression is increased in steatotic states (20), as we have described in mouse models of acute (i.e. in the present study) and chronic hepatosteatosis (19). Based on work presented here, we propose that CREBH processing and apoA-IV up-regulation may be an important pathway to facilitate export of excess liver TG in hepatosteatotic individuals.

In summary, we provide evidence for a pathway that integrates increased lipid flux into the ER with increased VLDL particle expansion through activation of CREBH processing, which up-regulates apoA-IV expression. This pathway appears to function in chronic steatosis, induced by high fat diet or genetic induction of lipogenesis, and in acute physiological (overnight fasting) or pathophysiological (ketogenesis) states to protect the liver from lipid overload by promoting TG incorporation into nascent lipoproteins and secretion into the circulation. This integrated lipid transport pathway has dual beneficial purposes, protecting the liver from ectopic lipid accumulation while generating fewer atherogenic plasma apoB lipoprotein particles. Hence, stimulating this pathway would be expected to exert multiple beneficial effects on both fatty liver disease and atherosclerotic cardiovascular disease.
Experimental Procedures

Plasmid Construction

pCMV5-hMTP—hMTP cDNA was obtained by PCR reverse transcription of HuH7 mRNA using primers containing HindIII or XbaI restriction sites (underlined): forward primer, 5′-AGAAAGCCTGCTGTTCAATATGATTCTTTCG-3′; reverse primer, 5′-AGATCTGATACAGGCTAGTTC-AAAAACATCC-3′. The PCR product was cleaved with HindIII and XbaI restriction enzymes, fractionated by agarose gel electrophoresis, and cloned into HindIII/XbaI double-digested pCMV5 expression vector.

pShuttle-hMTP—The pCMV5-hMTP plasmid was linearized with HindIII, blunt ended with T4 DNA polymerase, and then digested with XbaI to release the hMTP cDNA insert. The pShuttle vector (Clontech, catalog no. 631513) was digested with NheI, blunt-ended with T4 DNA polymerase, and then digested with XbaI. The hMTP cDNA was then ligated to pShuttle vector.

pSIREN-apoB shRNA—ApoB shRNA oligonucleotides were synthesized by Integrated DNA Technologies. The oligonucleotide sequences for apoB shRNA are 5′-ATTCTAAAAAACGAGCACACTGGAATAGTATTTCCAAGTGCTTGTCTGCG-3′ and 5′-GATCCCGACACCACTTTGAGATTACATTCAAGCTTAGTAATCTCAAATGG-3′. Non-targeting control shRNA oligonucleotides were from Clontech (catalog no. 631527). The oligonucleotides were annealed to form double-stranded oligonucleotides and inserted into RNAi-Ready pSIREN-Shuttle vector (Clontech, catalog no. 631527). The entire plasmid insert and flanking regions were verified by DNA sequence analysis.

Recombinant Adenovirus Production

The pShuttle gene expression cassettes for human MTP and LacZ (Clontech, catalog no. 631513) and pSIREN-Shuttle vector apoB and control shRNA oligonucleotide-encoding cassettes were released from their respective vectors by restriction enzyme digestion with PI-SceI and I-CeuI and subcloned into LacZ (Clontech, catalog no. 631513) and pSIREN-Shuttle vector (Clontech, catalog no. 631527). The entire plasmid insert and flanking regions were verified by DNA sequence analysis.

Animals

C57BL/6 mice (stock number 000664) were obtained from the Jackson Laboratory. Liver-specific MTP knock-out (MTPKO) mice were generated by crossing MTP floxed mice (Mtpflox/flox), obtained from Dr. Lawrence Chan (Baylor College of Medicine) (28) with albumin Cre recombinase-expressing mice (Jackson Laboratory; B6.Cg-Tg(Alb-cre)21Mgn/J). Genotypes were verified by PCR analysis of tail genomic DNA (42). Primers for detecting the loxP site in intron 4 of MTP (28) were

5′-ACAGAGTTATGGGATCTTCATCAGTCC-3′ and 5′-ATACAGCTAATCTGACGTTGAGAT-3′, and primers for detecting Cre recombinase were 5′-ACCTGAGATGTGCAGTTATC3′ and 5′-ACCTGACGTTGAGAT-3′. Mice homozygous for the MTP floxed allele and harboring a Cre allele were used for studies (i.e. MTPKO). Mice were fed ad libitum either a cereal-based rodent chow diet or a keto diet containing 93.4% fat, 4.7% protein, and 1.8% carbohydrate (F-3666, Bio-Serv) for 6 days. All mice were housed in a pathogen-free animal facility in plastic cages in a temperature-controlled room (22 °C) with a 12-h light and 12-h dark cycle. For some experiments, mice were fasted overnight for 16 or 20 h, as indicated, with free access to water. All animal procedures were conducted in conformity with United States Public Health Service policies and were approved by the institutional animal care and use committee of Wake Forest School of Medicine. Mice were deeply anesthetized with ketamine/xylazine before exanguination via heart puncture. Blood was placed into a tube containing a protease inhibitor mixture (Sigma, catalog no. P2714) dissolved in 0.05% EDTA, 0.05% NaN3, and plasma was obtained by low speed centrifugation (6000 rpm) for 15 min at 4 °C. Livers were perfused with ice-cold saline and cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80 °C.

Adenoviral Transduction of Mice

Retro-orbital injection of adenovirus was administered under isofluorane sedation. Three days after injection, mice were euthanized, and plasma and liver tissue were harvested as described above.

In Vivo Hepatic TG Secretion

In vivo hepatic TG secretion was measured after detergent block of TG lipolysis, as detailed previously with minor modifications (43). Briefly, mice (n = 5/group) were fasted for 20 h to induce acute steatosis, followed by a third dose of MTP inhibitor. Four hours later, mice were sacrificed, and liver and plasma were harvested for analyses.
RNA Extraction and mRNA Quantification

RNA was extracted from frozen liver samples using TRIzol as recommended by the manufacturer (Invitrogen). Total RNA was reverse transcribed into cDNA with random primers using qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). A typical PCR (20 μl) contained 10 μl of 2× Fast SYBR Green Master Mix (Roche Applied Science), 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 GAPDH, 5′-TGTGTCGCTGGATGATCGA-3′; reverse GAPDH, 5′-CTGTGCTCACCACCTGTGAGAG-3′; forward apoA-IV, 5′-TTCCTGAGAGCAGGTGCTGCT-3′; reverse apoA-IV, 5′-CTGTGCTCACCACCTGTGAGAG-3′; forward apoB, 5′-GCTCACTCAGGGTACCGTGTA-3′; reverse apoB, 5′-AGGGTGACTGCAAGGTTG-3′; forward CREBH, 5′-TCTCTGAGTGTTGCAAGA-3′; reverse CREBH, 5′-TCTCGAGTTGCAAGA-3′; forward apoC-III, 5′-GGCTAGGCTTCCCTTTGAG-3′; reverse apoC-III, 5′-GGCTAGGCTTCCCTTTGAG-3′; forward LaminB, 5′-TCCTCGCAGTGTTCGCAAG-3′; reverse LaminB, 5′-TCCTCGCAGTGTTCGCAAG-3′; forward apoA-V, 5′-GCCATTGACCTGGACATGT-3′; reverse apoA-V, 5′-GCCATTGACCTGGACATGT-3′; forward apoC-I, 5′-CTGTTAGGCTTCCCTTTGAG-3′; reverse apoC-I, 5′-CTGTTAGGCTTCCCTTTGAG-3′; forward CREBH, 5′-GGCCATTGACCTGGACATGT-3′; reverse CREBH, 5′-GGCCATTGACCTGGACATGT-3′; forward CREBH, 5′-GGCCATTGACCTGGACATGT-3′; reverse CREBH, 5′-GGCCATTGACCTGGACATGT-3′.

Immunoblot Analysis

For immunoblot analysis, ~50 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, and 10 μg/ml aprotinin). Liver protein lysates or plasma were mixed with concentrated SDS-PAGE loading buffer, boiled for 5 min, and then separated by SDS-PAGE. Proteins were electrophoretically transferred to a PVDF membrane, which was then incubated sequentially with primary antibody, HRP-conjugated secondary antibody, and SuperSignal West Pico chemiluminescent substrate. The membrane was then exposed to a Fuji LAS3000 Imaging System or with X-film. Chemiluminescence was detected by direct visualization with a Fuji LAS3000 Imaging System or with X-film. The following primary antibodies were used in this study: rabbit anti-serum raised against purified mouse apoA-IV (19); mouse monoclonal antibody against mouse MTP (BD Transduction Laboratories, catalog no. 612022); rabbit antisera raised against hMTP (44); goat anti-human apoB (Academy Bio-Medical Co., catalog no. 20A-G1b); and mouse monoclonal antibodies against apoB (45), calnexin (Enzo, catalog no. ADI-SPA-865), LaminB (Santa Cruz Biotechnology, Inc., catalogue no. SC-56145), and CREBH (22).

Analysis of Plasma and Liver Lipids

Plasma TG concentration and liver TG content was quantified using an enzymatic colorimetric assay (Triglycerides/GB kit, Wako) as described previously (46).

Statistical Analysis

Results are presented as means ± S.D., unless otherwise noted. Significance differences in outcome parameters among the different mouse strains and experimental conditions were determined by one-way analysis of variance with Tukey post-hoc analysis.

Author Contributions—D. C. helped design experiments, generated and analyzed data, and wrote the first draft of the manuscript. X. X. performed CREBH processing assays in Figs. 2–5 and analyzed the Western blotting data. T. S. managed mouse colonies and generated the Triton block data in Fig. 1. F.-I. Z. D. designed shRNA-apoB sequences. M. V. generated some preliminary data. E. B. helped with the mouse sacrifice procedures and Triton block experiments. A.-H. L. and G. S. S. conceived the project, designed experiments, interpreted results, and edited the manuscript. R. B. W. and J. S. P. designed experiments, interpreted results, and edited the manuscript. J. S. P. provided overall supervision for the studies. All authors approved the final version of the manuscript.

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