Hepatic Apolipoprotein M (ApoM) Overexpression Stimulates Formation of Larger ApoM/Sphingosine 1-Phosphate-enriched Plasma High Density Lipoprotein*

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Mingxia Liu†, Jeongmin Seo‡, Jeremy Allegood§, Xin Bi,‡ Xuewei Zhu‡, Elena Boudyguina‡, Abraham K. Gebre‡, Dorit Avni§, Dharika Shah‡, Mary G. Sorci-Thomas‡, Michael J. Thomas‡, Gregory S. Shelnitz‡, Sarah Spiegel‡, and John S. Parks‡,¶

From the Departments of Pathology-Lipid Sciences and Biochemistry, Wake Forest School of Medicine, Winston-Salem, North Carolina 27157 and the Department of Biochemistry and Molecular Biology, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298

Background: ApoM overexpression in nonhepatic cells generates larger nascent HDLs.

Results: Hepatocyte-specific apoM transgenic mice have larger plasma HDLs and hepatocytes that generate larger nascent HDLs and increased S1P secretion.

Conclusion: Hepatocyte-specific apoM overexpression facilitates large apoM/S1P-enriched HDL formation by promoting large nascent HDL formation and stimulating sphingolipid synthesis and S1P secretion.

Significance: Hepatic apoM regulates HDL and S1P production.

Apolipoprotein M (apoM), a lipocalin family member, preferentially associates with plasma HDL and binds plasma sphingosine 1-phosphate (S1P), a signaling molecule active in immune homeostasis and endothelial barrier function. ApoM overexpression in ABCA1-expressing HEK293 cells stimulated larger nascent HDL formation, compared with cells that did not express apoM; however, the in vivo role of apoM in HDL metabolism remains poorly understood. To test whether hepatic apoM overexpression increases plasma HDL size, we generated hepatocyte-specific apoM transgenic (APOM Tg) mice, which had an ~3–5-fold increase in plasma apoM levels compared with wild-type mice. Although HDL cholesterol concentrations were similar to wild-type mice, APOM Tg mice had larger plasma HDLs enriched in apoM, cholesteryl ester, lecithin:cholesterol acyltransferase, and S1P. Despite the presence of larger plasma HDLs in APOM Tg mice, in vivo macrophage reverse cholesterol transport capacity was similar to that in wild-type mice. APOM Tg mice had an ~5-fold increase in plasma S1P, which was predominantly associated with larger plasma HDLs. Primary hepatocytes from APOM Tg mice generated larger nascent HDLs and displayed increased sphingolipid synthesis and S1P secretion. Inhibition of ceramide synthases in hepatocytes increased cellular S1P levels but not S1P secretion, suggesting that apoM is rate-limiting in the export of hepatocyte S1P. Our data indicate that hepatocyte-specific apoM overexpression generates larger nascent HDLs and larger plasma HDLs, which preferentially bind apoM and S1P, and stimulates S1P biosynthesis for secretion. The unique apoM/S1P-enriched plasma HDL may serve to deliver S1P to extrahepatic tissues for atheroprotection and may have other as yet unidentified functions.

Cardiovascular disease is the leading cause of death in developed countries. Numerous studies have observed an inverse association between plasma HDL cholesterol (HDL-C) concentrations and increased cardiovascular disease risk (1). However, recent evidence suggests that HDL function, not HDL-C concentration, may best predict its atheroprotective capacity (2, 3). One function of HDL is to facilitate macrophage reverse cholesterol transport (RCT), a process by which cholesterol in arterial plaque macrophages is transported to the liver for secretion into bile and excretion in feces (4). Although RCT is presumed to be HDL’s most important anti-atherogenic role, these particles also have anti-inflammatory and anti-oxidative activities and carry cardioprotective molecules (5–7). However, because our understanding of the atheroprotective roles of HDL is incomplete, additional focus on HDL function and metabolism is merited.

HDL formation initiates when the ATP-binding cassette transporter A1 (ABCA1) effluxes free cholesterol (FC) and phospholipid (PL) to lipid-free apoA-I, forming nascent HDL particles. The liver is quantitatively the most important tissue for nascent HDL formation (8). After initial assembly, nascent

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Pathology/Section on Lipid Sciences, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157. Tel.: 336-716-2145; Fax: 336-716-6279; E-mail: jparkes@wakehealth.edu.

§ The abbreviations used are: HDL-C, HDL-cholesterol; apoM, apolipoprotein M; S1P, sphingosine 1-phosphate; APOM Tg, hepatocyte-specific apoM transgenic; RCT, reverse cholesterol transport; ABCA1, ATP-binding cassette transporter A1; FC, free cholesterol; PL, phospholipid; LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl ester; SR-BI, scavenger receptor class B, type I; hA-I Tg, human apoA-I transgenic; TG, triglyceride; DH-S1P, dihydrosphingosine 1-phosphate; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2; SPTLC1, serine palmitoyltransferase long chain subunit 1; SGPP1, S1P phosphatase 1; FB1, fumonisin B1.
HDL particles undergo maturation to become spherical HDL particles via the actions of lecithin:cholesterol acyltransferase (LCAT), which increases core cholesterol ester (CE) content (9), and phospholipid transfer protein (PLTP) (10), which transfers PL to the HDL surface. To complete the process of RCT, HDL-C is taken up by the liver, mostly via scavenger receptor class B, type I (SR-BI), for secretion into bile and excretion in feces (11).

ApoM, a 25-kDa plasma apolipoprotein, belongs to the lipocalin family and contains a small hydrophobic binding pocket (12, 13). The identity of human (NP_061286.1) and mouse apoM (NP_061286.1) is over 80%, and both contain a signal peptide anchor and hydrophobic binding pocket (12, 13). The identity of human (NP_061974.2) and mouse apoM (NP_061286.1) is over 80%, and both contain a hydrophobic binding pocket (12, 13). The identity of human (NP_061974.2) and mouse apoM (NP_061286.1) is over 80%, and both contain a hydrophobic binding pocket (12, 13). The identity of human (NP_061974.2) and mouse apoM (NP_061286.1) is over 80%, and both contain a hydrophobic binding pocket (12, 13). The identity of human (NP_061974.2) and mouse apoM (NP_061286.1) is over 80%, and both contain a hydrophobic binding pocket (12, 13).

HDL particles contain apoM (15). HDL-bound apoM can also transfer PL to the HDL surface. To complete the process of RCT, HDL-C is taken up by the liver, mostly via scavenger receptor class B, type I (SR-BI), for secretion into bile and excretion in feces (11).

Apom knock-out mice have a 17–21% decrease in HDL-C, whereas overexpression of human APOM increases HDL-C by 13–22% and protects against atherogenesis (17, 18). The antiatherogenic function of apoM has been attributed to its ability to promote preβ-HDL formation (17), stimulate cholesterol efflux from macrophage foam cells (18, 19), and increase the antioxidant activity of HDL (20). We previously demonstrated that apoM stimulates the generation of larger nascent HDL particles by HEK293 cells that overexpress ABCA1 (21). However, whether hepatic overexpression of apoM affects plasma HDL size, composition, and function (i.e. RCT) in vivo is unknown.

In addition to its role as an HDL-binding protein, which can impact HDL metabolism, apoM is also a sphingosine 1-phosphate (S1P) carrier (22). Apom knock-out mice have decreased HDL-S1P, whereas APOM transgenic mice display an increase in HDL-S1P (22). Adenovirus-mediated overexpression of APOM also increases plasma apoM and S1P concentrations (23). Because S1P signaling helps maintain endothelial integrity (22, 24) and immune homeostasis (25), HDL apoM may also be atheroprotective by transporting S1P through the plasma compartment to endothelial and immune cell receptors (26). Previous studies demonstrated that tissue sources of plasma S1P include erythrocytes (27), vascular endothelial cells (28), and platelets (29, 30). Hepatocytes can also generate and secrete S1P (23, 28). Because apoM is mainly produced by the liver and its overexpression in mice increased plasma S1P levels (22), the liver may play an important role in whole body S1P distribution and homeostasis. However, to date, there are limited data regarding the extent to which hepatic apoM expression regulates hepatocyte S1P formation and secretion (23).

The goal of this study was to address unanswered questions regarding the role of hepatic apoM expression in HDL, apoM, and S1P metabolism. Based on our previous studies showing that HEK293-ABCA1-expressing cells form larger nascent HDL particles, we explored whether hepatic overexpression of apoM resulted in increased nascent and mature plasma HDL particle size and S1P content in vivo. We also wished to determine how hepatic apoM expression affects plasma HDL composition and the ability of HDL to promote in vivo macrophage RCT. Finally, we investigated the effect of hepatic apoM overexpression on hepatic sphingolipid metabolism and S1P production and secretion. Collectively, our results suggest that hepatic apoM overexpression enhances sphingolipid recycling and secretion of S1P from hepatocytes and also generates larger nascent HDLs and apoM/S1P-enriched plasma HDL particles.

**EXPERIMENTAL PROCEDURES**

**Generation of Hepatocyte-specific Human APOM Transgenic (APOM Tg) Mice**—A full-length human APOM cDNA (GenBank™ accession number BC020683) was purchased from the National Institutes of Health mammalian gene collection, human (Invitrogen, Clone ID 4733993), and used to construct a C-terminal FLAG-tagged APOM cDNA as described previously (21). To create APOM Tg mice, MfI and MluI sites were introduced into the 5′- and 3′-flanking ends, respectively, of C-terminal FLAG-tagged APOM cDNA by PCR amplification using the following primers: forward, 5′-CTGCCAATTTGAAGATGTTCCACCAAATTTGGGC-3′; reverse, 5′-ACGACCGGGTTCATCTTGTCGTCGTCCCTTTGATGTCGTTATTGG-3′. Amplified cDNA was digested with MfI and MluI and then cloned into the same sites in the pLiv-11 plasmid (31), a liver-specific transgenic expression vector (32–34). The transgene cassette containing the human apoE promoter, C-terminal FLAG-tagged human APOM cDNA insert, and human apoE 3′ hepatic control region was released from the plasmid construct by Spel/Sall digestion, followed by agarose gel electrophoresis and purification of the excised gel fragment with the Elutip kit (Whatman/GE Healthcare). Pronuclear microinjection of the transgene cassette into fertilized mouse oocytes from B6D2F1 (C57BL/6×DBA/2) mice and subsequent implantation of microinjected embryos into foster mothers were conducted at the Transgenic Mouse Core Facility of Wake Forest School of Medicine. Founders were identified by PCR screening of tail genomic DNA and immunoblotting of plasma with antiFLAG monoclonal antibody M2 (Sigma). PCR primers used to determine the presence and integrity of the transgene cassette were as follows: 5′-CAGCTGGCAGTTGATGACAG-3′ and 5′-CTCACAGGCTCTTGATTC-3′ for the human APOM gene; 5′-CATGAGGTTAGGAGAAGGGAC-3′ and 5′-AGGGCGG GTTCATCTGCAAGGGAC-3′ for the human apoE promoter region; 5′-CGCCTTCACTTGAGAAGAC-3′ and 5′-CTCTCAAGGCTCTTGATTC-3′ for the human apoE hepatic control region, and 5′-GGCAGTGGTTAGGAGAAGGGAC-3′ and 5′-AGGGCGG GTTCATCTGCAAGGGAC-3′ for the VLDL receptor, used as a load control. Upon breeding of transgenic mice with WT mice, ~50% of offspring were transgenic, confirming hemizygosity of the transgene.

**Animals**—All animal procedures were approved by the Institutional Animal Care and Use Committee of Wake Forest School of Medicine. Mice were housed in the Wake Forest School of Medicine animal facility with a 12-h light/12-h dark cycle and fed a commercial chow diet ad libitum. Wild-type (C57BL/6) and homozygous human apoA-I transgenic mice on a pure C57BL/6J background (hA-I Tg) were obtained from Jackson Laboratory (C57BL/6-Tg(APOA1)Rub/J). APOM Tg mice were backcrossed into the C57BL/6J background for at least five generations. hA-I Tg/APOM Tg mice were generated by...
crossing APOM Tg mice with hA-I Tg mice. All APOM Tg mice studied herein were compared with littermate controls.

Plasma Lipid and Lipoprotein Quantification—Mouse plasma was harvested by tail bleeding or cardiac puncture of anesthetized mice at sacrifice. Plasma total and free cholesterol, phospholipid, and triglyceride concentrations were measured by enzymatic assays (total cholesterol, FC, TG, and phospholipid (PC), Wako). Plasma samples were either fractionated using two Superose 6 FPLC columns (1 × 30 cm) in series (flow rate 0.5 ml/min) or by a high resolution Superose 6™ FPLC column (10/300GL, Amersham Biosciences; flow rate 0.5 ml/min) with an on-line cholesterol analyzer (35). Lipoprotein fractions eluted from FPLC columns were collected to determine cholesterol, TG, and PL concentrations by enzymatic assay (Wako). In some experiments, lipoprotein fractions were pooled for Western blot analysis. Lipids were extracted by the Bligh-Dyer method, and some experiments, lipoprotein fractions were pooled for Western blot analysis. Lipids were extracted by the Bligh-Dyer method, and concentrations were measured by enzymatic assays (36, 37). ApoA-I concentrations were determined by ELISA (38).

Hepatic Lipid Analysis—Livers were perfused, snap-frozen in liquid nitrogen, and stored at −80 °C. Lipid content was quantified using detergent-based enzymatic assays as described previously (36, 37).

In Vivo Macrophage Reverse Cholesterol Transport—In vivo macrophage RCT assays were conducted as described by Rader and co-workers (39) with minor modifications (40). J774 mouse macrophages were radiolabeled and loaded with [3H]cholesterol and acetylated LDL, respectively. Cells were then injected into the peritoneal cavity of recipient mice, and plasma samples were collected at 6, 24, and 48 h after injection. Feces were collected throughout the 48-h study. At necropsy, tissues were harvested, and 3H tracer levels in plasma, liver, and feces were quantified after lipid extraction and liquid scintillation counting. Aliquots of plasma were also fractionated by FPLC, and cholesterol mass and radiolabel distribution among lipoprotein fractions were quantified.

In Vivo VLDL TG Secretion Rate Determination—Tyloxapol (500 mg/kg body weight) was injected intravenously into 4-h fasted and anesthetized mice to block TG lipolysis acutely (41). Blood was collected before (0 min) and 30, 60, 120, and 180 min after injection for measurement of plasma triglyceride (TG) concentrations by enzymatic assay. VLDL-TG secretion rate was determined by calculating the slope of the time versus plasma TG concentration plot for each animal using linear regression analysis.

Primary Hepatocyte Isolation, Nascent HDL Particle Formation, and S1P Production—Primary hepatocytes were isolated as described previously (42) with minor modifications. After isolation from liver, hepatocytes were centrifuged at 50 × g for 5 min in 50% Percoll-containing Williams’ media E to pellet live cells. Cells were then washed with Williams’ media E before seeding in 100-mm dishes at a density of 2 × 10⁶ cells per dish. Cells were incubated at 37 °C for 4 h before experiments were initiated. Primary hepatocytes were incubated with 10 μg/ml 125I-apoA-I (10⁵ cpm/μg) for 24 h in serum-free DMEM using conditions described previously (43). After incubation, the conditioned media were harvested and fractionated on a Superdex-200HR FPLC column (Amersham Biosciences) eluted with 0.15 M NaCl, 0.01% EDTA, pH 7.4, at a flow rate of 0.3 ml/min. Fractions were collected and analyzed for 125I radioactivity to monitor the size distribution of nascent HDL particles.

Quantification of Sphingolipids by Liquid Chromatography and Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS)—Plasma samples were freshly collected and stored in methanol before extraction. Livers were perfused via the portal vein with PBS, and liver tissues were collected and snap-frozen. HDL FPLC fractions were collected, supplemented with phosphatase inhibitors (Roche Applied Science, catalog no. 04906845001), and stored frozen. Hepatocytes from WT and APOM Tg mice were isolated and cultured in Williams’ media E for 3 h, washed, and preincubated with serum-free DMEM for 2 h. Fresh serum-free DMEM was then replaced; hepatocytes were incubated for 6 h before media, and cells were collected for analysis. Media were centrifuged to pellet cell debris, and supernatants were harvested with addition of phosphatase inhibitors. Cells were washed with PBS and scraped off the plates in methanol. Sphingolipid content/concentration of plasma, liver, HDL, hepatocytes, and hepatocyte-conditioned medium was determined using liquid chromatography mass spectrometry. HPLC grade solvents from VWR (West Chester, PA) were used for all steps of the procedure. Extraction and quantitation of each sphingolipid species were essentially as described previously (44) using a Shimadzu LC-20 AD coupled to an ABI 4000 quadrupole/linear ion trap (QTrap, Applied Biosystems, Foster City, CA) operating in a triple quadrupole mode as described previously (44).

In some experiments, S1P concentrations in plasma and FPLC-isolated plasma lipoprotein fractions were also measured essentially as described by Berdyshev et al. (45) using 25-μl aliquots of mouse plasma containing 50 pmol of C₁₇S₁P as an internal standard. After extraction and derivatization, bisacetylated S1P was analyzed on a YMC-Pack ODS-AQ column (100 × 1.0 mm inner diameter, 3-μm particle size) and detected using a ThermoFinnigan TSQ Quantum Discovery Max Triple Quadrupole mass spectrometer in negative ion mode. Bisacetylated sphingolipids were eluted using the following gradients: 2 min hold of solvent A/solvent B (50:50), ramp to 100% solvent B over 0.1 min, hold at 100% solvent B for 8.5 min and then regenerate the column with solvent A/solvent B (50:50) for 30 min with the following settings: ion spray voltage −3000 V, ion transfer capillary temperature 200 °C, collision gas 1 millitorr of argon with a collision energy of 11 V. Multiple reaction monitoring transitions monitored were C₁₇S₁P m/z 448/388 and S1P m/z 462/402.

Analysis of Hepatocyte S1P Synthesis and Secretion—S1P synthesis from radiolabeled sphingosine was measured as described previously (47). Primary hepatocytes were isolated from WT and APOM Tg mice, seeded in 35-mm dishes, and incubated in serum-free media for 2 h at 37 °C before addition of 0.225 μCi of [3H]sphingosine (PerkinElmer Life Sciences) and 1.5 mm sphingosine (Cayman Chemical) per dish for 10 min to radiolabel cells. Medium was then switched to fresh serum-free DMEM, and cells were incubated at 37 °C for 2 h.
before and cells were collected for differential lipid extraction (47). Cell and media [3H]S1P radiolabels were quantified by liquid scintillation spectroscopy after extraction. Data are presented as [3H]S1P/total 3H radiolabel × 100% in cells or media, normalized to cellular PL content as measured by phosphorus assay (48).

**Myriocin and Fumonisin B1 Treatment**—Primary hepatocytes were isolated and seeded for 2 h before they were washed and incubated with serum-free media for 2 h. Myriocin (Sigma) dissolved in methanol and fumonisin B1 (Sigma) dissolved in ethanol were added to hepatocytes at a final concentration of 10 and 25 μM, respectively, and incubated for 6 h before media were collected and supplemented with phosphatase inhibitors. Control dishes were incubated with the appropriate vehicle. Cells were washed and collected in methanol. Both cells and media were subjected to LC-ESI-MS/MS analysis. Replicate dishes in each group were used to determine protein content by BCA assay.

**Western Blotting**—Proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell) (250 mA, 2 h). Membranes were blocked with 5% non-fat dry milk in TBST buffer, incubated with primary antibody at 4 °C overnight, washed three times, and then incubated with secondary antibody for 1 h at room temperature. Blots were incubated with SuperSignal West Pico chemiluminescence substrate (Pierce) and visualized with a Fujifilm LAS-3000 camera. Antibodies used for Western blots included the following: anti-FLAG monoclonal antibody M2 (Sigma); anti-apoE and anti-LCAT (generated in-house); anti-mouse apoM (Lifespan); anti-human and mouse apoM (Abcam); anti-mouse apoA-I (Biodesign); anti-GAPDH (Sigma), and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare). Band intensities were quantified using MultiGauge software (Fujifilm).

**Gene Expression Analysis**—RNA was isolated from liver and hepatocytes using TRIzol (Invitrogen) and reverse-transcribed into cDNA using Omniscript RT kit (Qiagen). Expression levels were analyzed by quantitative real time PCR (49). Primers used for indicated gene expression are: GAPDH, forward, TGTGTCGGCTGTTGAATCTG; and reverse, CCTGCTTACACCTTCTGTGAT; SPTLC1, forward, AGTGGTGGGAGAGTCCCCTTT; and reverse, CAGTGGACCAAAACCTGTAG; SPHK2, forward, CGGATGGCCATTGGTTCTCT; and reverse, TGAGCAAAGCTGCAACCCG; SGPP1, forward, GGAGGTTGCTGAATGCTG; and reverse, GCAAATGGCGTTTCAACAGAGCA; acid SMase, forward, ATGCCCTCACCACCTAGAAA; and reverse, AGCAGGATCTGGAGGTGA; SR-BI, forward, GGCTGCTTTTGTGCG; and reverse, GTCTGGATGAGGGGAG.

**Statistics**—Results were reported as mean ± S.E. Two-tailed Student’s t tests were used to detect statistically significant differences between wild-type and APOM Tg mice. Two-way analysis of variance and the Bonferroni post hoc test were used to analyze data from myriocin and fumonisin B1 inhibition experiments. A p value <0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism software.

### RESULTS

**Generation of Hepatocyte-specific Human APOM Transgenic Mice**—Hepatocyte-specific APOM transgenic (APOM Tg) mice were generated using the pLiv11 vector (32–34). PCR of mouse tail genomic DNA was used to identify transgenic positive mice. To estimate the level of overexpression of human apoM, WT and APOM Tg mouse plasma were analyzed by Western blot analysis using an antibody (Abcam) that detects both mouse and human apoM (Fig. 1A). The mass of apoM observed in 2 μl of WT and Tg mouse plasma is consistent with ~3–5-fold overexpression of apoM in the Tg mice.

**Quantification of Plasma and Liver Lipids in APOM Tg Mice**—We examined plasma lipid and lipoprotein cholesterol concentrations in APOM Tg mice in both WT and human apoA-I Tg (hA-I) backgrounds. Two different backgrounds were examined because WT mice have relatively uniformly sized HDL particles, whereas hA-I Tg mice have polydisperse HDL subfractions that are similar in size to human plasma HDL particles (50). In both WT and hA-I backgrounds, male APOM Tg mice had significantly increased plasma total cholesterol (24% increase in WT and 10% increase in hA-I background), PL (29% increase in WT and 19% increase in hA-I background), and triglyceride (TG) (4.5-fold increase in WT and 6.7-fold increase in hA-I background) concentrations relative to their non-apoM transgenic counterparts (Fig. 1B). Plasma FC (WT, 35.55 ± 0.84 vs. APOM Tg, 49.14 ± 4.17 mg/dl; p < 0.05) and CE (WT, 111.2 ± 2.5 vs. APOM Tg, 130.2 ± 5.8 mg/dl; p < 0.05) were both increased in APOM Tg mice, relative to the WT control. FPLC fractionation of plasma revealed that the increased plasma cholesterol level in APOM Tg mice was mostly attributed to increased plasma VLDL (7.6-fold increase in WT and 15.8-fold increase in hA-I background) and LDL concentrations (no increase in WT and 1.7-fold increase in hA-I background) (Fig. 1C). In contrast, HDL-C did not differ between APOM Tg mice and their control counterparts in both backgrounds (Fig. 1C). We observed a similar lipid and lipoprotein phenotype in female mice (data not shown).

Whole plasma apoB concentrations were also increased in APOM Tg mice, suggesting an increase in plasma apoB-containing lipoproteins (Fig. 2, A and B). To determine whether liver-specific overexpression of human apoM increased hepatic VLDL-TG secretion, we acutely inhibited intravascular TG lipolysis using a detergent block procedure and observed similar rates of plasma TG accumulation in WT and APOM Tg mice (Fig. 2, C and D), suggesting that hepatic VLDL-TG secretion was not altered by overexpression of human apoM. Thus, in agreement with a previous study (51), increased plasma VLDL-C and TG likely resulted from delayed VLDL catabolism. In contrast to plasma values, hepatic levels of FC, CE, and TG were similar in WT and APOM Tg mice (data not shown), except for a significant decrease in hepatic CE in male APOM Tg mice (WT, 0.52 ± 0.07 vs. APOM Tg 0.27 ± 0.07 μg per mg liver weight, p < 0.05).

**APOM Tg Mice Generated Larger Plasma HDLs Enriched in CE, ApoE, LCAT, and ApoM**—We previously reported that apoM overexpression in a nonhepatic cell line (HEK293-ABCA1) generated larger nascent HDL particles (21). To deter-
mine whether liver-specific overexpression of human apoM increased HDL particle size in vivo, we studied plasma lipoprotein distribution by FPLC size exclusion chromatography. Interestingly, in both WT and hA-I backgrounds, HDL from APOM Tg mice eluted from the FPLC column at an earlier position relative to their WT counterparts, suggesting increased HDL particle size (Fig. 3A). To further explore this, we measured the lipid content of the FPLC-isolated HDL and normalized the results to apoA-I content, as determined by ELISA. As shown in Table 1, HDLs from APOM Tg mice contained significantly more CE and PL per apoA-I molecule, likely contributing to the increased HDL size in APOM Tg mice. The HDL ratio of surface lipid and protein to core lipids (FC/H11001 PL/H11001 apoA-I)/CE was significantly decreased in APOM Tg versus WT mice (Table 1), also agreeing with increased HDL size in APOM Tg mice. Finally, we examined the protein composition of the FPLC-isolated HDL fractions by Western blot analysis. In both genetic backgrounds, APOM Tg mice had HDLs with more apoE and LCAT than their WT counterparts (Fig. 3, B and C). Endogenous mouse apom expression was lower in APOM Tg mice in both backgrounds (Fig. 3, B and C), presumably due to displacement by the more abundant human apoM. In general, apoE-containing HDLs are larger than non-apoE-containing HDL particles (52). The enrichment of LCAT on HDLs from APOM Tg mice likely leads to enhanced conversion of FC to CE, resulting in increased HDL size and CE content in APOM Tg mice.

**Hepatic ApoM Stimulates Formation of Large S1P-enriched HDL**

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**FIGURE 1.** Lipid and lipoprotein characteristics of APOM Tg mice. A, Western blot analysis of 2 μl of WT and APOM Tg mouse plasma using an antibody that detects both mouse and human apoM. Band intensities were quantified using MultiGauge software. The intensity of the least intense band for WT and APOM Tg plasma was set to 1, and other bands were normalized to its intensity and are shown above the bands. B, plasma total cholesterol (TC), phospholipid (PL), and triglycerides (TG) from male mice were analyzed by enzymatic assays. C, plasma lipoproteins were fractionated by FPLC, and cholesterol concentration in VLDL, LDL, and HDL was determined by enzymatic assays. B and C, n = 13, 9, 11, and 12 for WT, APOM Tg, hA-I, and hA-I/APOM Tg mice, respectively. *, p < 0.05, APOM Tg mice versus their control (WT or hA-I) counterparts. hA-I = human apoA-I transgenic mouse.

**FIGURE 2.** APOM Tg and WT mice have comparable hepatic TG secretion. A, plasma samples from mice of the indicated genotypes were Western-blotted for apolipoprotein B. B, Western blot results were quantified using MultiGauge software. Total apoBs from apoB100 and apoB48 were calculated, normalized to total apoB in WT sample on the 1st lane as fold change, and plotted; *, p < 0.05. C and D, mice fasted for 4 h were retro-orbitally injected with tyloxapol (500 mg/kg) to block TG lipolysis acutely. Blood was collected before injection (0) and 30, 60, 120, and 180 min after injection. C, plasma TG levels at each time point were determined by enzymatic assay and plotted; n = 5. D, slope of the line of best fit for each mouse was calculated by linear regression analysis using GraphPad Prism software; *, p < 0.05.

**ApoM Predominantly Associates with Larger Sized HDL**

Because we observed a similar increase in HDL size in APOM Tg mice for both WT and hA-I backgrounds, all subsequent studies were performed using APOM Tg mice in the WT background. To determine the relationship between HDL particle size and apoM content, we examined apoM distribution across HDL fractions eluted from an FPLC column. The cholesterol distribution for WT and APOM Tg mouse plasma fractionated by FPLC is shown in Fig. 4A; the distribution of mouse and human apoM and mouse apoA-I, determined by Western blot analysis, is shown in Fig. 4B. Using mouse apoA-I as a protein
marker of HDL particle size distribution, we observed that mouse and human apoM distribution was shifted to earlier eluting fractions relative to apoA-I, demonstrating that both endogenous mouse apoM and transgenic human apoM preferentially associate with larger HDL particles.

**Primary Hepatocytes from APOM Tg Mice Produce Larger Nascent HDL Particles**—To determine whether increased plasma HDL size in APOM Tg mice resulted from assembly of larger nascent HDL by hepatocytes, we isolated primary hepatocytes from WT and APOM Tg mice and examined nascent HDL formation by incubating hepatocytes with 125I-apoA-I for 24 h, followed by size exclusion chromatography. As shown in Fig. 5, hepatocytes from APOM Tg mice generated a similar number of small nascent HDL particles but relatively more particles of larger sizes as compared with WT mice. This agreed with our previous finding using the nonhepatic HEK293-ABCA1-expressing cell line, which showed that apoM overexpression increased nascent HDL particle size (21). Because nascent HDL particles undergo maturation by LCAT and plasma HDL particles from APOM Tg mice were enriched in LCAT (Fig. 3B), it appears that HDL generated by APOM Tg mouse hepatocytes may better recruit LCAT, thereby leading to production of
larger sized CE-enriched plasma HDLs. The increase in HDL particle size is probably not attributable to decreased HDL catabolism, because hepatic levels of SR-BI mRNA and protein levels of SR-BI and LDL receptor-related protein (data not shown), both of which are involved in HDL catabolism (53–55), were similar in both genotypes. Additionally, HDL isolated from APOM Tg and WT mice had similar protein binding capacity (215.3 ± 29.7 versus 232.4 ± 21.3 ng of HDL protein/2 h per mg of cell protein) and HDL cholesterol ester uptake (12.9 ± 0.3% versus 13.6 ± 0.3%) in IdA cells overexpressing SR-BI (56). Overall, our data suggest that larger nascent HDL particles generated from hepatocytes overexpressing apoM lead to generation of larger mature plasma HDL.

Increased HDL Size in APOM Tg Mice Did Not Promote in Vivo Macrophage Reverse Cholesterol Transport—A previous study showed that apoM-enriched HDL from APOM Tg mice increased cholesterol efflux from macrophage foam cells compared with WT mouse HDL, although macrophage RCT was not correspondingly increased in vivo (19). However, in this previous study, no increase in plasma HDL size was reported. To determine whether the larger plasma HDL particles observed in our APOM Tg mice increased RCT, [3H]cholesterol-loaded macrophages were injected into the peritoneal cavity of WT and APOM Tg mice, followed by periodic blood sampling. Mice were sacrificed 48 h after injection, and liver, plasma, and feces were collected for [3H]cholesterol quantification. At all times up to 48 h after injection, APOM Tg and WT mice had comparable levels of plasma [3H]cholesterol radiolabel (Fig. 6A), even though the FPLC distribution of radiolabel among plasma lipoproteins revealed more radiolabel in larger HDL of APOM Tg versus WT mice (Fig. 6B). These data suggest a similar ability of plasma from WT and APOM Tg mice to efflux macrophage-radiolabeled cholesterol. Furthermore, the radiolabel in liver (Fig. 6C) and feces (Fig. 6D) was similar for WT and APOM Tg mice, suggesting that larger apoM-enriched HDL particles in APOM Tg mice do not enhance in vivo macrophage RCT.

Large HDL in APOM Tg Mice Preferentially Transport S1P—Because recent studies suggested that S1P is transported in plasma by apoM, and its plasma concentration is increased in apoM transgenic mice and decreased in apoM knock-out mice (22, 57), we explored the extent to which the larger HDL particles in our APOM Tg mice increased S1P levels. Plasma S1P concentrations were increased ~5-fold in APOM Tg mice (~3.5 μM) versus WT (~0.7 μM) (Fig. 7A). Dihydrosphingosine 1-phosphate (DH-S1P), a product of dihydrosphingosine phosphorylation by sphingosine kinases, was also increased ~2-fold in APOM Tg mouse plasma (Fig. 7B). S1P was significantly increased in plasma VLDL, LDL, and HDL of APOM Tg mice, but the largest increase and the predominant lipoprotein fraction containing S1P was large HDL (Fig. 7C). Large HDL was also most enriched in apoM (Figs. 4 and 7D). Collectively, these
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FIGURE 8. APOM Tg mice have increased hepatic S1P production and secretion. A, liver tissues were subjected to lipid extraction, and S1P and DH-S1P were analyzed by LC-ESI-MS/MS. B and C, hepatocytes from WT and APOM Tg mice were incubated in 1 ml of serum-free medium for 6 h. Lipids were extracted from cells (B) and media (C), and sphingolipids were analyzed by LC-ESI-MS/MS. A–C, data were normalized to liver or cellular protein content; n = 3 for each genotype; *, p < 0.05. D and E, primary hepatocytes were isolated from WT and APOM Tg mice and incubated in serum-free media for 2 h at 37 °C before addition of 0.225 μCi of [3H]sphingosine and 1.5 mM sphingosine for 10 min to radiolabel cells. Medium was replaced with serum-free DMEM, and cells were incubated at 37 °C for 2 h before media and cells were collected for lipid extraction. Cell (D) and media (E) [3H]S1P radiolabel were quantified by liquid scintillation spectroscopy after differential extraction (48). Data are presented as [3H]S1P/total [3H] radiolabel × 100%, normalized to cellular PL content measured by phosphorus assay. n = 3/genotype; *, p < 0.05.

Data suggest that hepatocyte-specific apoM overexpression generates larger size HDL particles that appear optimal for primary hepatocytes overexpressing apoM compared with their WT counterparts (Fig. 8C), whereas sphingoid bases levels in the medium were similar (data not shown). To further examine the effect of apoM overexpression on hepatocyte S1P synthesis and secretion, primary mouse hepatocytes were incubated with [3H]sphingosine. Although both WT and APOM Tg hepatocytes produced similar amounts of [3H]S1P (Fig. 8D), APOM Tg hepatocytes secreted significantly more [3H]S1P (Fig. 8E), further supporting the notion that apoM overexpression enhances secretion of S1P from hepatocytes.

Increased Sphingolipid Content in Hepatocytes from APOM Tg Mice—Hepatocytes from APOM Tg mice also had significantly increased cellular ceramide and sphingomyelin content compared with those from WT mice (Fig. 9A). The major ceramide species in hepatocytes are the very long chain ceramides, C22:0, C24:1, and C24:0, followed by the long chain C16 species (Fig. 9B). All of these ceramide species were increased to similar extents by overexpression of apoM, suggesting that cellular ceramide content does not increase because of changes in activity of specific ceramide synthases with different fatty acyl-CoA specificity. In contrast, ceramide and sphingomyelin content in the media of hepatocytes overexpressing apoM was decreased (Fig. 9C). Taken together, these results suggest that apoM overexpression in hepatocytes specifically promotes the secretion of S1P and DH-S1P but not other sphingolipid species. In addition, comparable levels of ceramide and sphingomyelin were found in plasma and HDL fractions from APOM Tg and WT mice (data not shown).

Sphingosine kinase 1 and 2 (Sphk1 and Sphk2) catalyze the production of S1P and DH-S1P from sphingosine and Dihydrosphingosine, respectively (Fig. 9D). To determine whether Sphk1 and Sphk2 were up-regulated in APOM Tg mice, leading to increased cellular S1P and DH-S1P levels in hepatocytes, we measured mRNA expression in liver and primary hepatocytes. Expression of Sphk1 was barely detectable in liver and hepatocytes (Fig. 9, E and F), whereas Sphk2 was slightly up-regulated in liver (Fig. 9E), but not in primary hepatocytes (Fig. 9F), from APOM Tg mice. Sphk2 protein expression was similar in WT and APOM Tg mouse primary hepatocytes (data not shown). Furthermore, the major hepatocyte sphingosine kinase activity was Sphk2, with minimal Sphk1 activity (Fig. 9G). Although there was no apparent difference in Sphk2 protein expression, its activity was slightly but significantly increased (29%) in hepatocytes from APOM Tg mice. We also analyzed liver expression of other key enzymes involved in generation or breakdown of S1P (Fig. 9E), including SPTLC1, acid sphingomyelinase, and SGPP1; only acid sphingomyelinase was slightly increased. Overall, these data indicate that there are no major changes in the expression of genes involved in sphingolipid metabolism in APOM Tg mice.

Ceramide Synthase Inhibition Induces Cellular Levels, but Not Secretion, of S1P and DH-S1P in Hepatocytes from APOM Tg Mice—Ceramide, the precursor of sphingosine and S1P, is produced in cells by de novo biosynthesis, which is initiated by the condensation of serine with palmitoyl-CoA by serine palmitoyltransferase or by recycling of sphingosine via a salvage pathway (Fig. 9D). Dihydrosphingosine, but not sphingosine, is an intermediate in the de novo biosynthesis pathway. To examine
the pathways involved in the generation of increased levels of S1P and long chain ceramides in apoM-overexpressing hepatocytes, we used myriocin (ISP-1), a specific inhibitor of serine palmitoyltransferase (SPT) sphingolipid synthesis, and fumonisin B1 (FB1), an inhibitor of CoA-dependent dihydroceramide/ceramide synthase (58). Myriocin treatment did not significantly decrease cellular ceramide levels in WT and apoM-overexpressing hepatocytes (Fig. 10A), yet, as expected, it did decrease levels of dihydroceramides (Fig. 10B) and dihydro sphingosine (data not shown). Although S1P levels in hepatocytes overexpressing apoM were slightly decreased by myriocin treatment (Fig. 10C), this decrease did not affect secretion of S1P or DH-S1P (Fig. 10D).
Collectively, these data suggest that de novo sphingolipid synthesis only plays a minor role in elevating cellular ceramide and S1P in APOM Tg hepatocytes and does not influence secretion of S1P or DH-S1P.

In agreement with previous studies (60), FB1, which inhibits all six ceramide synthases, reduced ceramide levels (Fig. 11A). Importantly, it also reduced cellular ceramide levels in apoM-overexpressing hepatocytes (Fig. 11A). Decreases in cellular ceramides were accompanied by increased levels of sphingosine and dihydrosphingosine (data not shown), as well as their phosphorylated forms, S1P and DH-S1P (Fig. 11B). These data suggest that recycling of sphingosine to the ceramide biosynthetic pathway is enhanced by apoM overexpression. However, although FB1 induced a large increase in cellular S1P and DH-S1P, it had no effect on their secretion into the medium, even in hepatocytes overexpressing apoM (Fig. 11C). Taken together, these results suggest that apoM is rate-limiting in mobilizing cellular S1P (and DH-S1P) for secretion.

DISCUSSION

ApoM is a low abundance apolipoprotein that associates with ∼5% of plasma HDL particles (13) and is suggested to be atheroprotective (17, 18). Although overexpression of apoM in HEK293 cells leads to generation of larger nascent HDLs (21), it is unclear whether in vivo hepatic overexpression of apoM increases HDL particle size. Here, we show that liver-specific human apoM transgenic mice had larger plasma HDL particles that were enriched with CE, LCAT, apoE, apoM, and S1P. Despite the presence of larger plasma HDL particles, APOM Tg mice displayed plasma HDL-C concentrations and in vivo macrophage reverse cholesterol transport similar to WT mice. Primary hepatocytes from APOM Tg versus WT mice generated larger nascent HDL and had increased sphingolipid synthesis and secretion of S1P. Inhibition of ceramide synthase in hepatocytes greatly increased cellular S1P levels, but not secretion, suggesting that apoM is rate-limiting in the export of hepatocyte S1P. Collectively, these data indicate that liver apoM facilitates the generation of larger nascent HDL and stimulates the synthesis and secretion of hepatocyte S1P, both
leading to the presence of large apoM/S1P-enriched plasma HDL. These unique HDL particles may serve to deliver S1P to extrahepatic tissues for atheroprotection or other as yet unidentified functions.

**ApoM Expression and HDL Particle Size**—The appearance of the unique HDL particle enriched in CE, LCAT, apoE, apoM, and S1P in APOM Tg mice was observed in both WT and hA-I backgrounds and in the absence of any detectable increase in plasma HDL-C concentrations. In contrast, Christoffersen et al. (18) reported that transgenic mice with 10-fold overexpression of apoM had a 13–22% increase in HDL-C compared with WT mice, but HDL particle size was unaffected. In mice deficient in hepatic nuclear factor α or treated with apoM siRNA, both of which result in low apoM expression, HDL particles were larger than their control counterparts (17). However, this phenotype was not confirmed in later studies using apoM knock-out mice (18). The discrepancy regarding HDL particle size between our study and the study by Christoffersen et al. (18) may be related to the sensitivity of methods used to detect HDL size changes and differences in APOM Tg mouse lines (liver-specific promoter versus endogenous promoter). Very recently, another study was published showing that adenoviral overexpression of apoM in mice resulted in larger plasma HDL (23). Here, we show that apoM was predominantly associated with larger sized HDL both in APOM Tg mice and WT mice, suggesting that large plasma HDL particles are optimal for binding of apoM and S1P regardless of the level of hepatic apoM expression. In contrast to our results in mice, a study by Lee et al. (61) suggested that human plasma S1P was more concentrated in smaller sized HDL₃ compared with larger sized HDL₂ and that HDL₃-S1P is more biologically active in stimulating plasminogen activator inhibitor-1 release from adipocytes than HDL₂-S1P. Although Lee et al. (61) did not analyze apoM levels, apoM distribution on HDL particles was likely similar to that of S1P, suggesting that apoM is associated with small-sized HDL in human plasma. Differences between our mouse study and the human study could be attributable to cholesterol ester transfer protein remodeling of human HDL. Cholesterol ester transfer protein, which is present in human, but not mouse plasma, exchanges CE in HDL for TG in VLDL and LDL, resulting in TG-enriched HDL particles that are substrates for hepatic lipase and endothelial lipase-mediated remodeling to smaller HDL (62). This process may lead to the conversion of large S1P-containing HDL to smaller S1P-containing HDL. Additional studies will be necessary to define the compositional elements of HDL that dictate optimal apoM/S1P binding.

**ApoM and Nascent HDL Formation**—Primary hepatocytes from APOM Tg mice generated larger nascent HDL particles compared with hepatocytes from WT mice, agreeing with previous findings that apoM overexpression in ABCA1-expressing HEK293 cells leads to formation of larger nascent HDL (21). After secretion, nascent HDL particles undergo maturation by several lipid transfer proteins and modifying enzymes. LCAT converts FC to CE in HDL, generating spherical HDL particles (9). It is likely the larger nascent HDL particles secreted by APOM Tg mouse hepatocytes are the precursor particles for the large plasma HDL particles that are enriched in CE, LCAT, apoE, and apoM/S1P (Fig. 3 and Table 1). Christoffersen et al. (18) also observed an increase in HDL apoE content in APOM Tg mice, although HDL size and LCAT activity remained unchanged. The LCAT enrichment of HDL likely resulted in increased generation of CE that would help drive HDL particle enlargement, which, in turn, may allow more efficient apoE binding. Previous studies showed that HDL enrichment with SM decreased LCAT-mediated CE formation (63). Consistent with this finding, hepatocyte medium from APOM Tg mice had decreased SM concentrations (Fig. 9C). These combined results are compatible with the hypothesis that ABCA1-mediated nascent HDL particle assembly in the presence of apoM overexpression results in decreased SM, which in turn allows for more efficient LCAT-mediated cholesterol esterification and HDL particle maturation to larger spherical plasma HDL enriched in apoM/S1P.

**ApoM Overexpression Increases Liver and Hepatocyte S1P Synthesis and Secretion**—S1P is rapidly turned over in plasma (t½ ~15 min), suggesting that a high capacity biosynthetic source of S1P is necessary to maintain plasma S1P concentrations (28). Previous studies have suggested that erythrocytes, platelets, and vascular endothelial cells are major contributors to plasma S1P. Platelets have high sphingosine kinase activity and no S1P lyase or phosphatase activity, resulting in a high cellular content of S1P (29, 30). However, a mouse model with virtually no circulating platelets had normal plasma S1P levels, suggesting the existence of other cellular sources of plasma S1P (27). Results from adoptive transplantation of WT bone marrow into mice without plasma S1P due to conditional ablation of SphK1 and SphK2 demonstrated that hematopoietic cells are a major source of plasma S1P (27). Furthermore, using mice with deletion of SphK1 and SphK2 in hematopoietic cells, it was conclusively demonstrated that erythrocytes are a major source of plasma S1P (27). Surprisingly, however, mice that were thrombocytopenic, anemic, or leukopenic did not show an appreciable reduction in plasma S1P, again indicating the existence of other tissue sources of S1P (28). Adenoviral overexpression of SphK1 in livers of SphK1 whole body knock-out mice restored plasma S1P levels, which was attributed to enhanced secretion of S1P from liver endothelial cells, not hepatocytes, because cultured endothelial cells secreted more S1P than primary hepatocytes (28). However, because there are many more hepatocytes than endothelial cells in liver, this conclusion may have been premature. In this study, we show that hepatocytes secrete significant amounts of S1P and DH-S1P and that overexpression of apoM enhances their secretion. Furthermore, apoM, the major carrier for plasma S1P (22), is mostly expressed in the liver and kidney, and its expression levels directly correlate with levels of plasma S1P (22), as well as its secretion from hepatocytes (Figs. 8, 10, and 11). Hence, it appears, based on this study and other evidence, that hepatocytes are an important source of plasma S1P.

Our data suggest that in addition to its role in maintaining plasma S1P levels and as a carrier of intravascular S1P to extrahepatic tissues, apoM also stimulates hepatic S1P production. Interestingly, apoM overexpression in hepatocytes also enhanced levels of ceramide, most likely resulting from enhancement of the salvage/recycling pathway, because inhibition of ceramide synthases...
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with FB1, but not inhibition of de novo sphingolipid synthesis with myriocin, reduced ceramide elevations. Although FB1 treatment increased cellular S1P and DH-S1P, it did not affect their secretion (Fig. 11), suggesting that hepatocyte apoM production is rate-limiting in S1P secretion.

Hepatic Sphingolipid Production and Cholesterol Metabolism—Hepatocytes from APOM Tg mice had increased cellular ceramide and SM content (Fig. 9) and secreted larger nascent HDL particles (Fig. 5), suggesting that there is coordination between sphingolipid and cholesterol metabolism. Sphingolipids and cholesterol not only serve as essential structural components of membranes, especially lipid microdomains (64), but also regulate the metabolism of one another. For example, induction of cellular sphingolipid storage stimulated cholesterol synthesis by activating sterol-responsive element-binding protein 2 (SREBP2), a major transcriptional regulator of genes involved in cholesterol uptake and biosynthesis. Conversely, sphingo-

myelin depletion inhibited SREBP2 activation (65), resulting in cholesterol synthesis inhibition (66). Ceramide treatment of nonhepatic cell lines also promoted ABCA1-mediated cholesterol efflux (67). In mice deficient in S1P lyase, an enzyme responsible for S1P degradation, plasma HDL-C and LDL-C were significantly increased, as were plasma and liver S1P (68), suggesting a sphingolipid intermediate or S1P may regulate HDL and LDL production. These data support the hypothesis that increased hepatocyte ceramide, sphingomyelin, and/or S1P content in APOM Tg mice may contribute to the generation of larger nascent HDLs.

In conclusion, our study demonstrates that hepatocyte-speci-
fic apoM overexpression facilitates formation of large apoM/ S1P-enriched HDL by promoting formation of large nascent HDL and stimulating sphingolipid synthesis and S1P secretion. These unique HDL particles may serve to deliver S1P to extrahepatic tissues for atheroprotection or other functions.

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