

# Microsomal Triglyceride Transfer Protein Transfers and Determines Plasma Concentrations of Ceramide and Sphingomyelin but Not Glycosylceramide\*<sup>§</sup>

Received for publication, April 15, 2015, and in revised form, September 1, 2015. Published, JBC Papers in Press, September 8, 2015, DOI 10.1074/jbc.M115.659110

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**Background:** It is unknown how sphingolipids arrive at plasma.

**Results:** Plasma ceramide and sphingomyelin are low in microsomal triglyceride transfer protein (MTP)-deficient humans and mice. MTP does not play a role in sphingolipid synthesis.

**Conclusion:** MTP transfers and assists in the secretion of ceramide and sphingomyelin with apoB-containing lipoproteins.

**Significance:** MTP is a major and a partial determinant of plasma ceramide and sphingomyelin, respectively.

Sphingolipids, a large family of bioactive lipids, are implicated in stress responses, differentiation, proliferation, apoptosis, and other physiological processes. Aberrant plasma levels of sphingolipids contribute to metabolic disease, atherosclerosis, and insulin resistance. They are fairly evenly distributed in high density and apoB-containing lipoproteins (B-lps). Mechanisms involved in the transport of sphingolipids to the plasma are unknown. Here, we investigated the role of microsomal triglyceride transfer protein (MTP), required for B-lp assembly and secretion, in sphingolipid transport to the plasma. Abetalipoproteinemia patients with deleterious mutations in MTP and absence of B-lps had significantly lower plasma ceramide and sphingomyelin but normal hexosylceramide, lactosylceramide, and different sphingosines compared with unaffected controls. Furthermore, similar differential effects on plasma sphingolipids were seen in liver- and intestine-specific MTP knock-out (*L,I-Mttp*<sup>-/-</sup>) mice, suggesting that MTP specifically plays a role in the regulation of plasma ceramide and sphingomyelin. We hypothesized that MTP deficiency may affect either their

synthesis or secretion. MTP deficiency had no effect on ceramide and sphingomyelin synthesis but reduced secretion from primary hepatocytes and hepatoma cells. Therefore, MTP is involved in ceramide and sphingomyelin secretion but not in their synthesis. We also found that MTP transferred these lipids between vesicles *in vitro*. Therefore, we propose that MTP might regulate plasma ceramide and sphingomyelin levels by transferring these lipids to B-lps in the liver and intestine and facilitating their secretion.

Because of their hydrophobicity, lipids are transported in the aqueous milieu (plasma) of blood associated with proteins (e.g. albumin) or lipoproteins (1, 2). There are two types of plasma lipoproteins as follows: apoB-containing lipoproteins (B-lps)<sup>4</sup> and apoB-free high density lipoproteins (HDLs). B-lps and HDLs transport different lipids in the plasma compartment (1). Triglycerides are transported with B-lps, whereas phospholipids and cholesterol are transported with both B-lps and HDL.

B-lps are assembled in the endoplasmic reticulum (ER) and Golgi of hepatocytes and enterocytes in a two-step process that requires microsomal triglyceride transfer protein (MTP) (3, 4). Kinetic studies suggest that MTP has two lipid transfer sites as follows: a slow phospholipid transfer site that is hypothesized to be in the N-terminal domain and a fast bulk lipid (triglyceride, phospholipid, and cholesterol ester) transfer site in the C-ter-

\* This work was supported, in whole or in part, by National Institutes of Health Grants HL-95924 and R56DK046900-17A1 (to M. M. H.) and Grants HL-38180, DK-56260, and DK-52574 (to N. O. D.). This work was also supported by Veterans Affairs Merit Award BX001728 (to M. M. H.), American Heart Association grant-in-aid (to J. I.), and Veterans Affairs Merit Award BX000978 (to R. L. K.).

<sup>§</sup> This article contains supplemental Tables 1–9.

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<sup>2</sup> Supported in part by the Lipidomics Shared Resource, Hollings Cancer Center, Medical University of South Carolina Grant P30 CA138313 and National Institutes of Health Grant P20 RR017677 (to the Lipidomics Core, Lipidomics and Pathobiology COBRE, Medical University of South Carolina for sphingolipid analysis).

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<sup>4</sup> The abbreviations used are: B-lps, apoB-containing low density lipoprotein; ABCA1, ATP cassette-binding family A protein 1; ABL, abetalipoproteinemia; ER, endoplasmic reticulum; HE ABL, heterozygous ABL; HO ABL, homozygous ABL; MTP, microsomal triglyceride transfer protein; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); MTPi, MTP inhibitor; HE ABL, heterozygous ABL; SMSi, sphingomyelin synthase inhibitor.

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minal domain (5). In the first step, MTP physically associates with apoB to help translocation across the ER membrane and transfers triglycerides, phospholipids, and cholesterol esters to nascent apoB to form primordial lipoproteins. In the second step, primordial lipoproteins fuse with luminal lipid droplets to form mature particles. It is possible that MTP may also participate in the formation of luminal lipid droplets and their fusion with the primordial particles (3, 4).

In humans, MTP deficiency results in abetalipoproteinemia (ABL), which is a rare autosomal recessive disorder with an estimated frequency of <1 in 1,000,000 characterized by the absence of plasma B-lps, fat-soluble vitamin deficiency, stunted growth, and acanthocytosis (6). ABL patients have low plasma levels of glycerolipids (triglycerides and phospholipids) and cholesterol, but the concentrations of sphingolipids in their plasma are unknown.

Germ line MTP ablation in mice is embryonic lethal (7, 8). In postnatal animals, however, liver-specific ablation of MTP reduces plasma cholesterol and triglycerides by 60–80% and increases their hepatic concentrations (8–10). Intestinal MTP gene deletion in mice also reduces (~40–50%) plasma triglycerides and cholesterol levels and enhances triglyceride and free fatty acid accumulation in the intestine (11–13). However, the effect of tissue-specific MTP deficiency on plasma and tissue sphingolipids in mice is unknown.

Sphingolipids include hundreds of distinct molecular species consisting of the common 18-carbon amino-alcohol backbone, sphingosine. Cellular sphingolipids are biologically active in signaling, differentiation, apoptosis, stress responses, inflammation, atherosclerosis, and lipoprotein metabolism (14–16). Different species of sphingolipids carry out distinct functions. Ceramides act as tumor suppressors by augmenting apoptosis, autophagy, and cell cycle arrest (17). Their accumulation in the muscle, adipose tissue, and heart increases insulin resistance, inflammation, and atherosclerosis (16, 18). In contrast to ceramides, glucosylceramides support cell proliferation and differentiation, and the ablation of genes involved in their synthesis results in embryonic lethality (19). In *ob/ob* mice, glucosylceramides are increased, and inhibition of their synthesis improves glucose homeostasis, adipocyte function, and insulin sensitivity (19). Atherosclerotic plaques contain large amounts of lactosylceramide, which appear to stimulate smooth muscle cell proliferation and increase expression of adhesion molecules in vascular endothelial cells (20). Sphingomyelins are concentrated in lipid rafts, and these membrane microdomains perform specialized functions such as cell signaling, lipid and protein sorting, cholesterol efflux, and an inflammatory response (21, 22).

*De novo* sphingolipid synthesis begins in the ER with the condensation of serine and palmitoyl-CoA to form 3-ketodihydrosphingosine followed by its reduction to dihydrosphingosine. Dihydrosphingosine is acylated to dihydroceramide and reduced to ceramide. Ceramides are then transported to the Golgi via ceramide transfer protein, four-phosphate adaptor protein 2 (FAPP2), or transport vesicles for the synthesis of sphingomyelin, hexosylceramide, lactosylceramide, and other complex sphingolipids. In the salvage pathway, sphingomyelin is hydrolyzed to sphingosine and transported to the ER for cer-

amide synthesis. Ceramide can also be generated on the plasma membrane by sphingomyelinases.

Although intracellular transport of sphingolipids is widely studied, mechanisms of their transport to the plasma are unknown. Sphingolipids are present in plasma associated with both B-lps and HDLs in nearly equal concentrations (23–25). Sphingosines and their 1-phosphates are associated with albumin and HDL (26). Higher levels of plasma sphingolipids are associated with various pathologies. Total plasma ceramide levels are increased in cardiovascular disease (27), type 2 diabetes (28, 29), obesity (28), and insulin resistance (16). Plasma sphingomyelin levels are higher in hypercholesterolemia and increase the risk for coronary artery disease (30, 31). Thus, there is a need to know the origin and mechanisms involved in the regulation of plasma sphingolipids.

Studies in rat primary hepatocytes have suggested that palmitic acid treatment increases cellular and secreted ceramide (32, 33). However, secretion of sphingomyelin was equivocal. Secreted ceramide was mainly associated with VLDL (32). Palmitic acid also increased cellular MTP and ABCA1 (33). Therefore, it has been speculated that MTP and ABCA1 might play a role in ceramide secretion (33). Here, we explored the role of MTP and B-lps in the transport of various sphingolipids from the liver and intestine to the plasma.

### Experimental Procedures

**Materials**—[<sup>3</sup>H]Triolein (22 Ci/mmol), [<sup>3</sup>H]sphingomyelin (60 Ci/mmol), [<sup>3</sup>H]dihydrosphingosine (60 Ci/mmol), and [<sup>14</sup>C]ceramide (50 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc. NBD-ceramide (catalog no. 6224) was purchased from Setareh Biotech, and NBD-sphingomyelin (catalog no. 229573) was from Avanti Polar Lipids.

Human plasma samples from heterozygous (HE, *n* = 4) and homozygous (HO, *n* = 9) ABL subjects were collected at the University of Pennsylvania, University of Modena and Reggio (Emilia, Italy), The Robarts Research Institute (Canada), and the Washington University School of Medicine (St. Louis) after obtaining approval from the universities' institutional review boards and consent from subjects and/or parents. Frozen samples were shipped to the State University of New York Downstate Medical Center where different analyses were performed. Additionally, appropriate aliquots of these samples were shipped from the State University of New York Downstate to the Lipidomics Shared Resource at the Medical University of South Carolina for sphingolipid analyses using LC-MS/MS as described previously (24).

**Animals**—Chow fed, male, 10-week-old mice on a C57BL/6J background were used in these studies. *ER<sup>T2</sup>-villin-Cre;Mttp* (*Mttp<sup>f/f</sup>*) mice have been described previously (12, 34). These mice were generated by breeding mice that carry floxed *Mttp* gene with *ER<sup>T2</sup>-villin-Cre* mice. To generate liver- and intestine-specific MTP-ablated (*L,I-Mttp<sup>-/-</sup>*) mice, 8-week-old *Mttp<sup>f/f</sup>* mice were injected once intravenously with 10<sup>9</sup> pfu of either AAV-TBG-Luc (control) or AAV-TBG-Cre viruses (to ablate hepatic floxed genes) in 100 μl of sterile PBS. Additionally, mice injected with AAV-TBG-Luc were injected intraperitoneally with 200 μl of corn oil for 3 alternate days to serve as controls. Mice injected with AAV-TBG-Cre were injected with

0.5 mg of tamoxifen (induces Cre-recombinase expression in the intestine to ablate floxed genes) in 200  $\mu$ l of corn oil for 3 alternate days to obtain *L,I-Mttp*<sup>-/-</sup> mice. Plasma and tissues were harvested 7 days after the last tamoxifen injection. All experiments were approved by the Institutional Care and Use Committee at State University of New York Downstate Medical Center.

**MTP Lipid Transfer Assays**—MTP lipid transfer assays were performed as described before (35–37). Donor phosphatidylcholine vesicles were prepared by sonicating 1.8  $\mu$ mol of phosphatidylcholine, 135 nmol of cardiolipin, and 1  $\mu$ Ci of radiolabeled [<sup>3</sup>H]triolein, [<sup>14</sup>C]ceramide, or [<sup>3</sup>H]sphingomyelin in 4.5 ml of buffer II (15 mM Tris, 40 mM NaCl, 1 mM EDTA, 0.02% NaN<sub>3</sub>, pH 7.4) for 20 min. Acceptor vesicles were prepared by sonicating 10.8  $\mu$ mol of phosphatidylcholine and 21.6 nmol of triolein in 4.5 ml of buffer II for 40 min. Donor and acceptor vesicles were centrifuged in Beckman Coulter ultracentrifuge for 60 min at 50,000 rpm and 10 °C. The top 4 ml were transferred to a different tube. For blank and totals, 50  $\mu$ l of donor and 50  $\mu$ l of acceptor vesicles were incubated with 200  $\mu$ l of BSA stock (10 mg of fatty acid-free BSA/ml buffer II). To measure MTP activity, 50  $\mu$ l of donor vesicles, 50  $\mu$ l of acceptor vesicles, and 200  $\mu$ l of BSA stock were incubated with purified MTP or tissue homogenates. After 1 h, 200  $\mu$ l of DE52 (1:1 v/v suspension in buffer containing 15 mM Tris, 1 mM EDTA, 0.02% NaN<sub>3</sub>, pH 7.4) was added in blank and MTP samples (totals received only the above buffer), mixed for 5 min by rotation, and centrifuged for 10 min at 12,000  $\times$  *g* and 4 °C. To determine the amounts of radiolabeled lipids transferred to acceptor vesicles, supernatants (200  $\mu$ l) were added to 5 ml of a scintillation mixture and counted in a scintillation counter. Amounts of radiolabel transferred to acceptor vesicles were quantified and expressed as % transfer/h.

To determine IC<sub>50</sub> values, donor vesicles containing different radiolabeled lipids were incubated with 1  $\mu$ g of purified MTP with indicated concentrations of lomitapide. After 1 h, DE52 was added to precipitate donor vesicles. Amounts of radioactivity transferred to acceptor vesicles were quantified. Non-linear regression analysis (Graphpad Prism) was used to calculate IC<sub>50</sub> values.

**Cell Culture Studies**—To study the effect of MTP inhibitor on sphingolipid synthesis, Huh-7 cells were incubated with fumonisin B1 (50  $\mu$ M) or lomitapide (500 nM) for 2 h, washed, and then incubated with [<sup>3</sup>H]dihydrospingosine (0.5  $\mu$ Ci) and 15  $\mu$ M unlabeled dihydrospingosine for 1 or 3 h in the presence and absence of different inhibitors. Lipids from cells were extracted with 100  $\mu$ l of isopropyl alcohol and separated on thin layer silica plates (catalog no. 44931, Analtech, Inc.) using a CHCl<sub>3</sub>/CH<sub>3</sub>OH/C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>/NH<sub>4</sub>OH/H<sub>2</sub>O (40:40:20:0.4:1.6, ratios by volume) solvent system.

To determine whether MTP inhibition affects sphingolipid secretion, cells were incubated with fumonisin B1 (50  $\mu$ M) or lomitapide (500 nM) and [<sup>3</sup>H]dihydrospingosine (0.5  $\mu$ Ci) for 16 h. Lipids from media were extracted with CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, v/v) (38). Lipids were evaporated, dissolved in 100  $\mu$ l of isopropyl alcohol, and separated by TLC as described above.

**Ceramide Synthesis**—Liver or intestinal homogenates (250  $\mu$ g of protein) from wild-type and *L,I-Mttp*<sup>-/-</sup> mice were incu-

bated with [<sup>3</sup>H]dihydrospingosine (0.05  $\mu$ Ci), dihydrospingosine (15  $\mu$ M), fatty acid-free BSA (20  $\mu$ M), and different fatty acyl-CoAs (50  $\mu$ M) in the presence or absence of fumonisin B1 (50  $\mu$ M) or lomitapide (500 nM) in a total volume of 200  $\mu$ l reaction for 30 min at 37 °C. The reaction was stopped by adding 200  $\mu$ l of CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, v/v). Lipids were extracted and subjected to TLC.

**Sphingomyelin Synthesis**—Liver tissues from wild-type and *L,I-Mttp*<sup>-/-</sup> mice were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5% sucrose, pH 7.4, and a mixture of protease inhibitors. The homogenate was centrifuged in a tabletop centrifuge 5415D (Eppendorf) at 5,000 rpm for 10 min at 4 °C. The supernatant (250  $\mu$ g of protein) was added to assay buffer containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, C6-NBD ceramide (3.3  $\mu$ g/ml), and phosphatidylcholine (100  $\mu$ g/ml) in a total volume of 200  $\mu$ l reaction and incubated for 2 h at 37 °C. The reaction was stopped by adding 200  $\mu$ l of CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, v/v). Lipids were extracted and subjected to TLC.

Enterocytes from wild-type and *L,I-Mttp*<sup>-/-</sup> were isolated as described previously (39). Enterocytes or Huh-7 cells were incubated with C6-NBD ceramide (3.3  $\mu$ g/ml) and phosphatidylcholine (100  $\mu$ g/ml) in the presence or absence of lomitapide (500 nM), D609 (500  $\mu$ M), an inhibitor of sphingomyelin synthesis, for 2 h. Lipids were extracted from cells with isopropyl alcohol and separated by TLC.

**Quantification of Lipids and Apolipoproteins**—Kits were used to measure triglycerides (Thermo Fisher Scientific, catalog no. TR22421), phospholipids (Wako Diagnostics, catalog no. 433-36201), and cholesterol (Thermo Fisher Scientific, catalog no. TR13421) in plasma, liver, and intestinal samples according to the manufacturer's instructions. Sphingolipids were quantified by LC-MS/MS as described before (24). Human apoB and apoAI were quantified by ELISA (40, 41).

**Quantification of Sphingolipids**—Plasma (100  $\mu$ l) from homozygous (ABL, *n* = 9) or heterozygous (HE ABL, *n* = 4) ABL subjects, as well as *L,I-Mttp*<sup>-/-</sup> (*n* = 3) and *Mttp*<sup>f/f</sup> (*n* = 4) mice (150  $\mu$ l), were used for the quantification of different species of ceramide, sphingomyelins, hexosylceramide, and lactosylceramide using HPLC-MS/MS (24). Amounts of different sphingolipid species were combined to obtain total plasma levels. Two milligrams of liver or intestinal tissue homogenates were used to quantify intracellular sphingolipids. Feces collected over a 48-h period were dehydrated and crushed with a mortar and pestle. Sphingolipids were measured in 1 g of dried feces.

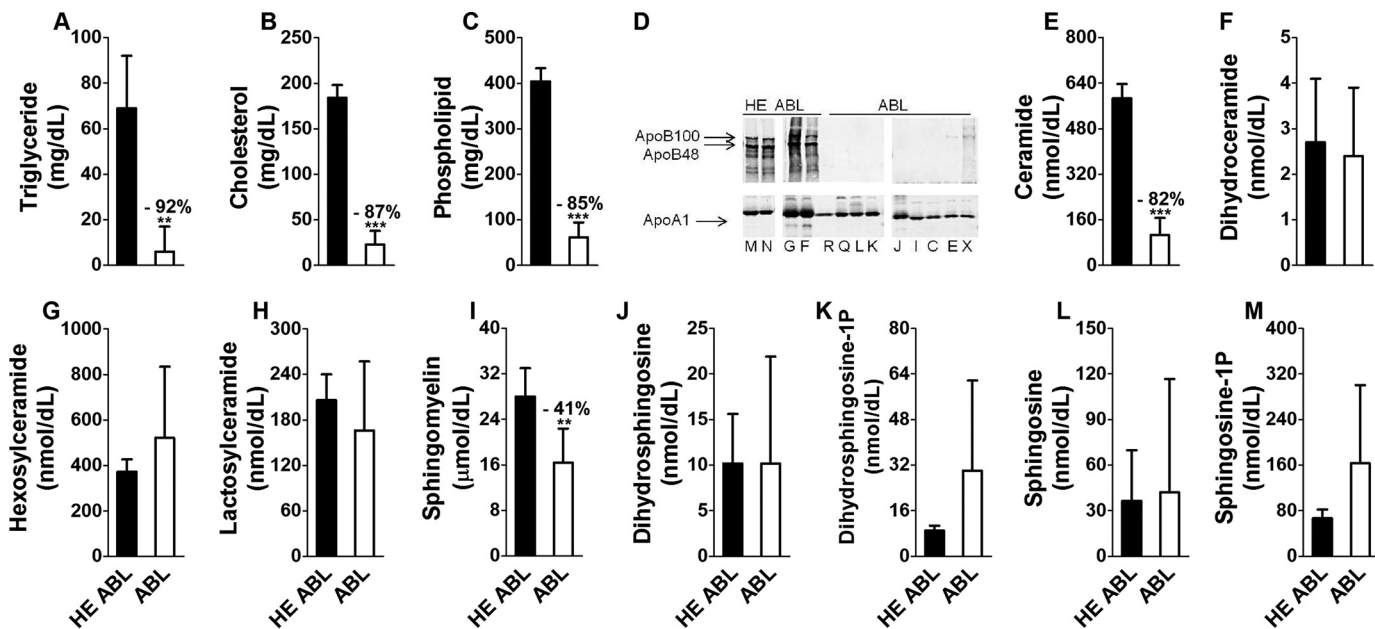
**Statistics**—Comparisons between wild-type and different knock-out mouse models were made by Student's *t* test. Comparisons among HE ABL and HO ABL subjects were evaluated using Student's *t* test using Graphpad Prism. Significance among different treatments in Figs. 4–6 was determined by one-way analysis of variance followed by the Dunnett's test between untreated cells or homogenates and each condition.

## Results

**Plasma Ceramide and Sphingomyelin Concentrations Are Significantly Reduced in ABL Patients**—We measured the concentrations of the traditional plasma lipids and apolipoproteins, as well as those of multiple sphingolipid classes, in the



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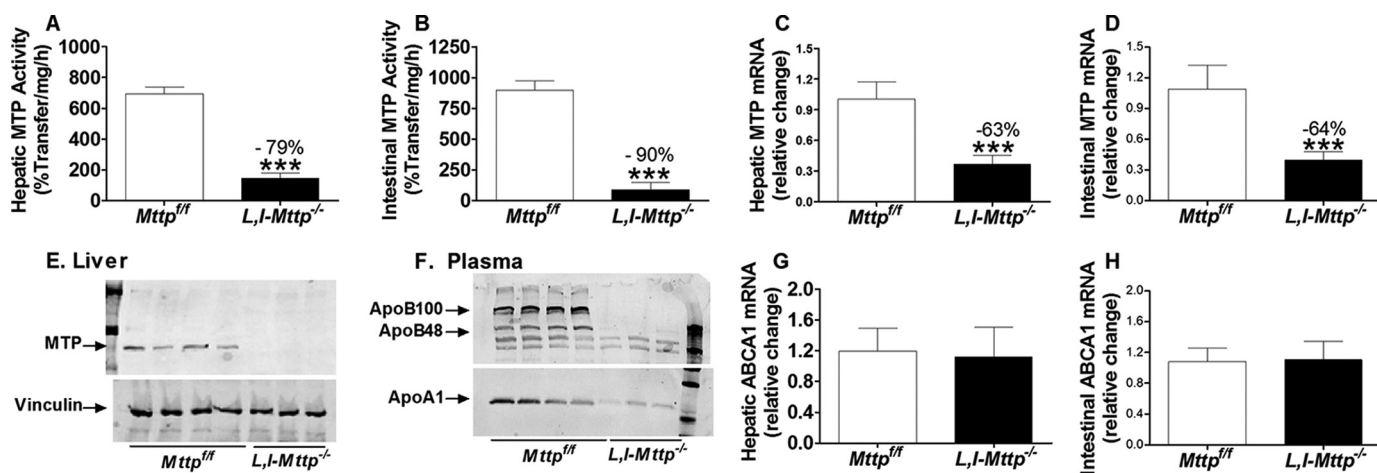
**FIGURE 1. Total plasma lipids in HE ABL and homozygous ABL subjects.** A–C, plasma samples from HE ( $n = 4$ ) and HO ( $n = 9$ ) ABL were used to measure triglyceride, phospholipid, and cholesterol using commercial kits. D, plasma ( $0.5 \mu\text{l}$ ) was separated on 4–15% polyacrylamide gradient gels, transferred to nitrocellulose, and blotted with goat anti-human apoB polyclonal antibodies (Academy Biomedical, catalog no. 205-G2; 1:1,000 dilution). Blots were then incubated with Alexa Fluor<sup>®</sup> 633-labeled rabbit anti-goat antibodies (Invitrogen, catalog no. A-21082; 1:10,000 dilution) and visualized using a phosphorimager (Storm 860, Molecular Dynamics, 635 nm). For apoA1, plasma ( $0.25 \mu\text{l}$ ) was separated on 10% polyacrylamide gels and blotted using goat anti-human apoA1 (Academy Bio-Medical Co., Inc., catalog no. 115-G2, 1:2,000 dilution). Next, membranes were incubated with Alexa Fluor<sup>®</sup> 633 donkey anti-goat IgG (Life Technologies, Inc., catalog no. A-21082, 1:10,000 dilution) and visualized using a phosphorimager. The letters at the bottom correspond to individual samples in the supplemental Tables S1–S5. E–M, various species of ceramide, sphingomyelin, hexosylceramide, and lactosylceramide in the plasma of these subjects were measured using HPLC-MS/MS. Amounts of different species of sphingolipids were combined to obtain total concentrations. Individual values are in supplemental Tables S1–S5.  $p$  values are after two-tailed Student's  $t$  test. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

plasma of ABL subjects and compared them with their unaffected HE ABL siblings and parents. ABL subjects had significantly lower total plasma triglyceride, cholesterol, phospholipid, apoB, and apoA1 (Fig. 1, A–D, and supplemental Table S1). ABL subjects also had significantly lower total plasma ceramide (–82%, Fig. 1E) and sphingomyelin (–41%, Fig. 1I). All other sphingolipids were not significantly different from HE ABL (Fig. 1, F–H, and J–M, and supplemental Table S1). All ceramide species were lower in ABL compared with HE ABL; however, hexosylceramide, lactosylceramide, and sphingosine species were similar in these two groups (supplemental Tables S2–4). Except for C18, C24:1, C26, and C26:1, all sphingomyelin species were lower in ABL (supplemental Table S5). Thus, ABL subjects exhibited significantly lower plasma concentrations of ceramide and sphingomyelin, but the plasma concentrations of other quantified sphingolipids were not reduced.

**Plasma Ceramide and Sphingomyelin Levels Are Significantly Lower in Liver- and Intestine-specific MTP-deficient ( $L,I$ -Mtp<sup>-/-</sup>) Mice**—To generate a mouse model of ABL, we conditionally and simultaneously deleted the *Mtp* gene in the liver and small intestine ( $L,I$ -Mtp<sup>-/-</sup>) of C57BL/6J mice. MTP activity, mRNA, and protein levels were reduced by >60% in the livers and intestines of  $L,I$ -Mtp<sup>-/-</sup> mice compared with controls (Fig. 2, A–E). Furthermore, apoB100 and apoB48 were absent in the plasma of these mice (Fig. 2F). Although plasma apoA1 levels were reduced (–84%) in  $L,I$ -Mtp<sup>-/-</sup> mice compared with controls, *Abca1* mRNA expression in the liver and intestine was not affected (Fig. 2, G and H). Plasma concentrations of triglyceride, phospholipid, cholesterol, and ceramide

were reduced by >90% in  $L,I$ -Mtp<sup>-/-</sup> mice compared with *Mtp*<sup>f/f</sup> control mice (Fig. 3A). Plasma sphingomyelin levels were reduced by ~73% in the MTP-deficient mice, but levels of hexosylceramide, lactosylceramide, dihydrosphingosine, and dihydrosphingosine 1-phosphate (dihydrosphingosine-1P) concentrations were not. Plasma sphingosine concentrations in  $L,I$ -Mtp<sup>-/-</sup> mice were increased by >2-fold compared with *Mtp*<sup>f/f</sup> mice. In contrast, plasma levels of sphingosine 1-phosphate (sphingosine-1P) were significantly decreased by 32% in the same animals (Fig. 3A). The concentrations of all the major ceramide species (supplemental Table S6) were significantly reduced in the plasma of  $L,I$ -Mtp<sup>-/-</sup> mice. Except for decreases in levels of C22 and increases in C22:1 hexosylceramide, other species were not significantly different (supplemental Table S7). The concentrations of C16 and C24:1 lactosylceramide were significantly decreased in the plasma, but levels of other species were not (supplemental Table S8). All species of sphingomyelin were significantly reduced in  $L,I$ -Mtp<sup>-/-</sup> mice (supplemental Table S9). Thus, intestinal and hepatic MTP deficiency reduces total plasma concentrations of ceramide and sphingomyelin in mice. These changes were similar to those seen in ABL subjects.

Hepatic lipid analysis revealed that tissue concentrations of triglyceride and phospholipid were significantly reduced in the  $L,I$ -Mtp<sup>-/-</sup> mice compared with *Mtp*<sup>f/f</sup> mice, but those of cholesterol, sphingomyelin, dihydrosphingosine, and sphingosine 1-phosphate were not (Fig. 3B). In contrast, ceramide, dihydroceramide, hexosylceramide, lactosylceramide, and sphingosine levels were significantly increased in the livers of



**FIGURE 2. MTP and apoB levels in the liver, intestine, and plasma of *L,I-Mttp<sup>-/-</sup>* mice.** In *L,I-Mttp<sup>-/-</sup>* mice, the *Mttp* gene was conditionally deleted in the liver and intestine as described under "Experimental Procedures." *A* and *B*, liver and intestinal homogenates from *Mttp<sup>+/+</sup>* ( $n = 5$ ) and *L,I-Mttp<sup>-/-</sup>* ( $n = 5$ ) mice were used to measure MTP activity. *C* and *D*, MTP mRNA levels in the liver and intestine of MTP deficient and control mice. *E*, liver homogenates (25  $\mu$ g of protein) were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, probed with mouse anti-mouse MTP (BD Transduction Laboratories<sup>TM</sup>, catalog no. 612022, 1:1,000 dilution) followed by Alexa Fluor<sup>®</sup> 633-labeled goat anti-mouse IgG (Life Technologies, Inc., catalog no. A-21050, 1:10,000 dilution). For control, an identical blot was probed with anti-vinculin (Sigma, catalog no. V9131, 1:2,000 dilution). *F*, to detect apoA1 and apoB, plasma (0.5  $\mu$ l) was run on a 5–14% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was cut in half. One-half was probed with rabbit anti-mouse apoB (Meridian Life Science, catalog no. K23300R, 1:1,000 dilution) and then incubated with Alexa Fluor<sup>®</sup> 633 goat anti-rabbit IgG (Life Technologies, Inc., catalog no. A-21070, 1:10,000 dilution). The other half was probed with goat anti-apoA1 (Academy Bio-Medical Co., Inc., catalog no. 115-G2, 1:2,000 dilution) followed by Alexa Fluor<sup>®</sup> 633 donkey anti-goat IgG (Life Technologies, Inc., catalog no. A-21082, 1:10,000 dilution). Bands were visualized in a phosphorimager. *G* and *H*, Abca1 mRNA levels in the liver and intestine of *L,I-Mttp<sup>-/-</sup>* mice.

*L,I-Mttp<sup>-/-</sup>* mice. Significant hepatic accumulation of several sphingolipids in *L,I-Mttp<sup>-/-</sup>* mice suggest that liver MTP might play an important role in the control of hepatic sphingolipid metabolism.

Analyses of different species of ceramide indicated that livers of *L,I-Mttp<sup>-/-</sup>* mice accumulated significant amounts of C14, C16, C18, C18:1, C24:1, and C26:1 ceramide, whereas amounts of C20, C22, C24, C26 ceramide were reduced compared with the levels in control animals (supplemental Table S6). Increases in the concentrations of total hepatic hexosylceramide resulted mainly from accretions of the C16, C18, and C24:1 species despite a significant decrease in the C22 species (supplemental Table S7). Significant increases in hepatic lactosylceramide levels were due to accumulations of C16, C22, and C24:1 species (supplemental Table S8). Although total hepatic sphingomyelin levels were not different in the livers of *L,I-Mttp<sup>-/-</sup>* and control mice, there were significant differences in the accretions of different sphingomyelin species in *L,I-Mttp<sup>-/-</sup>* mice; tissue levels of C18 and C26:1 sphingomyelin were increased, whereas those of the C20, C22, C22:1, C24, and C26 species were significantly reduced (supplemental Table S9). These data indicate that MTP might play a significant role in the transport of sphingolipids with C16, C18, C18:1, C24:1, and C26:1 fatty acyl chains. The reasons for the observed decreases in the concentrations of the C22, C24, and C26 species of ceramide and sphingomyelin species in the livers of *L,I-Mttp<sup>-/-</sup>* mice are unclear.

Intestinal lipid analysis showed increased levels of triglyceride, phospholipid, and cholesterol, whereas sphingomyelin levels were decreased in *L,I-Mttp<sup>-/-</sup>* mice compared with controls (Fig. 3C). No significant accretions of different species of ceramide, dihydroceramide, hexosylceramide, lactosylceramide, dihydrosphingosine, and sphingosine were observed in the intestines of *L,I-Mttp<sup>-/-</sup>* mice (supplemental Tables S6

–S9). Thus, glycerolipids and cholesterol accumulate selectively in the intestines of *L,I-Mttp<sup>-/-</sup>* mice, but sphingolipids do not. These studies highlight tissue-specific roles of MTP in the regulation of glycerolipids and sphingolipids. Furthermore, these results suggest that intestinal MTP activity might contribute significantly to plasma glycerolipid and cholesterol levels, whereas MTP activity in the liver contributes to plasma sphingolipid levels (see "Discussion").

Analysis of fecal lipids revealed increased excretions of phospholipid, cholesterol, ceramide, and sphingomyelin in *L,I-Mttp<sup>-/-</sup>* mice but not of glycosylceramide (Fig. 3D). Concentrations of other lipids were not different in *L,I-Mttp<sup>-/-</sup>* mice and *Mttp<sup>+/+</sup>* mice. Increases in fecal ceramide were correlated with significant enhancements in C18, C20:1, and C24:1 species, whereas those in sphingomyelin were due to increases in C16 and C18:1 species (supplemental Tables S6–S9). Other sphingolipid species were not significantly enhanced.

**MTP Does Not Play a Role in Ceramide and Sphingomyelin Synthesis but Is Required for Their Secretion**—The above studies indicated that MTP deficiency significantly reduces plasma ceramide and sphingomyelin in humans and mice. Therefore, we wanted to find out how MTP regulates plasma ceramide and sphingomyelin levels. We hypothesized that the low plasma concentrations of ceramide observed during MTP deficiency might be secondary to reduced synthesis of these lipids in the liver. The liver expresses several ceramide synthases that prefer different fatty acyl-CoA substrates (22, 42). The mouse liver homogenates converted >90% of [<sup>3</sup>H]dihydrosphingosine to dihydroceramide when incubated with lignoceroyl-CoA (Fig. 4A) consistent with this being the major hepatic ceramide synthase activity (42). Fumonisin B1, an inhibitor of ceramide synthesis (43), inhibited this process by >75%, but an MTP inhibitor (MTPi; lomitapide) did not. Similarly, MTPi did not inhibit synthesis of dihydroceramide in the presence of palmitoyl-

## MTP Deficiency Reduces Plasma Spingolipids

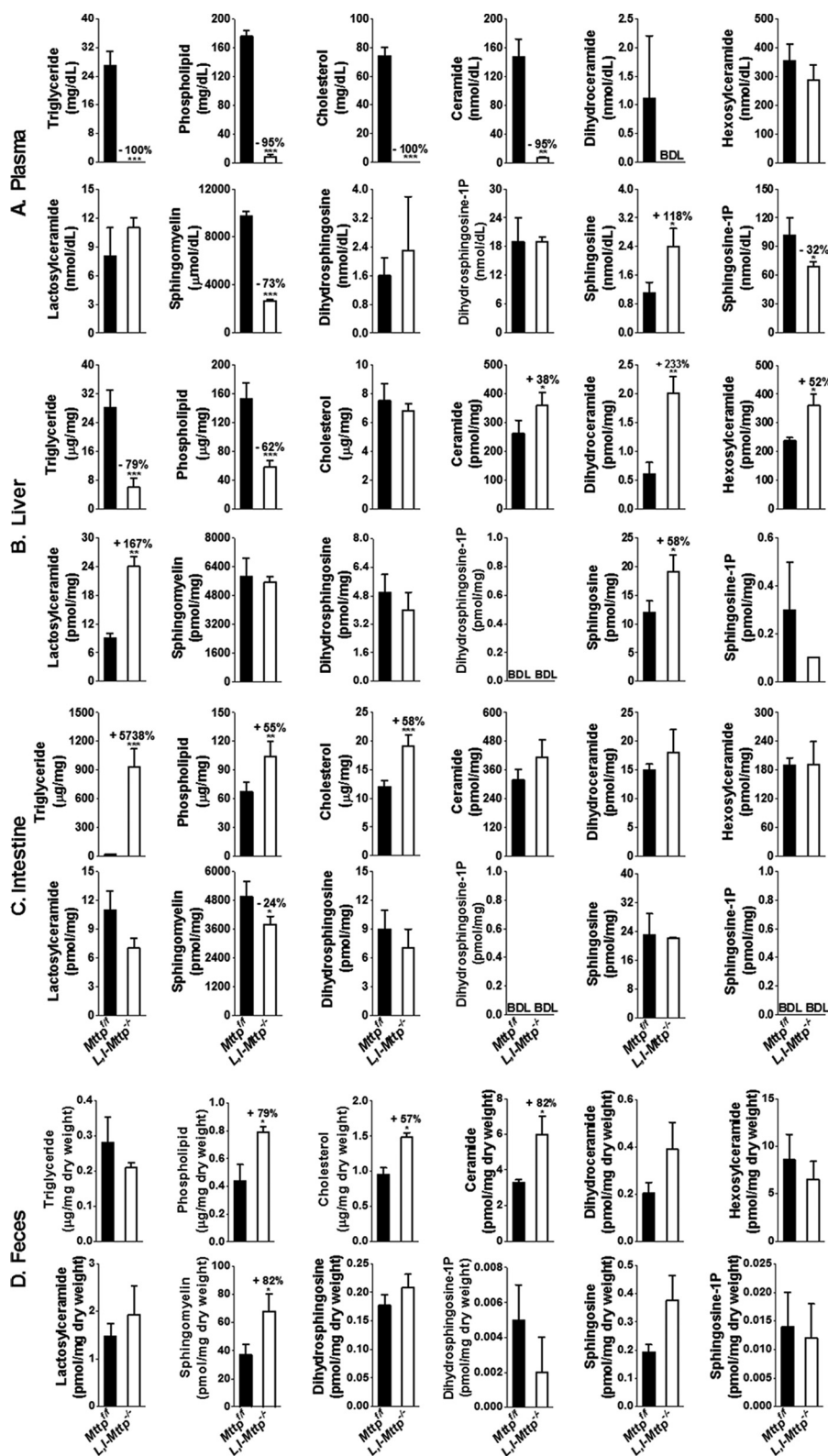
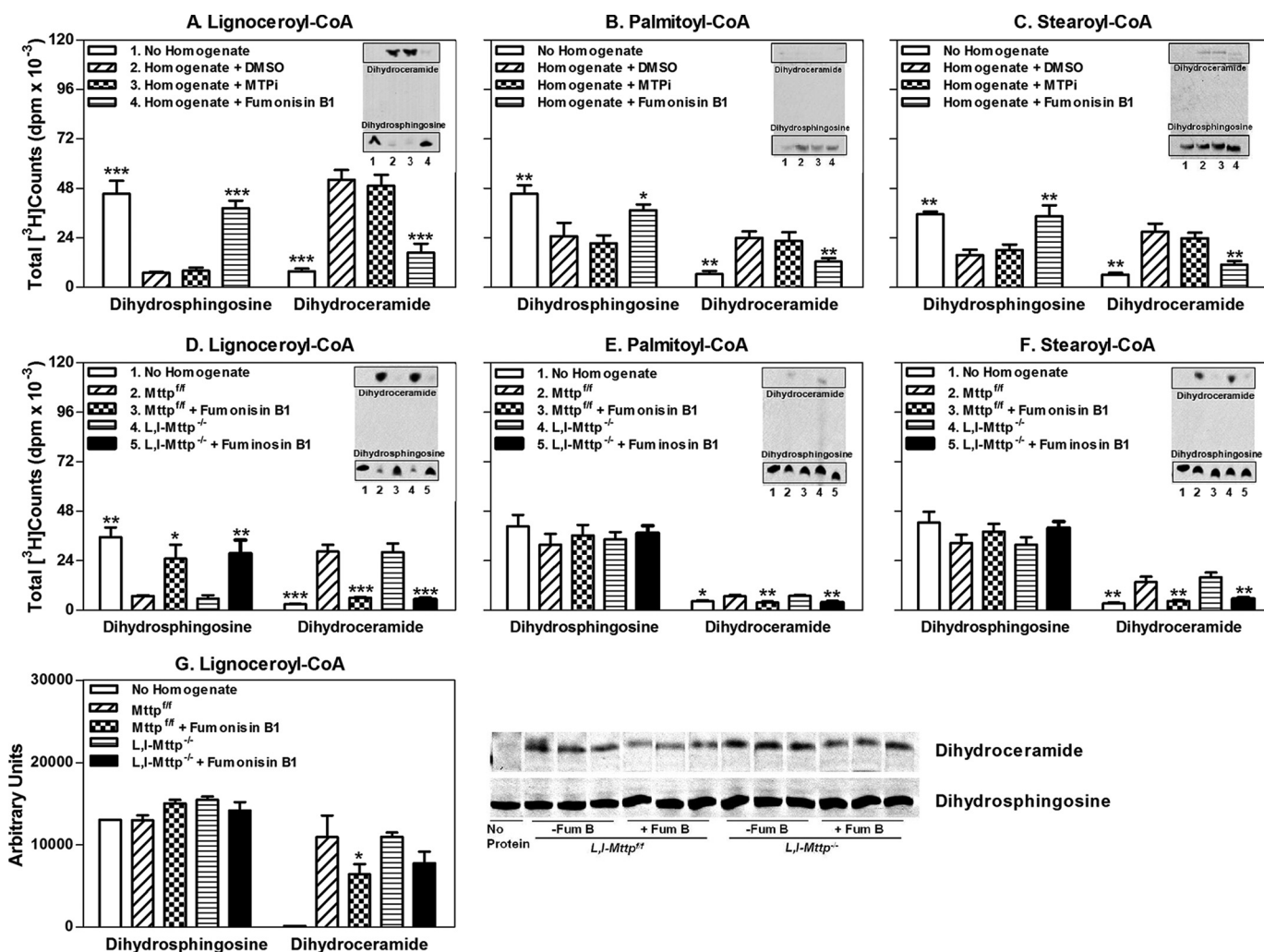


FIGURE 3. **Effect of hepatic and intestinal MTP deficiency on plasma and tissue lipids in mice.** Chow-fed 8-week-old male *Mtp<sup>+/+</sup>* mice were injected once intravenously with  $10^9$  pfu of either AAV-TBG-Luc ( $n = 4$ ) or AAV-TBG-Cre viruses ( $n = 3$ ) in 100  $\mu$ l of sterile PBS. Next, mice injected with AAV-TBG-Luc were injected intraperitoneally with 200  $\mu$ l of corn oil for 3 alternate days. Mice injected with AAV-TBG-Cre were injected with 0.5 mg of tamoxifen in 200  $\mu$ l of corn oil for 3 alternate days to obtain *L1-Mtp<sup>-/-</sup>* mice. Seven days after the last injection, mice were fasted for 16 h, and lipids were measured in plasma (A), liver (B), and intestine (C). In a separate experiment, feces were collected over a period of 48 h from *Mtp<sup>+/+</sup>* ( $n = 3$ ) and *L1-Mtp<sup>-/-</sup>* ( $n = 3$ ) mice (D). Individual values for different sphingolipid species are in supplemental Tables S6–S9.  $p$  values are after two-tailed Student's  $t$  test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .





**FIGURE 4. Effect of MTP and ceramide synthase inhibition on the synthesis of ceramide.** A–C, *in vitro* conversion of dihydrosphingosine to dihydroceramide. Liver homogenates (250  $\mu$ g of protein) from wild-type mice were incubated with [ $^3$ H]dihydrosphingosine (0.05  $\mu$ Ci), dihydrosphingosine (15  $\mu$ M), fatty acid-free BSA (20  $\mu$ M), and lignoceroyl-CoA (50  $\mu$ M), palmitoyl-CoA (50  $\mu$ M), or stearoyl-CoA (50  $\mu$ M) in the presence or absence of MTP inhibitor (500 nM) or fumonisin B1 (50  $\mu$ M) for 30 min at 37  $^{\circ}$ C. The reaction was stopped by adding 200  $\mu$ l of  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (2:1, v/v). Lipids were extracted and subjected to TLC. Radioactivity in the dihydrosphingosine and dihydroceramide bands was quantified. D–F, liver homogenates from *Mttp<sup>fl/fl</sup>* and *L,I-Mttp<sup>-/-</sup>* mice were incubated with [ $^3$ H]dihydrosphingosine (0.05  $\mu$ Ci), dihydrosphingosine (15  $\mu$ M), fatty acid-free BSA (20  $\mu$ M) and different indicated fatty acyl-CoAs (50  $\mu$ M) in the presence and absence of fumonisin B1 (50  $\mu$ M) or MTPi (500 nM) for 30 min at 37  $^{\circ}$ C. Lipids were extracted and separated on TLC. G, experiments were performed similarly to D–F with intestinal homogenates from *Mttp<sup>fl/fl</sup>* and *L,I-Mttp<sup>-/-</sup>* mice. Bands were quantified with ImageJ. Significance was determined by one-way analysis of variance followed by the Dunnett's test to determine significance compared with control.  $p \leq 0.05$ . Mean  $\pm$  S.D.,  $n = 3$ .

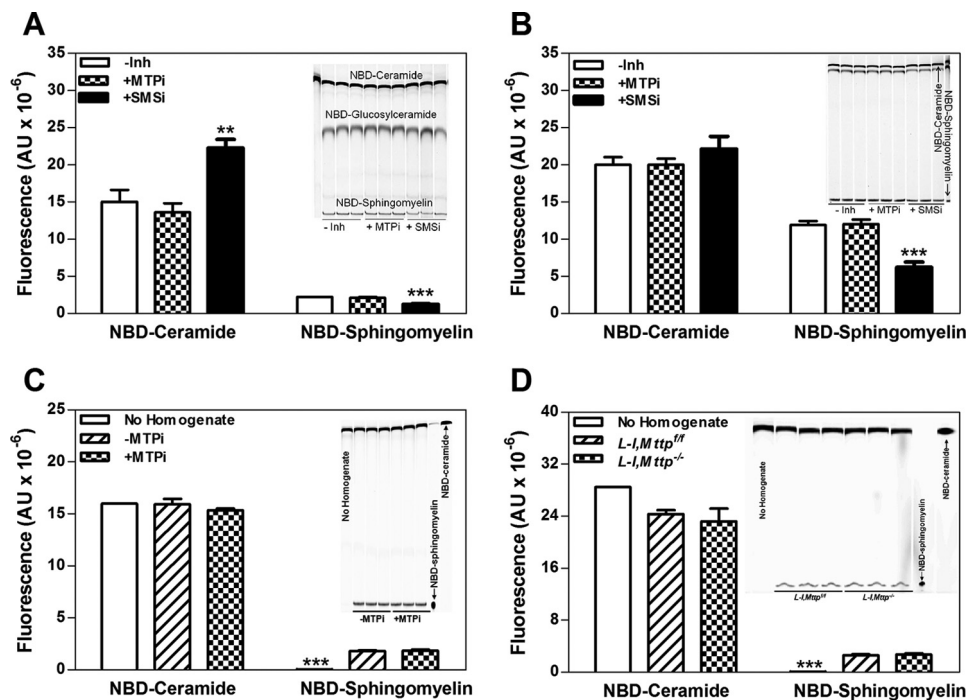
CoA, and stearoyl-CoA (Fig. 4, B and C). Moreover, synthesis of dihydroceramide in the presence of different fatty acyl-CoAs was not affected by MTP deficiency in the livers of *L,I-Mttp<sup>-/-</sup>* mice; however, the synthesis of dihydroceramide in *Mttp<sup>fl/fl</sup>* and *L,I-Mttp<sup>-/-</sup>* mice was inhibited by fumonisin B1 (Fig. 4, D–F). Thus, hepatic MTP deficiency does not affect dihydroceramide synthesis in the tissues.

Next, we determined whether MTP deficiency affected dihydroceramide synthesis in intestinal homogenates. Murine intestinal homogenates had lower conversion of dihydrosphingosine to dihydroceramide compared with the liver consistent with reduced expression of ceramide synthases in the intestine (44), and this conversion was inhibited by fumonisin B (Fig. 4G). There were no significant differences between the synthesis of dihydroceramide in *Mttp<sup>fl/fl</sup>* and *L,I-Mttp<sup>-/-</sup>* intestinal homogenates, indicating that MTP deficiency in the intestine does not affect dihydroceramide synthesis.

To study the effect of MTPi on sphingomyelin synthesis in intestinal and liver cells, murine primary enterocytes (Fig. 5A) and Huh-7 cells (Fig. 5B) were labeled with NBD-ceramide. In enterocytes, NBD-ceramide was converted to sphingomyelin and glucosylceramide. SMSi inhibited the synthesis of sphingomyelin but not of glucosylceramide. Moreover, MTPi had no effect on sphingomyelin and glucosylceramide syntheses. In Huh-7 cells, NBD-ceramide was mainly converted to sphingomyelin. SMSi significantly reduced sphingomyelin synthesis; however, MTPi had no effect. We then asked whether MTP plays a role in sphingomyelin synthesis in liver homogenates. Synthesis of sphingomyelin in liver homogenates incubated with NBD-ceramide was unaffected by MTPi (Fig. 5C) or *Mttp* gene ablations (Fig. 5D). These studies show that MTP deficiency has no effect on sphingomyelin synthesis.

To determine whether MTPi affects ceramide and sphingomyelin synthesis and secretion in cultured cells, we treated

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**FIGURE 5. Effect of MTP inhibition on the synthesis of sphingomyelin.** *A*, enterocytes were isolated from *Mttp<sup>flf</sup>* mice. Enterocytes were incubated with NBD-ceramide (3.3  $\mu\text{g/ml}$ ) and phosphatidylcholine (100  $\mu\text{g/ml}$ ) in the presence or absence of lomitapide (MTPi, 500 nM), D609 inhibitor (SMSi, 500  $\mu\text{M}$ ), or no inhibitor (*-Inh*) for 2 h. Lipids were extracted from the cells using isopropyl alcohol and separated on TLC. *B*, sphingomyelin synthesis was measured in Huh-7 cells as described above. *C*, *in vitro* synthesis of sphingomyelin. Liver homogenates (250  $\mu\text{g}$  of protein) from wild type mice were incubated in triplicate with NBD-ceramide (1  $\mu\text{M}$ ) for 30 min at 37  $^{\circ}\text{C}$  in the presence and absence of MTP inhibitor (500 nM), separated on TLC, and visualized with the phosphorimager. *D*, *in vitro* synthesis of sphingomyelin was performed in liver homogenates obtained from *Mttp<sup>flf</sup>* and *Lj-Mtp<sup>-/-</sup>* mice. Significance was determined by one-way analysis of variance followed by the Dunnett's test to determine significance of each group compared with untreated WT homogenate.  $p \leq 0.05$ . Mean  $\pm$  S.D.,  $n = 3$ .

Huh-7 cells with and without MTPi. As a positive control, cells were treated with fumonisin B1, a ceramide synthase inhibitor. MTPi reduced apoB secretion by 88% with no effect on apoAI secretion (Fig. 6, *A* and *B*). Fumonisin B1 had no effect on apoB and apoAI secretion (Fig. 6, *A* and *B*). When Huh-7 cells were labeled with [ $^3\text{H}$ ]dihydrosphingosine, a precursor for *de novo* ceramide synthesis, radiolabeled lipids migrating with triglyceride, ceramide, dihydrosphingosine, phosphatidylcholine, and sphingomyelin markers were present in control cells at both 1 and 3 h (Fig. 6*C*). In the presence of fumonisin B1, synthesis of radiolabeled ceramide and sphingomyelin was inhibited by  $\sim 96$  and 50% at 1 and 3 h, respectively, compared with controls, but the synthesis of triglyceride and phosphatidylcholine was not inhibited (Fig. 6*C*). MTPi had no effect on triglyceride, ceramide, phosphatidylcholine, and sphingomyelin synthesis. These studies indicate that MTP does not play a role in glycerolipid and sphingolipid synthesis.

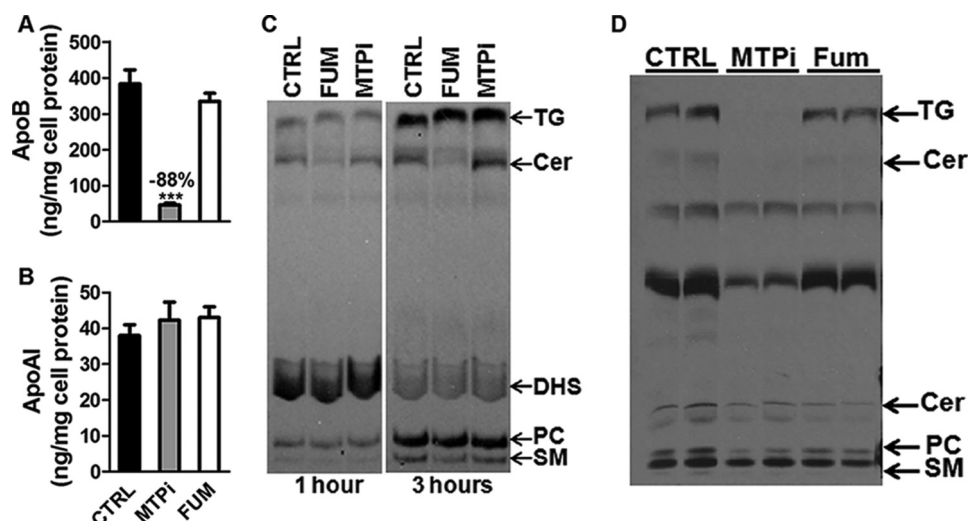
Next, we studied the effect of MTPi on the secretion of sphingolipids. Media of control Huh-7 cells incubated with [ $^3\text{H}$ ]dihydrosphingosine for 16 h contained triglyceride, ceramide, phosphatidylcholine, sphingomyelin, and other unidentified lipids (Fig. 6*D*). MTPi inhibited the secretion of radiolabeled triglyceride, ceramide, phosphatidylcholine, and sphingomyelin by 100, 90, 63, and 36%, respectively (Fig. 6*D*). Secretion of triglyceride, ceramide, phosphatidylcholine, and sphingomyelin was reduced by 20, 67, 34, and 37%, respectively, in the presence of fumonisin B1 (Fig. 6*D*). These studies show that MTPi significantly reduces ceramide secretion, although it has

partial inhibitory effect on sphingomyelin secretion. Therefore, ceramide secretion is more dependent on MTP activity than sphingomyelin secretion, and it is likely that sphingomyelin is transported to the plasma via multiple pathways.

**MTP Transfers Ceramide and Sphingomyelin**—We hypothesized that MTP participates in ceramide and sphingomyelin secretion by transferring these sphingolipids as it transfers glycerolipids. Radiolabeled triolein, ceramide, and sphingomyelin transfer increased with time from donor to acceptor vesicles in the presence, but not in the absence, of purified MTP (Fig. 7*A*). Transfer of these lipids also increased with increasing amounts of purified MTP and was inhibited by MTPi (Fig. 7*B*). Transfer of triolein appeared to saturate at  $\sim 0.5$   $\mu\text{g}$  of MTP, whereas the transfer of ceramide and sphingomyelin did not. It is likely that transfer of triglyceride might involve high affinity, low capacity binding sites, whereas sphingolipid transfer might involve low affinity, high capacity binding. Despite these differences in lipid transfer characteristics, MTPi inhibited the transfer of these lipids with similar  $\text{IC}_{50}$  values (Fig. 7*C*). Thus, time- and protein-dependent transfer of ceramide and sphingomyelin by MTP and inhibition of these transfer activities by MTPi suggest that MTP transfers these sphingolipids.

Next, we evaluated the transfer of these lipids by cellular MTP. Similar to purified MTP, mouse liver homogenates also transferred these lipids, and their transfer was inhibited by MTPi (Fig. 7*D*). Furthermore, the transfer of these lipids was significantly reduced in MTP-deficient liver homogenates compared with controls (Fig. 7*E*). These studies support the





**FIGURE 6. Effect of MTP and ceramide synthase inhibition on the synthesis and secretion of ceramide and sphingomyelin in Huh-7 cells.** *A* and *B*, Huh-7 cells were incubated with 50  $\mu$ M fumonisin B1 (*FUM*), 500 nM MTPi, or DMSO (*CTRL*) for 16 h. ApoB and apoA1 in media were measured using ELISA. *C*, Huh-7 cells were treated with 50  $\mu$ M fumonisin B1 or 500 nM MTPi for 2 h in DMEM containing 10% FBS. Then, 0.5  $\mu$ Ci of [ $^3$ H]dihydrosphingosine and 15  $\mu$ M dihydrosphingosine were added to the media. Cells were collected at 1 or 3 h, and lipids were extracted, separated on TLC, and autoradiographed. Pure lipid markers run in parallel were identified. Control cells received dimethyl sulfoxide (DMSO). *D*, Huh-7 cells were incubated with 0.5  $\mu$ Ci of [ $^3$ H]dihydrosphingosine, 15  $\mu$ M dihydrosphingosine, and 50  $\mu$ M fumonisin B1, 500 nM MTPi, or DMSO vehicle for 16 h. Lipids from the media were extracted and exposed to phosphorimager screens. Images in *C* and *D* are each representative of two independent experiments performed in duplicate. This experiment was repeated in HepG2 cells with similar results (data not shown). TG, triglyceride; Cer, ceramide; DHS, dihydrosphingosine; PC, phosphatidylcholine; SM, sphingomyelin.

notion that MTP transfers ceramide and sphingomyelin as it transfers triglycerides.

## Discussion

MTP is essential for the secretion of triglycerides with B-lps, but its role in the secretion of sphingolipids is unknown. Here, we demonstrated the following: 1) plasma levels of ceramide and sphingomyelin are significantly reduced in both ABL patients and *L, I-Mttp*<sup>-/-</sup> mice; 2) MTP is important for the secretion of these sphingolipids but not for their synthesis; and 3) purified and cellular MTP transfers these sphingolipids from donor to acceptor vesicles. Thus, it is likely that MTP assists in the secretion of ceramide and sphingomyelin by transferring them to B-lps in hepatocytes and enterocytes (Fig. 8*A*) and regulates their plasma levels.

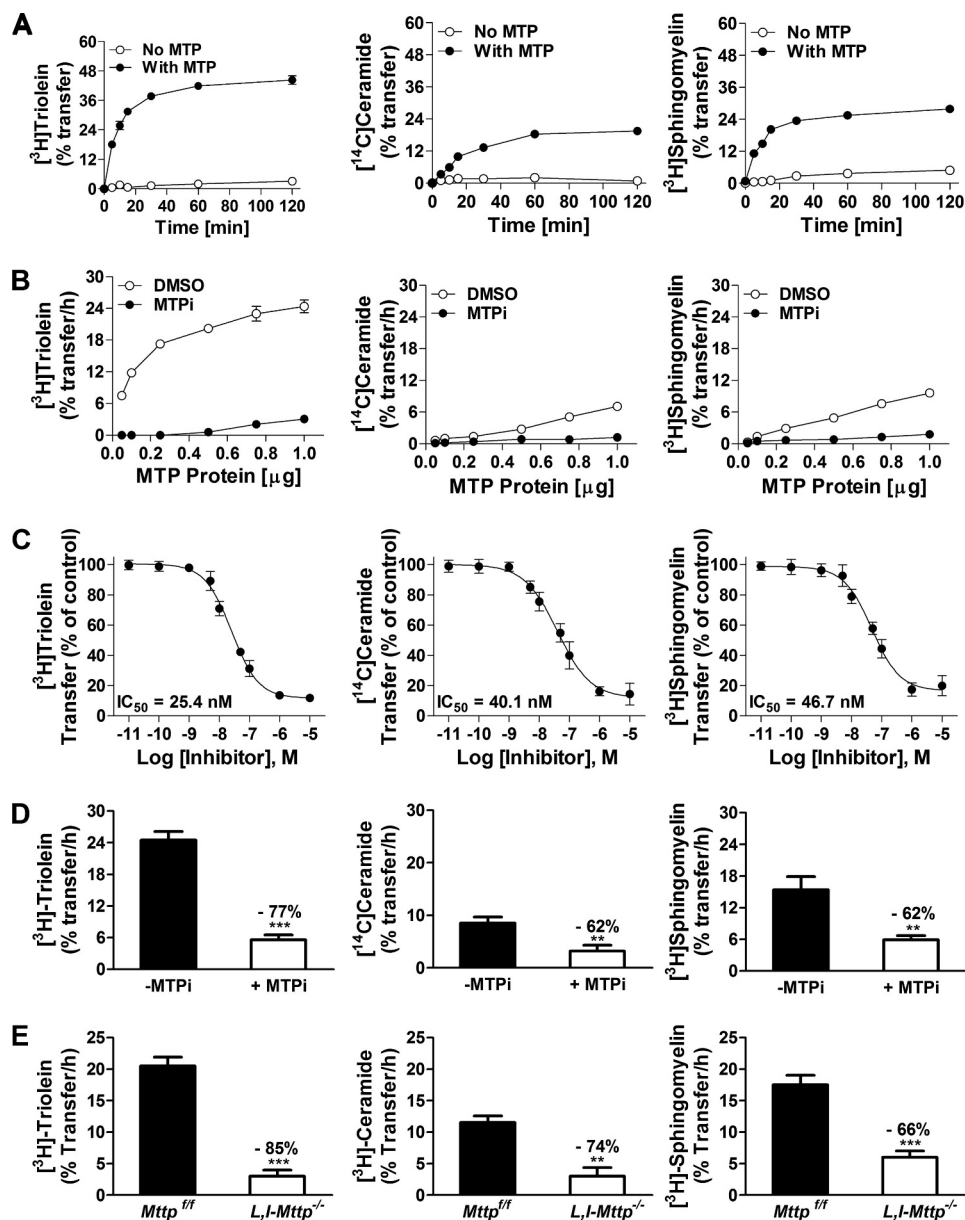
**Mechanisms Controlling Plasma Sphingolipids**—Our studies show that reductions in the biogenesis of B-lps secondary to MTP deficiency differentially affects plasma sphingolipids indicating that some, but not all, sphingolipids are exclusively secreted from the liver and intestine as part of B-lps. To determine whether MTP and B-lp biogenesis are required for sphingolipid secretion, we quantified plasma sphingolipids in MTP-deficient humans (ABL patients) and in mice deficient in liver and intestinal MTP (*L, I-Mttp*<sup>-/-</sup>). In general, we observed similar phenotypes in ABL patients and MTP-deficient mice; MTP deficiency markedly reduced plasma ceramide and partial reductions in sphingomyelin. In contrast to ceramide and sphingomyelin, plasma levels of hexosylceramide, lactosylceramides, dihydroceramide, dihydrosphingosine, dihydrosphingosine-1P, and sphingosine were not affected by MTP deficiency. The transport of sphingosine-1P may or may not be dependent on MTP; sphingosine-1P was reduced in the plasma of *L, I-Mttp*<sup>-/-</sup> mice but not in the plasma of ABL patients. Plasma levels of other quantified sphingolipids were not

affected by MTP deficiency, suggesting that they enter the plasma compartment via alternative mechanisms, possibly involving HDL, albumin, or other unidentified proteins. Hence, different complementary mechanisms exist for the transport of various sphingolipids from the intestine and liver to the plasma compartment. MTP specifically contributes to the majority of plasma ceramide and some of sphingomyelin.

Attempts to understand how MTP regulates plasma ceramide and sphingomyelin revealed that MTP does not play a role in their biosynthesis. No effect of MTP on sphingomyelin synthesis suggests that MTP-mediated ceramide transport via B-lps is not critical for its synthesis. These data also indicate that MTP deficiency has no effect on ceramide transfer protein-mediated transport of ceramide from the ER to Golgi that is essential for sphingomyelin synthesis. Our studies, however, show that MTP is important for the secretion of ceramide and sphingomyelin. MTP is known to transfer triglyceride, cholesterol ester, and phospholipid. We show for the first time that MTP can also transfer ceramide and sphingomyelin between vesicles. Therefore, it is possible that MTP could transfer these lipids intracellularly to nascent B-lps or lipid droplets in the lumen of the ER and Golgi and aid in their secretion (Fig. 8*A*). The observation that MTP can also transfer ceramide and sphingomyelin suggests that MTP might be a general lipid transfer protein. We speculate that it might recognize hydrophobic motifs rather than specific molecular or structural determinants and acts as a “general microsomal lipid transfer protein.”

Although MTP can transfer both ceramide and sphingomyelin, MTP deficiency has differential effects on their secretion. One possible explanation is that sphingomyelin is both secreted with B-lps and effluxed to HDL. Therefore, in the absence of B-lps, sphingomyelin can still be effluxed to HDL. Alternatively, this might be related to the amounts of MTP and sphingolipids

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**FIGURE 7. MTP transfers ceramide and sphingomyelin.** *A*, time course of lipid transfer by MTP. Different donor vesicles containing [ $^3\text{H}$ ]triolein, [ $^{14}\text{C}$ ]ceramide, or [ $^3\text{H}$ ]sphingomyelin were incubated with phosphatidylcholine acceptor vesicles and 1  $\mu\text{g}$  of purified bovine MTP in triplicate for the indicated times. Amounts of radiolabeled lipids transferred to acceptor vesicles were quantified in the absence (*no MTP*) or presence (*with MTP*) of purified MTP. *B*, lipid transfer increases with the amounts of purified MTP. Different indicated amounts of purified bovine MTP were incubated for 2 h in triplicate with donor vesicles containing [ $^3\text{H}$ ]triolein, [ $^{14}\text{C}$ ]ceramide, or [ $^3\text{H}$ ]sphingomyelins and acceptor vesicles. The reactions were carried out in the absence (DMSO) or presence of 1  $\mu\text{M}$  MTP inhibitor (MTPi, lomitapide). *C*, inhibition of MTP lipid transfer activities by lomitapide. Purified bovine MTP (1  $\mu\text{g}$ ) was incubated in triplicate with donor vesicles containing [ $^3\text{H}$ ]triolein, [ $^{14}\text{C}$ ]ceramide, or [ $^3\text{H}$ ]sphingomyelin and acceptor vesicles in the presence of different indicated amounts of lomitapide. After 1 h, DE52 was added, and donor vesicles were separated by centrifugation. Amounts of radiolabel transferred to acceptor vesicles were quantified and plotted. Non-linear regression analysis was used to calculate  $\text{IC}_{50}$  values. *D*, inhibition of cellular lipid transfer activities by MTPi. Mouse liver homogenates (100  $\mu\text{g}$  of protein) were incubated in triplicate in the presence and absence of MTPi (1  $\mu\text{M}$ ) for 1 h. Lipids transferred to acceptor vesicles were quantified. *E*, lipid transfer activities in the livers of different knock-out mice. Liver homogenates (100  $\mu\text{g}$  of protein) from different mice were used to measure transfer of [ $^3\text{H}$ ]triolein, [ $^{14}\text{C}$ ]ceramide, and [ $^3\text{H}$ ]sphingomyelin as described under "Experimental Procedures."

present in the ER and Golgi complex. MTP is mainly present in the ER, and it might efficiently transfer ceramide to B-lps. Much lower amounts of MTP are present in the Golgi complex, a site of sphingomyelin biosynthesis. Furthermore, sphingomyelin levels are  $\sim 20$ -fold higher than those of ceramide. Therefore, it is likely that MTP might be rate-limiting for the transfer of sphingomyelin in the Golgi complex, and other mechanisms might have evolved for its secretion.

The data presented here showed that MTP does not contribute to plasma hexosylceramide and lactosylceramide levels. The reasons for this are unclear. It is possible that these glycosylceramides are not transferred by MTP due to the presence of hydrophilic sugar residues. We were unable to test this hypothesis as the backgrounds of vesicles prepared with NBD-glycosylceramides were too high to perform transfer assays. Another possibility is that these sphingolipids are not available to MTP

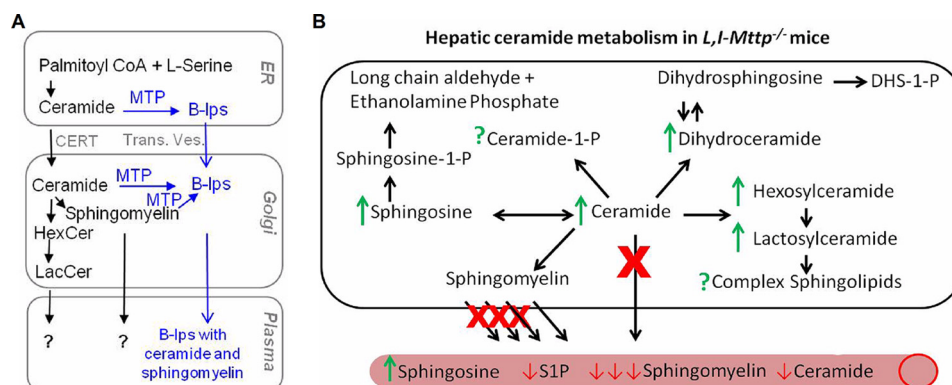


FIGURE 8. *A*, proposed model explaining the role of MTP in ceramide and sphingomyelin secretion. Ceramides are synthesized in the endoplasmic reticulum (ER) and transported by ceramide transfer protein (CERT) and transport vesicles (Trans. Ves.) to the Golgi, where they are converted to sphingomyelin and glycosphingolipids. We propose that MTP transfers ceramides to B-lps in the ER. These B-lps are then transported to the Golgi, where MTP transfers both ceramide and sphingomyelin before secretion into the plasma. Sphingomyelin may also enter the plasma compartment via unknown mechanisms. Mechanisms for secretion of hexosylceramides (HexCer) and lactosylceramide (LacCer) are unknown. *B*, simplified schematic representations of sphingolipid metabolism in MTP-deficient mice. Hepatocytes are shown to synthesize different sphingolipids starting from ceramide. In MTP-deficient mice, secretion of ceramides and sphingomyelin is severely curtailed. This results in the buildup of ceramides, hexosylceramides, lactosylceramide, and possibly other complex sphingolipids (green arrows). Arrows coming out of the cell represent secretion of ceramides and sphingomyelin involving an MTP-dependent process. The red cylinder represents a blood vessel.

for transfer. It is known that glycosylceramides are transferred by FAPP2 within intracellular organelles (45); this transfer may preclude the availability of these sphingolipids to MTP for transfer to B-lps.

*L,I-Mttp*<sup>-/-</sup> Mice Have Severe Hypolipidemia—Our data show that acute concurrent ablation of MTP in the liver and intestine results in a more severe hypolipidemia characterized by very low levels of triglyceride, phospholipid, and cholesterol than has been reported in liver-specific and intestine-specific MTP-deficient mice (9–12). Significant reductions in plasma cholesterol were unanticipated as the majority of cholesterol is transported with HDL in mice, and reductions in B-lps should not theoretically affect HDL cholesterol because *Abca1* mRNA levels did not change in MTP-ablated mice (Fig. 2, *G* and *H*). However, we observed that apoA1 levels were in fact markedly reduced. Because the hydrolysis of B-lps by lipoprotein lipase contributes to HDL biogenesis, it is possible that the absence of plasma B-lps contributes to lower production of HDL. Alternatively, the phenotype may be unique to the acute gene ablation model used in this study that perhaps does not allow compensatory changes in HDL biogenesis to take place. Chronic studies in these mice are required to fully understand the effects of intestinal and hepatic MTP deficiency on HDL metabolism.

*Role of Intestinal and Hepatic MTP in Tissue Lipid Homeostasis*—Our approach of comparing plasma, liver, and intestinal levels of different glycerolipids, cholesterol, and sphingolipids in MTP-deficient mice have provided new insights into the mechanisms of tissue lipid homeostasis. Combined intestinal and hepatic MTP deficiency significantly reduced plasma cholesterol levels but did not affect hepatic intracellular cholesterol levels. It is known that tissue cholesterol levels are tightly controlled by additional mechanisms independent of their secretion with lipoproteins.

Deficiencies of intestinal and liver MTP reduced plasma and liver triglycerides and phospholipids while increasing their concentrations in the intestine. Significant accumulations of triglyceride and phospholipid in the intestines of these mouse models suggest that intestinal transport might be a significant

determinant of triglyceride and phospholipid metabolism. This perhaps also indicates that the intestine may lack significant mechanisms to control cellular levels of these lipids. Alternatively, the main function of the intestine may be to either secrete or store these lipids for later transport.

Combined intestinal and hepatic MTP deficiency results in decreased accumulation of glycerolipids in the mouse liver, which differs from observations in liver- or intestine-specific MTP-deficient mice, as well as those seen in ABL subjects and humans treated with MTP inhibitors (9, 11, 12, 46). In liver- or intestine-specific MTP-deficient mice, triglycerides accumulate in the liver and intestine, respectively, suggesting that tissue-specific MTP ablation is associated with tissue-specific accretions of triglycerides. In humans, MTP deficiency is also associated with accumulation of both intestinal and hepatic lipids. It is possible that the phenotype observed in *L,I-Mttp*<sup>-/-</sup> is the early response due to acute ablation of the gene and that long term deletions might result in significant accumulation of lipids in both the liver and intestine.

In contrast to glycerolipids, sphingolipid accumulation in tissues showed an opposite effect. Sphingolipids did not accumulate in the intestines of *L,I-Mttp*<sup>-/-</sup> mice. Livers from *L,I-Mttp*<sup>-/-</sup> mice, however, had significantly higher amounts of ceramide, hexosylceramide, lactosylceramide, and sphingosines, but sphingomyelin levels did not change. We propose that hepatocytes tightly control cellular ceramide and sphingomyelin levels (Fig. 8*B*). Inhibition of secretory mechanisms may enhance conversion of ceramide to other metabolites, such as glycosylceramide, to avoid cellular toxicity and might explain their accumulation in the liver of these mice. It is unclear why plasma levels of glycosylceramides did not increase in these mice despite increases in their hepatic levels. It is possible that their secretion rates are limiting or they are stored for later release. As discussed above, combined intestinal and hepatic MTP deficiency had differential effects on tissue glycerolipids and sphingolipids. *L,I-Mttp*<sup>-/-</sup> mice accumulated significant amounts of glycerolipids in the intestine and sphingolipids in the liver. We speculate that in mice the intestine might be the



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major organ controlling glycerolipid metabolism, whereas the liver might be the major organ modulating sphingolipid homeostasis. It is likely that intestinal lipoproteins (chylomicrons) transport glycerolipids to the liver, and the liver re-circulates them via VLDL production. In the absence of MTP, these lipids mainly accumulate in the intestine. In contrast, ceramide and glycosylceramide in the liver of *L,I-Mttp*<sup>-/-</sup> mice may simply accumulate because they are not secreted with lipoproteins. More studies are needed to know why different ceramides accumulate in the liver but not in the intestine.

Cholesterol and phospholipids were significantly increased in the feces of *L,I-Mttp*<sup>-/-</sup> mice compared with control, presumably due to reduced absorption by intestinal cells. Similarly, ceramide and sphingomyelin levels were elevated in the feces of *L,I-Mttp*<sup>-/-</sup> mice compared with control. We hypothesize that the increase in sphingomyelin and ceramide in the feces of *L,I-Mttp*<sup>-/-</sup> mice is due to reduced hydrolysis and absorption of sphingomyelin and ceramide. Alternatively, the increase in sphingomyelin and ceramide excretion may be due to increased shedding of intestinal epithelial cells.

Individual ablation of the *Mttp* gene in the liver or intestine may yield different results than those seen in *L,I-Mttp*<sup>-/-</sup> mice. We expect to see a decrease in plasma ceramide and sphingomyelin in liver-specific MTP knock-out mice (*L-Mttp*<sup>-/-</sup>) compared with WT mice. We speculate that *L-Mttp*<sup>-/-</sup> mice would still accumulate sphingolipids (ceramide, lactosylceramide, and hexosylceramide) in their livers. However, it is possible that lactosylceramide and hexosylceramide may not accumulate in their livers because liver-specific MTP deficiency does not have a dramatic effect on HDL. Therefore, if glycosylceramide enters the plasma compartment with HDL and HDL biogenesis is normal, plasma lactosylceramides and hexosylceramides may be similar to WT mice. Because the intestine does not appear to be the main regulator of plasma sphingolipids, there may be only moderate decreases in plasma ceramide and sphingomyelin in intestine-specific MTP-deficient (*I-Mttp*<sup>-/-</sup>) mice. Analysis of plasma, hepatic, and intestinal sphingolipids in liver- and intestine-specific MTP knock-out mice may lend insight into the relative contribution of these two tissues to plasma sphingolipids.

*Similarities and Differences in Plasma Concentrations of Sphingolipids in Humans and Mice*—Comparison of plasma lipid concentrations between humans and mice in general indicates that plasma glycerolipids and sterols are ~50% less in mice (Figs. 1 and 3A). Plasma ceramide and sphingomyelin levels in mice are ~25% of those seen in humans. Plasma lactosylceramide levels are exceptionally low in mice. Surprisingly, plasma hexosylceramide, dihydrosphingosine-1P and sphingosine-1P levels are similar in humans and mice. Although low in both species compared with other sphingolipids, dihydrosphingosine levels are higher in mice. The reasons and physiological consequences of different levels of plasma lipids in the two species are not clear. However, our studies indicate that molecular mechanisms contributing to the modulation of plasma ceramide and sphingomyelin in both the species might be similar.

There were significant differences in the reduction of plasma sphingomyelin in ABL patients and in *L,I-Mttp*<sup>-/-</sup> mice. The sphingomyelin levels were significantly lower in *L,I-Mttp*<sup>-/-</sup>

mice than in ABL subjects. Because *L,I-Mttp*<sup>-/-</sup> mice have markedly reduced apoA1 levels compared with ABL patients, this may cause a greater decrease in plasma sphingomyelin in mice compared with humans.

In short, these studies provide evidence for the existence of several mechanisms for the transport of sphingolipids and the involvement of MTP in the transport of ceramide and sphingomyelin. MTP appears to be the major determinant of plasma ceramide. In contrast, it partially contributes to plasma sphingomyelin and is not involved in the control of plasma glycosylceramides. MTP may regulate plasma ceramide and sphingomyelin by transferring these lipids to B-lps and facilitating their secretion. Further investigations may unravel novel mechanisms involved in the secretion and transport of various sphingolipids.

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*Author Contributions*—J. I., M. T. W., and S. M. H. designed and performed experiments, analyzed data, interpreted results, discussed implications, and critically evaluated the manuscript. M. C., P. T., R. A. H., N. O. D., D. J. R., and R. L. K. processed human plasma samples and reviewed the manuscript. M. M. H. conceived the study, supervised the project, interpreted results, discussed implications, and wrote the manuscript.

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