

Hepatic overexpression of microsomal triglyceride transfer protein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B

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Abstract The microsomal triglyceride transfer protein (MTP) is essential for the hepatic secretion of apolipoprotein (apo) B-containing lipoproteins. Previous studies have indicated that inhibition of MTP results in decreased apoB plasma levels and decreased hepatic triglyceride secretion. However, the metabolic effects of overexpression of MTP have not been investigated. We constructed a recombinant adenovirus expressing MTP (AdhMTP) and used it to assess the effects of hepatic overexpression of MTP in mice. Injection of AdhMTP into C57BL/6 mice resulted in a 3-fold increase in hepatic microsomal triglyceride transfer activity compared to mice injected with Adnull. On day 4 after virus injection, AdhMTP-injected mice had significantly elevated plasma TG levels as compared to control virus (Adnull)-injected mice. Hepatic TG secretion rates were significantly greater in AdhMTP-injected mice (184 ± 12 mg/kg/h) compared with Adnull-injected mice (65 ± 9 mg/kg/h, $P < 0.001$). In addition, hepatic very low density lipoprotein (VLDL) apoB secretion in the AdhMTP-injected group was 74% higher than in the control virus group. Hepatic secretion of apoB-48 and apoB-100 contributed equally to this increase. These results provide the first data that hepatic overexpression of MTP results in increased secretion of VLDL-triglycerides as well as VLDL-apoB in vivo. These results suggest that MTP is rate-limiting for VLDL apoB secretion in wild-type mice under basal chow-fed conditions.—Tietge, U. J. F., A. Bakillah, C. Maugeais, K. Tsukamoto, M. Hussain, and D. J. Rader. **Hepatic overexpression of microsomal triglyceride transfer protein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B.** *J. Lipid Res.* 1999. 40: 2134–2139.

Supplementary key words recombinant adenovirus expressing MTP • apoB • triglyceride

Apolipoprotein B-100 (apoB-100) is the major structural apolipoprotein of liver-derived very low density lipoproteins (VLDL) and low density lipoproteins (LDL) (1, 2). The rate of secretion of apoB-containing VLDL by the liver is a major determinant of plasma triglycerides, LDL

cholesterol, and apoB levels and therefore substantially influences cardiovascular risk (3, 4). In general, apoB gene transcription is constitutive and the major mechanisms of regulation of apoB secretion occur at the posttranscriptional level (5–7). These involve mainly the variation of the proportion of apoB that is intracellularly degraded versus the proportion that is lipidated and finally secreted (8–12). Availability of adequate lipid substrate for lipoprotein assembly is one important factor in regulating apoB secretion rates (5, 6, 12, 13).

Another required factor for VLDL assembly and secretion is the microsomal triglyceride transfer protein (MTP) (14, 15). MTP is a heterodimeric protein consisting of a catalytic 97 kD subunit (further referred to as MTP in this study) and protein disulfide isomerase, a multifunctional ubiquitously expressed protein (16). MTP expression has been localized mainly to the endoplasmic reticulum (ER) of hepatocytes as well as enterocytes (14, 16, 17). Mutations in the MTP gene are the molecular defect in abetalipoproteinemia, an autosomal recessive disorder characterized by no detectable apoB-containing lipoproteins in plasma, indicating the requirement for MTP in apoB-lipoprotein secretion (15, 18). Transfection of a MTP cDNA into cells that normally do not secrete lipoproteins permits secretion of stably transfected apoB (19–22).

A variety of data indicate that reduction in MTP activity results in reduced secretion of apoB and apoB-containing lipoproteins. Pharmacologic inhibition of MTP in vitro resulted in a dose-dependent decrease of secretion of apoB-containing lipoproteins by HepG2 and CaCo2 cells (23–26), and reduced the oleate-induced secretion of apoB-

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; ER, endoplasmic reticulum; TG, triglycerides; FPLC, fast protein liquid chromatography.

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48-VLDL from transfected McA-RH7777 cells (27). Administration of an MTP inhibitor to WHHL rabbits (which lack functional LDL receptors) resulted in a reduction of plasma triglycerides, total cholesterol, and VLDL/LDL cholesterol levels in a dose-dependent manner (4). Rats administered the MTP inhibitor were demonstrated to have significantly reduced secretion rates of VLDL-TG (4). Homozygosity for the MTP gene knockout is lethal in mice (28). Heterozygous MTP-deficient mice have plasma apoB levels that are ~70% of those in wild-type mice and primary hepatocytes from heterozygous MTP knockout mice have reduced rates of apoB secretion (28). These results indicate that MTP is necessary for secretion of apoB-containing lipoproteins and that substantial inhibition of MTP reduces the rate of apoB secretion.

However, the question of whether increased MTP activity over basal levels of expression results in increased VLDL and apoB secretion in vivo has not yet been answered. Interestingly, a recent in vitro study suggested that MTP might be limiting for the secretion of apoB-containing lipoproteins (29). The same study reported that attempts to increase MTP activity by transfecting hepatoma cell lines with an expression plasmid encoding MTP were unsuccessful (29). Recently, adenovirus-mediated overexpression of MTP was shown to increase apoB secretion in HepG2 cells (30). The purpose of the present study was to test the hypothesis that hepatic overexpression of MTP using a recombinant adenovirus would increase VLDL-TG and apoB secretion by the liver in vivo. Our results demonstrate that increased MTP expression in the liver resulted in increased secretion of VLDL apoB and TG in wild-type mice on a chow diet. These data provide the first evidence that MTP is rate-limiting for hepatic VLDL assembly and secretion in vivo.

EXPERIMENTAL PROCEDURES

Construction of recombinant adenoviruses

The plasmid pRChMTP containing the full-length cDNA of the large subunit of human MTP (kindly provided by Dr. D. Gordon and Dr. J. Wetterau, Bristol-Myers-Squibb Pharmaceutical Research Institute, Princeton, NJ) was subcloned into the shuttle plasmid vector pAdCMVlink (31) generating the plasmid pAdCMVhMTP. Recombinant adenoviruses were produced with established methods (32) using adenoviral DNA containing a temperature-sensitive mutation (ts125) in the E2A region that renders the adenovirus incapable of replicating at 39°C (33). In this way two recombinant adenoviruses containing hMTP (AdhMTP) as well as no transgene (Adnull) were generated. Recombinant adenovirus was grown, screened by PCR, subjected to two subsequent rounds of plaque purification, purified, and stored as described (34).

MTP activity assay and Western blot

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM with 10% fetal bovine serum, and 1% antibiotic-antimycotic mixture (all reagents from Life Sciences). COS cells were infected with recombinant adenoviruses at a multiplicity of infection, of 750. At 48 h after infection, MTP activity was determined by measuring the

transfer rate of [¹⁴C]triglycerides from donor to acceptor small unilamellar vesicles using a preparation of microsomal proteins as described previously (23, 35). Assessment of MTP activity in the livers of mice injected with either AdhMTP or Adnull was performed using luminal proteins released by the treatment of microsomes with 100 mM sodium carbonate, pH 11.5, with the same triglyceride transfer assay (23, 35).

For Western blot analysis of hepatic MTP expression in the different experimental groups of mice, 5 μg of microsomal protein was electrophoresed under reducing conditions on a 10% polyacrylamide gel (UltraPure, Gibco BRL, Gaithersburg, MD) and subsequently blotted to nitrocellulose (Schleicher & Schuell, Keene, NH). MTP was identified with a polyclonal rabbit anti-bovine MTP antibody (19) in a dilution of 1:1000 (JW1 F, a kind gift from Dr. D. Gordon and Dr. J. Wetterau, Bristol-Myers-Squibb Pharmaceutical Research Institute, Princeton, NJ) and an appropriate second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) using the ECL (enhanced chemoluminescence) detection system (Amersham Corp., Arlington Heights, IL). This antibody cross-reacts with murine and human MTP (19, 28, 36).

Lipid and lipoprotein analysis

Plasma total cholesterol, triglycerides, phospholipids, and HDL cholesterol levels were measured enzymatically on a Cobas Fara (Roche Diagnostics Systems Inc., Nutley, NJ) using Sigma Diagnostics reagents (Sigma Diagnostics, St. Louis, MO). Pooled plasma samples from 4 mice (120 μl total volume) were subjected to fast protein liquid chromatography (FPLC) gel filtration using two Superose 6 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described (34). Lipoprotein fractions of 500 μl each were collected. Individual fractions were assayed for cholesterol concentrations using commercially available assay kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Animal studies

Female C57BL/6 mice, 6–8 weeks old, were purchased from Jackson Laboratory (Bar Harbor, ME) and fed a regular mouse chow diet (Diet 5010, PMI Nutrition International, Richmond, IN). Mice were bled from the retroorbital plexus after a 4-h fast using heparinized capillary tubes. Blood was drawn into tubes containing 2 mM EDTA, 0.2% NaN₃, and 1 mM benzamidine and placed immediately on ice. Aliquots of plasma were stored at –20°C until analysis. For the adenovirus studies, mice were injected with 1.5×10^{11} particles ($4.5\text{--}6 \times 10^9$ pfu) of AdhMTP, Adnull, or saline as indicated. To assess MTP expression in AdhMTP- and Adnull-injected mice, experimental animals were killed on day 3 after virus injection, perfused with ice-cold PBS, and livers were immediately frozen in liquid nitrogen. Tissues were then processed as described above.

Determination of hepatic triglyceride secretion

Mice (C57BL/6) that had been injected with either AdhMTP, Adnull, or PBS 3 days prior to the experiment were allowed to eat fat-free food for 2 h and were then injected via tail vein at 9 am with 20 mg of Triton WR1339 solution (Tyloxapol, Sigma, St. Louis, MO) in a total volume of 200 μl sterile PBS (37, 38). Prior to injection and at 30 min, 1 h, and 2 h after Triton WR1339 injection, 40 μl of blood was drawn by retroorbital bleeding, and plasma was separated and assayed for triglycerides. Based on pilot experiments that were performed to determine the linearity of the plasma triglyceride curve, we decided to use the 30-min time point for calculation of hepatic TG secretion. Therefore baseline TG levels were subtracted from the 30-min values in each of the mice and TG secretion rates expressed as mg/kg per h assuming the plasma volume to be 3.5% of the body weight.

Determination of hepatic apoB secretion

The measurement of hepatic VLDL apoB secretion was carried out by endogenous labeling under conditions of blocking VLDL catabolism with Triton WR1339. Under these conditions the rate of accumulation of endogenously labeled VLDL apoB is directly related to the rate of apoB-VLDL secretion. Therefore mice that had been injected with AdhMTP, Adnull, or PBS 3 days before were placed on a fat-free diet 2 h prior to a 40 μ l baseline bleed at 9 am. Then 200 μ l of a 10% Triton WR1339 solution containing 500 μ Ci of [³⁵S]methionine (NEN) was administered by tail vein injection. Blood was drawn by retroorbital bleeding at 5 min (10 μ l), 30 min, 1 h, and 2 h (40 μ l) after injection of the tracer solution. Based on previous experiments we used a 1 h time point for further processing of the samples in the experimental mice. To isolate VLDL, 20 μ l of mouse plasma was layered under 750 μ l of KBr solution (d 1.006 g/ml) and centrifuged at 90000 rpm at 10°C for 3 h (Beckman TL100 Ultracentrifuge). VLDL was recovered in the top fraction by tube slicing in a volume of 250 μ l. To 200 μ l of VLDL, 100 μ l of 3.6 mM deoxycholate and 100 μ l of TCA (4.9 M) were added, and the tubes were vortexed and placed on ice for 15 min to precipitate VLDL proteins. After centrifugation in a table-top centrifuge at top speed for 20 min, the supernatant was decanted and 90 μ l of Laemmli sample buffer (BioRad) and 10 μ l of 1 M NaOH were added. Samples were heated at 80°C for 10 min to dissolve the precipitate and 45 μ l was subjected to linear gradient SDS-PAGE (3–20%) (UltraPure, Gibco BRL). The gel was fixed and incubated in Amplify solution (Amersham Life Sciences) under gentle agitation for 30 min. After that the gel was dried under vacuum and exposed to an X-ray film (Fuji Medical Systems, Stamford, CT) at –80°C. From the autoradiography, relative positions of apoB-48 and apoB-100 were identified and the respective bands were cut out of the gel. The gel pieces were then placed in glass scintillation vials (Fisher Scientific) and 500 μ l of H₂O was added to rehydrate the gel. Then 500 μ l of Solvable (Packard, Meriden, CT) was added and the samples were incubated for 3 h at 50°C. Samples were cooled, 5 ml of Scintiverse BD (Fisher Scientific) was added, and the vials were vortexed and then counted on a scintillation counter (Beckman LS6500). The apoB counts were corrected for the injected dose of tracer in each mouse assessed as the plasma counts at 5 min after tracer injection. Therefore, individual counts in apoB were divided by the ratio of the respective 5 min plasma count and the highest 5 min plasma count in the experiment.

Statistical analysis

Values are presented as mean \pm SEM unless otherwise indicated. Results were analyzed by ANOVA and Student's *t* test for independent samples (two-tailed) using the GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance for all comparisons was assigned at $P < 0.05$.

RESULTS

COS-7 cells infected with AdhMTP exhibited significantly higher MTP lipid transfer activity than Adnull infected cells (1.45 ± 0.08 vs. $0.3 \pm 0.01\%$ TG transfer/100 μ g protein/h). We then injected the recombinant viruses AdhMTP or Adnull via the tail vein into C57BL/6 mice fed a chow diet. On day 3 after adenovirus injection, livers harvested from AdhMTP-injected mice had increased expression of MTP protein (**Fig. 1A**) and significantly higher microsomal triglyceride transfer activity than control virus-injected mice (6.5 ± 2.2 vs. $2.2 \pm 0.3\%$ TG transfer/h/50 μ g protein, resp., $P < 0.01$, Fig. 1B).

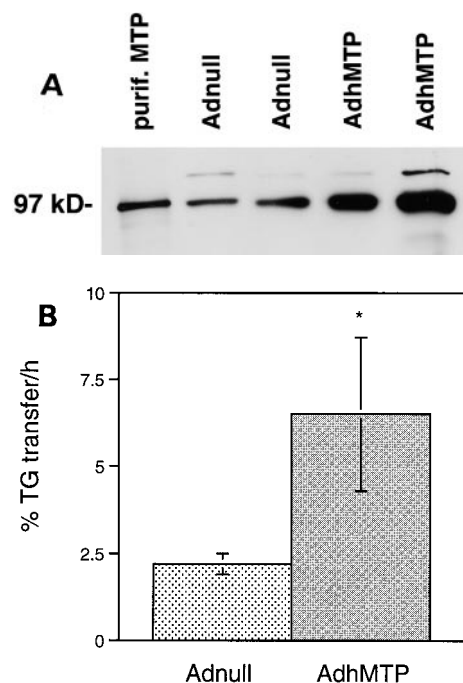


Fig. 1. Panel A: Western blot analysis of liver tissues from mice injected with control virus (Adnull) or MTP expressing adenovirus (AdhMTP) 3 days after injection. Five μ g of hepatic microsomal proteins was loaded and purified MTP was loaded as a positive control. Rabbit α -bovine MTP antiserum was used for immunoblotting. This antibody cross-reacts with mouse and human MTP. Panel B: TG transfer activity was quantitated on 50 μ g of microsomal protein from livers of mice injected with AdhMTP ($n = 3$) or Adnull ($n = 4$). AdhMTP-injected mice had significantly increased TG transfer activity (*, $P < 0.01$).

Next we assessed the effect of AdhMTP and Adnull on plasma lipids. Baseline lipid profiles in the two groups of experimental mice were not different. On day 4 after virus injection, triglycerides in the Adnull-injected mice were 79 ± 7 mg/dl as compared to 63 ± 3 mg/dl on day 0 ($P = n.s.$) demonstrating that virus injection itself had no effect on plasma triglyceride levels. In contrast, in the AdhMTP group, plasma TG levels increased from 59 ± 3 mg/dl at baseline to 110 ± 12 mg/dl on day 4 after injection ($P < 0.05$). This represents a mean 86% increase over baseline triglycerides (**Fig. 2**). The plasma levels of total cholesterol and HDL-cholesterol were not different between the experimental groups of mice (data not shown). At baseline, FPLC gel filtration separation of lipoproteins showed that the lipoprotein cholesterol profiles were identical with HDL being the major lipoprotein (data not shown). Separation of lipoproteins by FPLC on day 4 after virus injection revealed an increase in the small VLDL cholesterol peak in the AdhMTP-injected group while there was no change in this peak in the Adnull-injected mice (data not shown). The LDL and HDL cholesterol peaks were not different between the experimental groups.

To further investigate the metabolic basis for the changes in plasma triglycerides, we assessed the rate of hepatic triglyceride secretion in AdhMTP- and Adnull-injected mice. Hepatic triglyceride secretion rates in

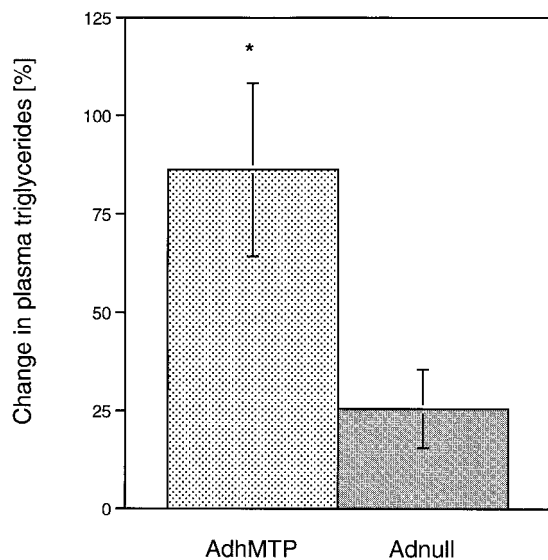


Fig. 2. Change in plasma triglycerides on day 4 after AdhMTP and Adnull injection ($n = 6$ each). Percent changes in plasma triglyceride levels on day 4 after injection of AdhMTP and Adnull into C57BL/6 mice on a chow diet as compared to day 0 levels. Plasma triglycerides were determined after a 4-h fast. AdhMTP-injected mice had a significant increase in plasma triglycerides as compared to Adnull-injected mice (*, $P < 0.05$).

AdhMTP-, Adnull-, and vehicle (PBS)-injected mice were determined after blocking VLDL TG catabolism with Triton WR1339. The TG secretion rate in AdhMTP-injected mice was 184 ± 12 mg/kg per h (Fig. 3). This was significantly increased as compared with the control virus-

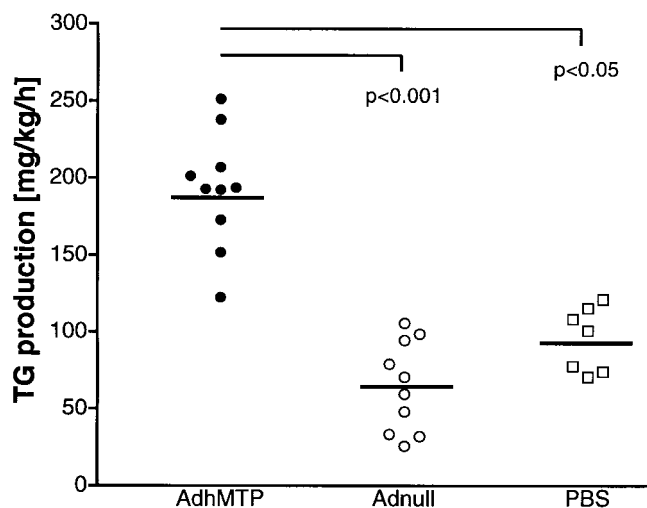


Fig. 3. Hepatic triglyceride secretion rates in AdhMTP-, Adnull- and vehicle-injected mice. Hepatic triglyceride secretion rates were determined on day 3 after injection of AdhMTP ($n = 10$), Adnull ($n = 10$), or PBS ($n = 7$) in C57BL/6 mice after blocking VLDL catabolism with Triton WR1339. Hepatic TG secretion rates were calculated from the difference in plasma triglycerides between baseline and the 30-min timepoint after Triton WR1339 injection. Triglyceride secretion in AdhMTP-injected mice was significantly higher than in the Adnull- and the PBS-injected groups ($P < 0.001$, $P < 0.05$, resp.).

injected group (65 ± 9 mg/kg/h, $P < 0.001$) and the PBS-injected control group (96 ± 8 mg/kg/h, $P < 0.05$). Hepatic TG secretion rates between the control virus and PBS-injected groups were not significantly different.

Because there was a significant increase in hepatic TG secretion in the AdhMTP overexpressing mice, we next determined hepatic VLDL apoB secretion rates using endogenous labeling with [35 S]methionine. Total hepatic apoB secretion in the AdhMTP-injected group was 74% higher than in the Adnull-injected and 101% higher than in the vehicle-injected groups of mice (Fig. 4A). The observed in-

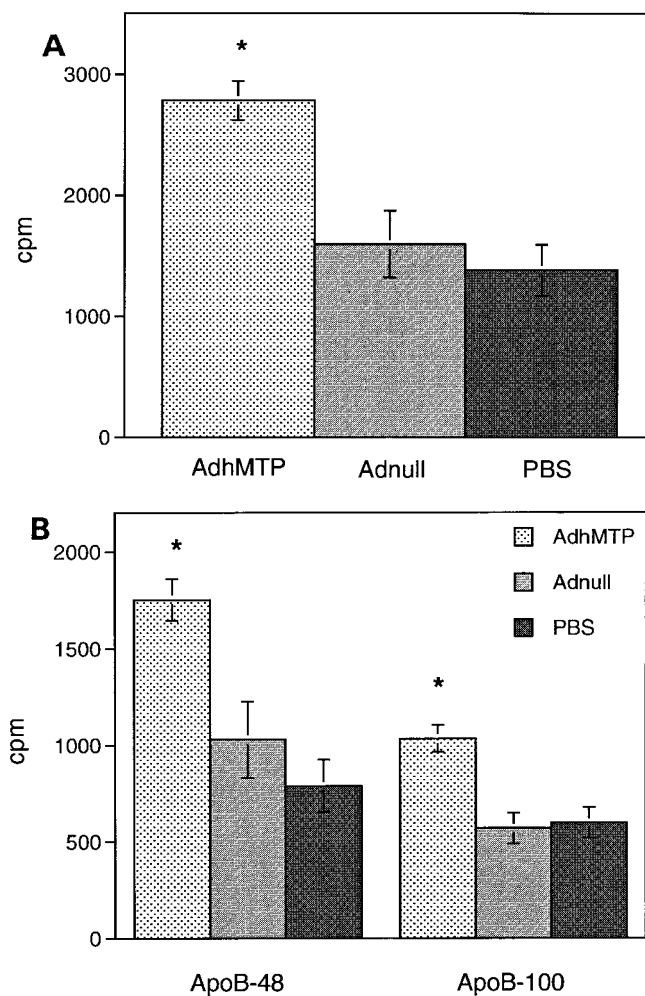


Fig. 4. Hepatic apoB secretion in AdhMTP-, Adnull-, and vehicle-injected mice. Hepatic apoB secretion was determined on day 3 after injection of AdhMTP ($n = 6$), Adnull ($n = 5$), or PBS ($n = 7$) using endogenous labeling with [35 S]methionine after blocking VLDL catabolism with Triton WR1339. Accumulation of labeled apoB in the plasma compartment for 1 h was taken as a measure of hepatic apoB secretion. VLDL was isolated by ultracentrifugation and apoB-48 as well as apoB-100 were separated by SDS-PAGE. The cpm values shown represent counts in VLDL apoB corrected for the injected dose in each experimental mouse. Total hepatic apoB secretion (Fig. 4A) was significantly higher in AdhMTP-injected mice (*) as compared to Adnull-injected ($P < 0.05$) and PBS-injected ($P < 0.01$) controls. The specific secretion rates of apoB-48 and apoB-100 (Fig. 4B) were both significantly increased in AdhMTP-injected mice (*) as compared to Adnull- (each $P < 0.05$) and PBS-injected (each $P < 0.01$) controls.

crease in total hepatic apoB secretion rates was due to both increased apoB-48 as well as increased apoB-100 secretion in the mice injected with the AdhMTP adenovirus (Fig. 4B). ApoB-48 secretion in the MTP overexpressing mice was 70% higher than in the Adnull group and 123% higher than in the group injected with vehicle. ApoB-100 secretion was 81% higher in the AdhMTP-injected mice as compared with the control virus-injected mice and 73% higher as compared with the PBS-injected group.

DISCUSSION

These data demonstrate for the first time that hepatic overexpression of MTP *in vivo* results in increased secretion of VLDL triglycerides and apoB by the liver. This finding is particularly interesting as our experimental model was the wild-type C57BL/6 mouse on normal chow without addition of lipid to the diet. Our results suggest that MTP is rate-limiting for hepatic VLDL secretion, as recently suggested by Jamil et al. (29) and Liao, Kobayashi, and Chan (30) based on *in vitro* studies. *In vitro* studies using pharmacological MTP inhibitors have established that decreased MTP activity results in a decrease of TG and apoB secretion (23–26). Reduction of MTP activity *in vivo* also results in reduced TG and apoB secretion (4, 28, 36). However, the physiologic effect of MTP overexpression on TG and apoB secretion *in vivo* had not been previously determined.

Current concepts on hepatic assembly and secretion of apoB-containing lipoproteins suggest a two-step process of apoB core lipidation, although the details of this mechanism remain incompletely understood (8, 14, 15). ApoB is transcribed constitutively, and transcriptional rates are apparently not regulated by dietary manipulations (1, 7). ApoB secretion appears to be primarily regulated at the posttranscriptional level. A significant portion of newly synthesized apoB is targeted for intracellular degradation (5, 12, 13). Lipidation of the newly synthesized apoB appears to prevent its degradation. In the presence of MTP and a sufficient intracellular lipid supply, cotranslational lipoprotein assembly is initiated (9, 10, 39). MTP is believed to be necessary for adding triglycerides as well as cholesteryl ester to the nascent apoB in a process that is incompletely understood. MTP may also promote the translocation of apoB across the ER membrane and have other effects in early VLDL assembly not directly related to lipid transfer (15, 16). Our finding that increased VLDL TG and apoB secretion is induced by overexpressing MTP in normal mice on a chow diet indicates that even under basal conditions MTP is rate-limiting in the process of VLDL assembly and secretion. Further studies will be necessary to elucidate the molecular mechanisms by which overexpression of MTP results in increased VLDL apoB secretion.

In summary, our results provide the first data on the effects of MTP overexpression *in vivo* and demonstrate that it causes increased secretion of VLDL apoB as well as VLDL triglycerides in normal mice on a chow diet. These results suggest the possibility that some forms of hepatic VLDL apoB overproduction, a common cause of familial

combined hyperlipidemia (40), could be related to increased MTP activity. Further studies will be required to test this hypothesis. Adenovirus-mediated overexpression of MTP will provide a useful tool to gain further insights in the role of MTP in the secretion of apoB-containing lipoproteins in various experimental settings. ■

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