Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER

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Abstract **Microsomal triglyceride transfer protein (MTP) is an intraluminal protein in the endoplasmic reticulum (ER) that is essential for the assembly of apolipoprotein B (apoB)-containing lipoproteins. In this study, we examine how the livers of mice respond to two distinct methods of blocking MTP function: *Cre*-mediated disruption of the gene for MTP and chemical inhibition of MTP activity. Blocking MTP significantly reduced plasma levels of triglycerides, cholesterol, and apoB-containing lipoproteins in both wild-type C57BL/6 and LDL receptor-deficient mice. While treating LDL receptor-deficient mice with an MTP inhibitor for 7 days lowered plasma lipids to control levels, liver triglyceride levels were increased by only 4-fold. Plasma levels of apoB-100 and apoB-48 fell by >90% and 65%, respectively, but neither apoB isoform accumulated in hepatic microsomes. Surprisingly, loss of MTP expression was associated with a nearly complete absence of apoB-100 in hepatic microsomes. Levels of microsomal luminal chaperone proteins [e.g., protein disulfide isomerase, glucose-regulated protein 78 (GRP78), and GRP94] and cytosolic heat shock proteins (HSPs) (e.g., HSP60, HSC, HSP70, and HSP90) were unaffected by MTP inhibition. These findings show that the liver responds rapidly to inhibition of MTP by degrading apoB and preventing its accumulation in the ER. The rapid degradation of secretion-incompetent apoB in the ER may block the induction of proteins associated with unfolded protein and heat shock responses.**—Liao, W., T. Y. Hui, S. G. Young, and R. Davis. **Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER. *J. Lipid Res.* 2003.** 44: **978–985.**

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VLDLs produced by the liver are the major source of LDLs in the plasma, which are causally related to the development of atherosclerotic cardiovascular disease (1). The importance of the hepatic secretion of apolipoprotein B (apoB) in cardiovascular disease was recognized in early studies of abetalipoproteinemic patients, who lack the ability to secrete apoB-containing lipoproteins (2) and are markedly resistant to cardiovascular disease (3). Subsequent studies showed that specific mutations in the microsomal triglyceride transfer protein (MTP) gene are responsible for abetalipoproteinemia (4, 5). MTP exists in a functional complex with protein disulfide isomerase (PDI) (6, 7). The loss of MTP function blocks the secretion of apoB-containing lipoproteins from both the liver and the intestine (5). Apart from neuropathy, which can be prevented by vitamin E supplements (8) and moderate steatosis in enterocytes and hepatocytes, abetalipoproteinemia is not usually associated with liver failure or cirrhosis (3). The absence of severe symptoms has suggested that inactivation of MTP might be a useful strategy for combating hyperlipidemia and cardiovascular disease.

Several chemical inhibitors of the lipid transfer activity of MTP have been developed (9–12). Many of these inhibitors lower plasma lipid levels in animal models (11–13). Especially encouraging results were obtained in Watanabe-heritable hyperlipidemic rabbits, a model of homozygous familial hypercholesterolemia (13). Administration of an MTP inhibitor to Watanabe-heritable hyperlipidemic rabbits reduced plasma lipid and lipoprotein levels to normal (13). Since many homozygous familial hypercholesterolemias are resistant to statin therapies (14), MTP inhibitors might provide a more attractive treatment to liver transplantation, which effectively reduces atherosclerosis in these patients (15).

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Abbreviations: ALLN, acetylated leucine, leucine, norleucal; ER, endoplasmic reticulum; GRP, glucose-regulated protein; HSP, heat shock protein; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase.

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MTP inhibitor drugs have provided the opportunity to examine the processes regulating the assembly, secretion, and degradation of apoB-containing lipoproteins. Studies of cultured cells suggest that the cotranslational translocation of apoB into the lumen of the endoplasmic reticulum (ER) governs the fate of newly synthesized apoB [reviewed in refs. (16–20)]. Unlike other proteins secreted by the liver, apoB-100 cannot be completely translocated into the ER lumen without MTP (21–23). MTP appears to facilitate both the folding and lipidation of apoB during translocation (24–32). In the absence of MTP, apoB translocation is abrogated, resulting in the rapid cotranslational degradation of the cytoplasmic, translocation-arrested C terminus of apoB (33) via the proteasome (34–36). Approximately 85 kDa of the N-terminal portion of translocation-arrested apoB is released from the ER membrane, enters the lumen, and can be secreted or degraded (21, 24, 37). The finding that plasma from abetalipoproteinemic patients was enriched with an 85 kDa N-terminal peptide compared with plasma from unaffected family members suggested that MTP-facilitated translocation occurs in the human liver (24). Several additional degradative processes act to ensure that secretion-incompetent apoB does not accumulate [reviewed in refs. (16–20)]. Preventing the accumulation of apoB within the secretory pathway may be essential in preventing an induction of unfolded-protein response (38–40).

To our knowledge, there have been no published studies that have examined how the liver in vivo responds to loss of MTP in regard to the accumulation of apoB and possible induction of unfolded protein and/or heat shock responses. Our studies revealed that the inactivation of MTP (by gene disruption or chemical inactivation) led to a block in the secretion of apoB and prevented its accumulation in the ER, as well as the induction of unfolded protein and/or heat shock responses.

EXPERIMENTAL PROCEDURES

Materials

The MTP inhibitor 8aR (11) was kindly provided by Dr. Gary Ksander (Novartis, Summit, NJ). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mice homozygous for both a conditional *Mttp* allele ($Mttp^{flox}$) and the Mx1-*Cre* transgene have been described (22). Wild-type C57BL/6 mice and C57BL/6 LDL receptor knockout mice ($Ldlr^{-/-}$) were obtained from The Jackson Laboratory (http://www.jax.org).

Animal procedures

Mice were maintained under standard conditions on a 12 h light-dark cycle (lights on from 0600 to 1800). To eliminate MTP expression in the liver, 8-week-old $Mttp^{flox/flox}Mx1\text{-}Cre^{+/+}$ mice were injected with polyinosinic-polycytidylic ribonucleic acid (polyIC; Sigma; 300 μg every two days for 5 times). Control mice received vehicle only. Three or six weeks after last injection, blood was drawn from the retro-orbital plexus into tubes containing EDTA, and plasma was separated by centrifugation. The mice were killed by cervical dislocation, and the livers were removed immediately and used to prepare microsomes (33). All protocols were approved by the Animal Care and Use Committee of San Diego State University.

MTP inhibitor experiments

The inhibitor 8aR was prepared as a water suspension in 3% cornstarch, and was administered orally at a dose of 50 mg/kg daily for 7 days, or using as a single dose of 100 mg/kg.

Preparation of microsomes

The liver was homogenized in 250 mM sucrose and 10 mM Hepes (pH 7.4) containing 1 mM phosphomethylsulfonylfluoride, 0.1 mM acetylated leucine, leucine-norleucal (ALLN), and 5 mM *N*-ethylmaleimide. Microsomes were obtained by ultracentrifugation and washing of fractions, as described (33). The protein concentration in the samples was measured using a protein assay kit (Bio-Rad, Hercules, CA).

Immunoblot analysis

Equal amounts of proteins from liver homogenates, microsomes, or plasma were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary antibodies as described (21). Blots were detected by enhanced chemiluminescence (ECL kit, Amersham). The relative intensity of the immunoblot bands was quantified with a Storm PhosphoImager, (Amersham Pharmacia Biotech, Piscataway, NJ).

Size fractionation of lipoproteins by fast protein liquid chromatography

Equal volumes of plasma from each mouse in each group were pooled (0.2 ml) and loaded onto a fast protein liquid chromatography (FPLC) system with two Superose 6B columns connected in series (HR10/30, Pharmacia FPLC System, Amersham Pharmacia Biotech) (41). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min with an elution buffer (15 mM NaCl, 0.01% EDTA, 0.02% sodium azide, pH 7.3).

Cholesterol and triglyceride assays

Cholesterol and triglycerides in plasma and FPLC fractions were assayed with commercial kits from Sigma, as described (41).

Statistical analysis

Results are given as the mean ± SD. Statistical significance was determined by Student's *t*-test with two-tailed *P* values. Differences were considered to be significant at $P < 0.05$.

RESULTS

Effects of disrupting *Mttp* in the liver

For our studies, we used $Mttp^{flox/flox}Mx1\text{-}Cre^{+/+}$ mice. *Cre* expression in these mice can be induced with polyIC, which eliminates exon 1 of *Mttp* and prevents the formation of a functional *Mttp* transcript (22). The absence of MTP expression by the liver did not affect body weight, but increased liver weight (**Fig. 1A**) and reduced the plasma levels of triglycerides (45%) and cholesterol (60%) (Fig. 1B). As a result, VLDL, IDLs, and LDLs were almost undetectable, and HDL levels were reduced by ~50% (Fig. 1B). Hepatic triglycerides were increased 2.5-fold, and hepatic cholesterol was increased by 65% (Fig. 1C). ApoB-100 was reduced to undetectable levels in plasma, and plasma levels of apoB-48 were reduced by ~65% (Fig. 1D). Surprisingly, loss of MTP expression was

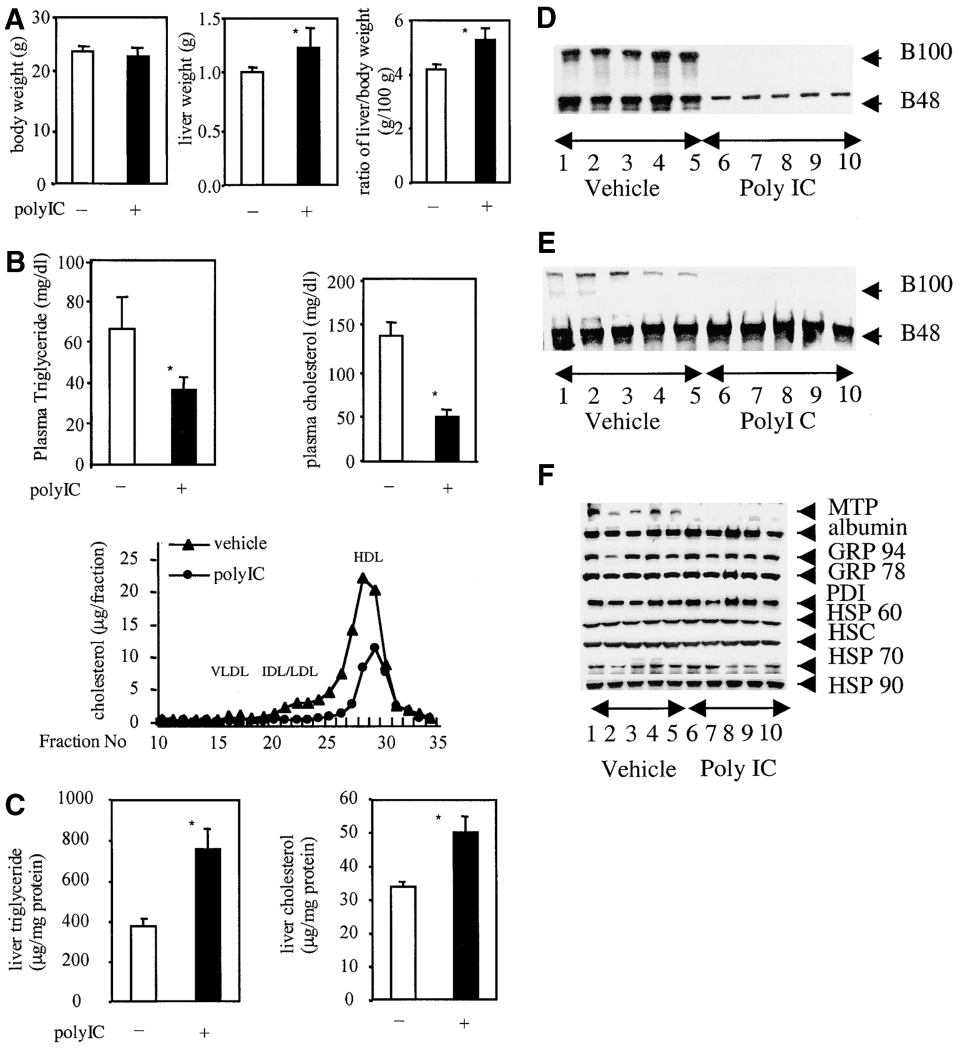

Fig. 1. Effect of liver-specific deletion of microsomal triglyceride transfer protein (MTP) on the plasma and liver levels of apolipoprotein B (apoB)-containing lipoproteins. Female *Mttp*$^{flox/flox}$*Mx1-Cre*$^{+/+}$ mice (8 weeks old) were injected with polyIC ip (300 μg every other day, five times) or saline only (control). Three weeks after the last injection, mice were sacrificed and plasma and liver were obtained and analyzed. A: Body and liver weights. B: Plasma triglyceride and cholesterol, and cholesterol in each fraction obtained from FPLC separation of pooled plasma (n = 5/group). C: Liver triglyceride and cholesterol. D: Immunoblot analysis of plasma detected with an anti-apoB antibody. E: Immunoblot analysis of liver microsomes detected with an anti-apoB antibody. F: Immunoblot analysis of liver microsomes detected with antibodies as indicated. Each bar graph value represents the mean ± SD. *$P < 0.02$.

associated with a nearly complete absence of apoB-100 in hepatic microsomes. The amount of apoB-48 was unaffected (Fig. 1E). The lack of MTP expression did not affect the levels of microsomal luminal chaparone proteins (e.g., PDI, glucose-regulated protein (GRP) 78 and GRP94) or cytosolic heat shock proteins (HSPs) (e.g., HSP60, HSC, HSP70, and HSP90) (Fig. 1F). Hepatic microsomes from mice treated for 6 weeks with polyIC contained no detectable MTP protein (Fig. 1F). Northern blots showed that apoB mRNA levels were unchanged by polyIC treatment (not shown). Thus, changes in apoB were the result of the absence of hepatic MTP expression. These data suggest that deletion of hepatic MTP expression blocks the assembly and secretion of apoB-containing lipoproteins without causing apoB-100 or apoB-48 to accumulate in the ER. This experiment was repeated twice with similar results (not shown).

Consequences of blocking MTP with chemical inhibitors

We next examined how mice responded to chemical inhibition of MTP. After 7 days of treatment with 8aR, the phenotype of C57BL/6 mice was essentially the same as that of mice lacking hepatic MTP. The liver weight increased (**Fig. 2A**), the plasma levels of triglyceride (50%) and cholesterol (70%) decreased (Fig. 2B), and the liver content of triglyceride (4-fold) and cholesterol (35%) increased (Fig. 2C). The MTP inhibitor markedly decreased plasma levels of apoB-48 (70%) and apoB-100 (95%) (Fig. 2D). Hepatic microsomes from 8aR-treated mice contained almost no apoB-100, and there was no accumula-

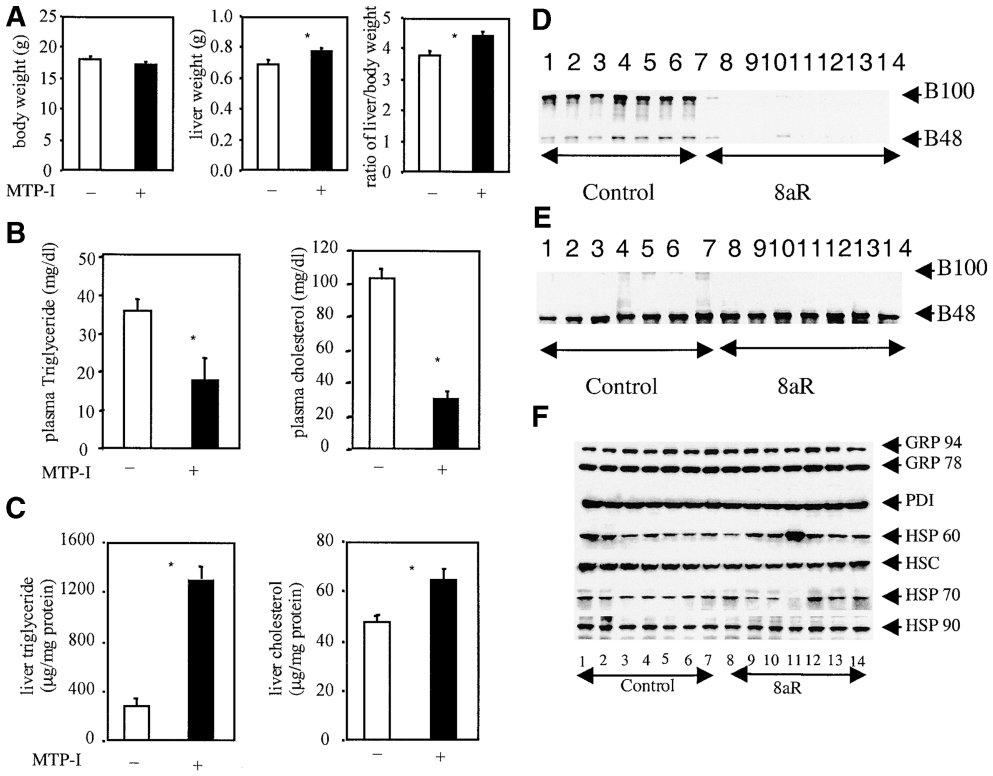

Fig. 2. Effect of 7 days of treatment with an oral MTP inhibitor on the plasma and liver levels of apoB-containing lipoproteins in C57BL/6 mice. Female C57BL/6 mice (8 weeks old) were given the MTP inhibitor 8aR (50 mg/day/kg) for 7 days at 0800 h or vehicle only (control). Mice were sacrificed at 1100 h, and plasma and livers were obtained and analyzed. A: Body and liver weights. B: Plasma triglyceride and cholesterol levels. C: Liver triglyceride and cholesterol. D: Immunoblot analysis of plasma detected with an anti-apoB antibody. E: Immunoblot analysis of liver microsomes detected with an anti-apoB antibody. F: Immunoblot analysis of liver microsomes detected with antibodies as indicated. Each bar graph value represents the mean ± SD. $*P < 0.02$.

tion of apoB-48 (Fig. 2E). The MTP inhibitor did not affect the levels of microsomal luminal chaperone proteins (Fig. 2F) or of cytosolic HSPs (Fig. 2F). These findings suggest that the liver adapts to inhibition of apoB secretion and prevents the accumulation of apoB in the ER.

We next examined the hepatic response to MTP inhibition in extremely hyperbetalipoproteinemic *Ldlr*$^{-/-}$ mice. Chemical inhibition of MTP did not increase liver weight (**Fig. 3A**), perhaps because *Ldlr*$^{-/-}$ mice already have enlarged livers; however, there were decreases in the plasma levels of cholesterol (85%), triglyceride (65%) (Fig. 3B), apoB-100 (70%), and apoB-48 (80%) (Fig. 3D). As a result, plasma VLDL, IDL, and LDL essentially disappeared, and HDL levels decreased by 65% (Fig. 3B). In the livers of mice, the stores of triglycerides increased 4-fold and cholesterol increased 2-fold (Fig. 3C). Despite the marked decrease in apoB secretion, hepatic microsomes contained almost no apoB-100, and there was no accumulation of apoB-48 (Fig. 3E). Microsomes from *Ldlr*$^{-/-}$ mice not treated with the MTP inhibitor contained markedly more apoB-100 than microsomes from untreated wild-type mice (Fig. 3F). Thus, the absence of the LDL receptor caused apoB-100 to accumulate in the ER of mice not treated with MTP. The MTP inhibitor did not affect the content of any microsomal luminal chaperone proteins (Fig. 3G) or the content of cytosolic HSPs (Fig. 3G). These data show that inhibition of MTP ameliorates severe hypercholesterolemia without causing accumulation of apoB in the ER, hepatic inflammation, or massive fatty liver.

To determine how rapidly apoB degradation is induced in response to MTP inhibition, we assessed the effects of a single dose of the MTP inhibitor. A single dose of 8aR did not alter liver weight (**Fig. 4A**). It did, however, reduce plasma levels of triglycerides (43%) and cholesterol (22%) (Fig. 4B), apoB-100 (95%), and apoB-48 (88%) (Fig. 4D). Neither apoB isoform accumulated in the microsomal fractions (Fig. 4E). Hepatic levels of markers of the HSP and unfolded protein responses were also unaffected (Fig. 4F). These findings show that the hepatic response to MTP inhibition and impaired apoB secretion is sufficiently rapid to prevent the apoB accumulation in the ER. This rapid response may help to explain the absence of an accumulation of misfolded proteins in the ER and an associated inflammatory response.

DISCUSSION

This study shows, for the first time, that blocking hepatic apoB secretion in vivo in mice results in a rapid ho-

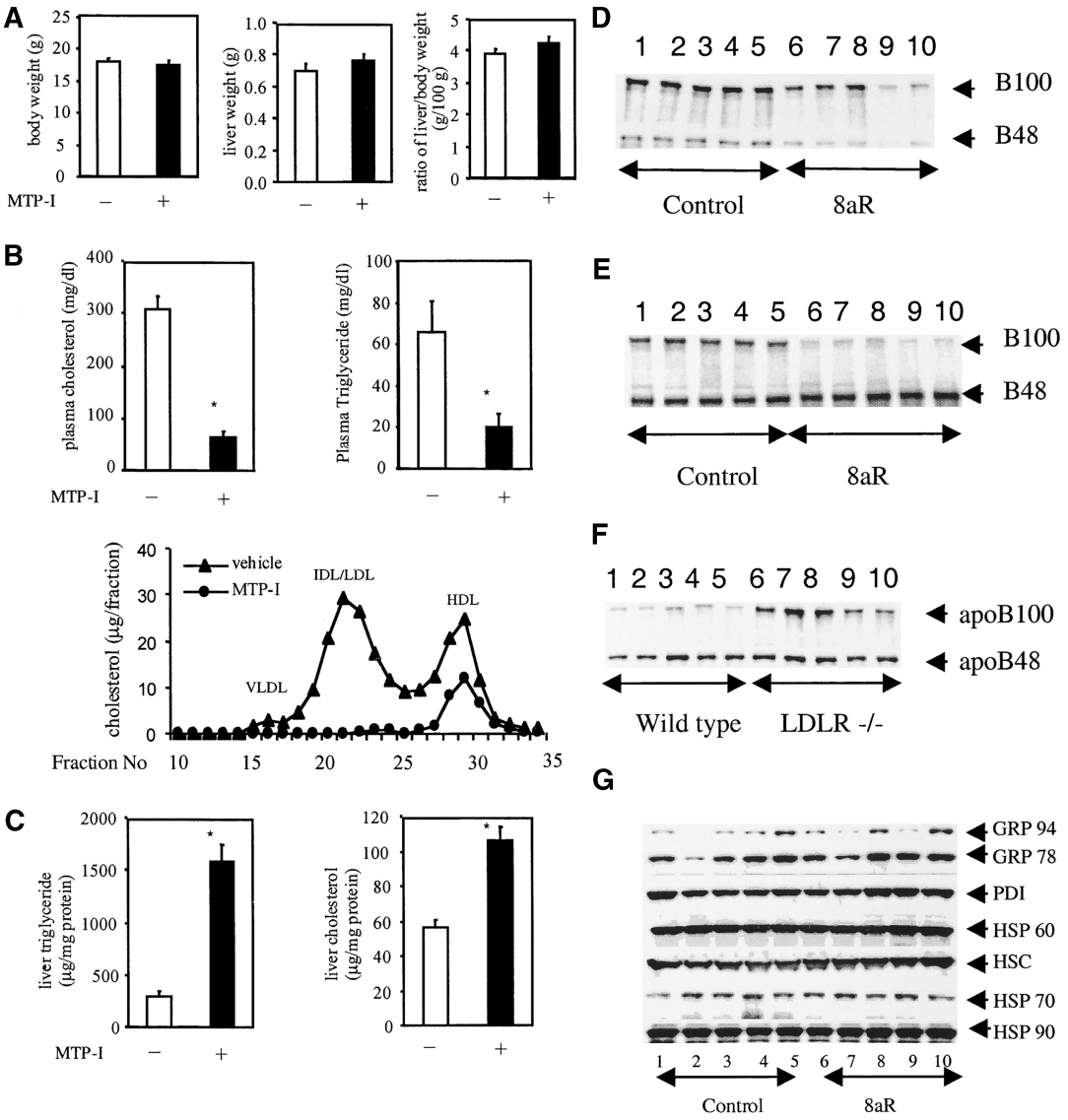

Fig. 3. Effect of 7 days of treatment with an oral MTP inhibitor on the plasma and liver levels of apoB-containing lipoproteins in *Ldlr*$^{-/-}$ mice. Female C57BL/6 mice (8 weeks old) were given the MTP inhibitor 8aR (50 mg/day/kg) for 7 days at 0800 h or vehicle only (control). Mice were sacrificed at 1100 h, and plasma and livers were obtained and analyzed. A: Body and liver weights. B: Plasma triglyceride and cholesterol, and cholesterol in each fraction obtained from FPLC separation of pooled plasma (five mice in each group). C: Liver triglyceride and cholesterol. D: Immunoblot analysis of plasma detected with an anti-apoB antibody. E: Immunoblot analysis of liver microsomes detected with an anti-apoB antibody. F: Immunoblot analysis of liver microsomes obtained from wild-type C57BL/6 and *Ldlr*$^{-/-}$ mice detected with an anti-apoB antibody. G: Immunoblot analysis of liver microsomes detected with antibodies as indicated. Each bar graph value represents the mean ± SD. *$P < 0.02$.

meostatic degradation pathway that prevents the accumulation of apoB in the ER without inducing heat shock or unfolded protein responses. Blocking MTP function by *Cre*-mediated gene disruption or chemical inhibition also reduced plasma lipid levels markedly without causing massive fatty liver. These findings suggest that MTP could be an effective therapeutic target for hyperlipidemia.

The processes through which apoB is assembled into lipoproteins and secreted by the liver are complex and controlled at many different levels throughout the secretory pathway [reviewed in refs. (16–20)]. Since MTP is localized to the proximal portion of the secretory pathway (i.e., the ER) (42), we have focused these studies on examining how blocking MTP affects the ER content of apoB and of lumenal proteins that participate in the unfolded protein response. Our findings are the first to show that in vivo deletion of MTP function (either by *Cre*-mediated gene disruption or chemical inhibition) blocked hepatic secretion of apoB-100 without causing apoB to accumulate in the ER. These findings are consistent with the notion that while loss of MTP function blocks apoB translocation, apoB does not accumulate in the ER (21, 25, 43–45) because it is cotranslationally degraded (30, 37, 46, 47). Our finding that interruption of MTP function results in the rapid and efficient degradation of secretion-incompetent apoB in vivo explains why apoB does not ac-

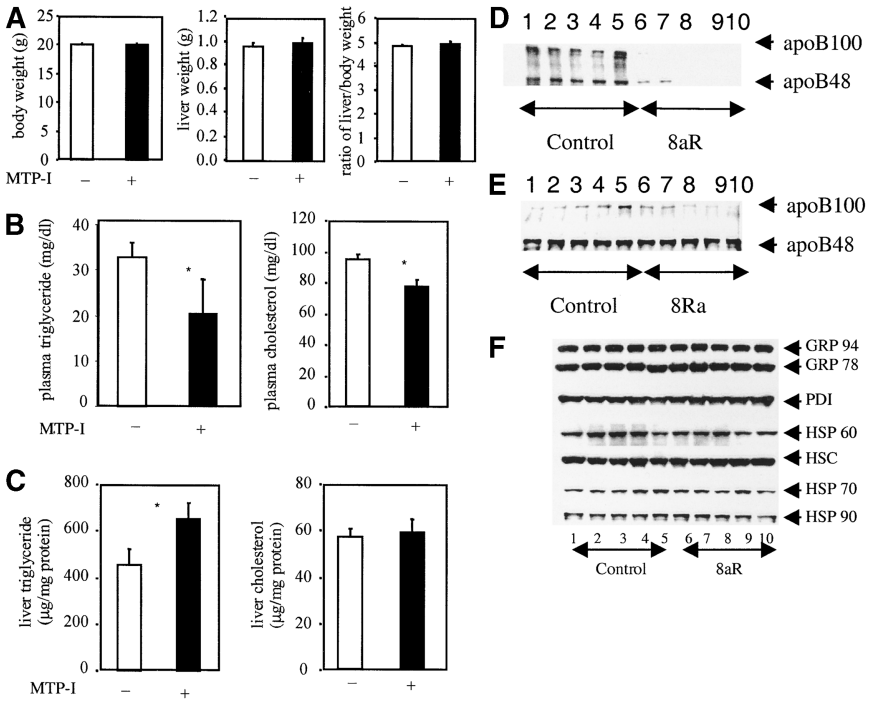

Fig. 4. Effect of a single dose of an oral MTP inhibitor on the plasma and liver levels of apoB-containing lipoproteins in C57BL/6 mice. Female C57BL/6 mice (8 weeks old) were given a single dose (50 mg/kg) of MTP inhibitor 8aR at 0800 h or vehicle only (control). Mice were sacrificed at 1100 h the following day. Plasma and liver were obtained and analyzed. A: Body and liver weights. B: Plasma triglyceride and cholesterol levels. C: Liver triglyceride and cholesterol. D: Immunoblot analysis of plasma detected with an anti-apoB antibody. E: Immunoblot analysis of liver microsomes detected with an anti-apoB antibody. F: Immunoblot analysis of liver microsomes detected with antibodies as indicated. Each bar graph value represents the mean ± SD. $*P < 0.02$.

cumulate in hepatic ER. Our findings may also explain why blocking the secretion of apoB-containing lipoproteins by the liver in mice is not associated with the inflammatory responses associated with the accumulation of secretion-incompetent protein in the secretory pathway (unfolded-protein and heat shock responses) (38–40). This proposal is further supported by the recent observation that deletion by itself of MTP expression in the livers of mice is not associated with hepatic inflammation or dysfunction (48).

The livers of *Ldlr*$^{-/-}$ mice contained more apoB-100 in the ER than livers of C57BL/6 mice (Fig. 3F). Since apoB mRNA expression was similar in the two groups of mice, this difference probably reflects differences in the degradation of apoB in the ER. Our findings are consistent with studies showing that hepatocytes from *Ldlr*$^{-/-}$ mice secrete more apoB-100 and degrade less of it in the ER than do hepatocytes from wild-type mice (49). Apparently, the association of the LDL receptor with nascent apoB-100 facilitates its removal from the ER or its degradation (50). The ligand-binding domain of the LDL receptor in the ER is in the lumen (51). Since the LDL receptor binding domain of apoB is located in the C terminus terminus (52, 53), it is likely that the LDL receptor associates with apoB after its translocation into the ER lumen is completed. Our finding that blocking MTP caused a similar decrease in the apoB-100 content in the ER indicates that the presence of the LDL receptor did not affect the degradation of apoB-100 induced in response to blocked secretion. These combined findings suggest the possibility that the absence of MTP function causes increased degradation of cytoplasmic (translocation-arrested) apoB-100, and the absence of the LDL receptor reduces the degradation of luminal (fully translocated) apoB.

The observation that some (54) [but not all (2, 8, 55)] abetalipoproteinemic patients develop liver disease implies that the response of the human liver to MTP inhibition may be influenced by genetic and environmental factors. In the same mice we used in this study, deletion of MTP did not, by itself, cause liver dysfunction, but it did increase susceptibility to toxin-induced liver injury (48). This increased susceptibility may be caused by impaired transport of essential lipid nutrients, such as vitamin E (8, 55, 56). The previous studies suggesting that MTP inhibitors corrected the marked hypercholesterolemia in Watanabe-heritable hyperlipidemic rabbits (13) provided support suggesting a possible alternative treatment for homozygous familial hypercholesterolemia, a lethal disorder corrected by liver transplantation (15). The studies in Watanabe-heritable hyperlipidemic rabbits did not provide data concerning liver function or inflammatory response (13). Our studies reported herein clearly show that inhibition of MTP corrected the hyperlipidemia in a mouse model of homozygous familial hypercholesterol-

emia without causing massive fatty liver or induction of heat shock or unfolded protein responses. Our findings provide further evidence that MTP may be a therapeutically useful target for reducing plasma levels of atherogenic lipoproteins in homozygous familial hypercholesterolemia.

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