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Blocking the Secretion of Hepatic Very Low Density Lipoproteins Renders the Liver More Susceptible to Toxin-induced Injury*

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Recently, we generated mice lacking microsomal triglyceride transfer protein (MTP) in the liver (*Mttp*$^{\Delta/\Delta}$) and demonstrated that very low density lipoprotein secretion from hepatocytes was almost completely blocked. The blockade in lipoprotein production was accompanied by mild to moderate hepatic steatosis, but the mice appeared healthy. Although hepatic MTP deficiency appeared to be innocuous, we hypothesized that a blockade in very low density lipoprotein secretion and the accompanying steatosis might increase the sensitivity of *Mttp*$^{\Delta/\Delta}$ livers to additional hepatic insults. To address this issue, we compared the susceptibility of *Mttp*$^{\Delta/\Delta}$ mice and *Mttp*$^{flox/flox}$ controls to hepatic injury from *Escherichia coli* lipopolysaccharides, concanavalin A, and *Pseudomonas aeruginosa* exotoxin A. At baseline, neither the *Mttp*$^{\Delta/\Delta}$ nor the *Mttp*$^{flox/flox}$ mice had elevated serum transaminases or histologic evidence of hepatic inflammation. After the administration of the toxins, however, the *Mttp*$^{\Delta/\Delta}$ mice manifested higher levels of transaminases and, unlike the *Mttp*$^{flox/flox}$ mice, developed histologic evidence of hepatic inflammation. The toxic challenge induced tumor necrosis factor-α to a similar extent in *Mttp*$^{\Delta/\Delta}$ and *Mttp*$^{flox/flox}$ mice, but other parameters of injury (*e.g.* chemokine transcript levels and lipid peroxides) were disproportionately increased in the *Mttp*$^{\Delta/\Delta}$ mice. Our results suggest that blocking lipoprotein secretion in the liver may increase the susceptibility of the liver to certain toxic challenges.

Microsomal triglyceride transfer protein (MTP)[1] is critical for the assembly and secretion of apolipoprotein (apo) B-containing lipoproteins, both in the intestine and in the liver (1, 2). A genetic absence of MTP causes abetalipoproteinemia, a disease characterized by intestinal fat malabsorption, a virtual absence of chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins in the plasma, and strikingly low plasma levels of triglycerides and cholesterol. The fact that a deficiency in MTP reduces the plasma levels of atherogenic lipoproteins has attracted the attention of the pharmaceutical industry. Many companies have established MTP programs, with the goal of identifying MTP inhibitors suitable for treating humans with hyperlipidemias (3, 4). Thus far, however, the efficacy and safety of these compounds in humans has not been documented.

To investigate the role of MTP in lipoprotein assembly and secretion, we inactivated the MTP gene (*Mttp*) in mice (5). Heterozygous knockout mice (*Mttp*$^{+/-}$) manifested slightly reduced levels of lipoprotein secretion, reduced levels of apoB100-containing lipoproteins in the plasma, and slightly increased levels of neutral lipids (triglycerides and cholesterol esters) in the liver. Homozygous knockout mice (*Mttp*$^{-/-}$) died during embryonic development. Subsequently, we used *Cre*/*LoxP* recombination techniques to produce mice lacking *Mttp* expression in the liver but not in the intestine (6). Those mice, designated *Mttp*$^{\Delta/\Delta}$ mice, exhibited strikingly reduced plasma levels of apoB100, sizable reductions in the plasma levels of cholesterol and triglycerides, and mild to moderate steatosis with increased levels of neutral lipids in the liver. The *Mttp*$^{\Delta/\Delta}$ mice were healthy and grew normally; their plasma transaminase levels were normal, and their livers were free of inflammatory infiltrates (6).

The fact that it was possible to eliminate hepatic *Mttp* expression in a mammalian model without noticeable side effects supported the concept that it might be possible to develop MTP inhibitors to treat hyperlipidemias. Also encouraging were studies by Wetterau *et al.* (7) that showed that MTP inhibitors could reduce plasma lipoprotein levels in low density lipoprotein receptor-deficient rabbits without causing elevated transaminases or histologic evidence of liver inflammation.

In this study, we further investigated the notion that it might be possible, with impunity, to inhibit MTP and block hepatic lipoprotein production. We were suspicious, based on several observations, that MTP inhibition might not be as safe as our original studies and those of Wetterau *et al.* (7) had

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[1] The abbreviations used are: MTP, microsomal triglyceride transfer protein; *Mttp*, the mouse gene for the large subunit of microsomal triglyceride transfer protein; apo, apolipoprotein; VLDL, very low density lipoprotein(s); Scd1, stearoyl-CoA desaturase 1; pI-pC, polyinosinic-polycytidylic ribonucleic acid; SREBP, sterol regulatory element-binding protein; LPS, lipopolysaccharide; PEA, *P. aeruginosa* exotoxin A; MIP, macrophage inflammatory protein; IL, interleukin: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TNF, tumor necrosis factor; TBARS, thiobarbituric acid-reactive substances; ConA, concanavalin A.

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implied. First, other human conditions associated with hepatic steatosis (*e.g.* diabetes mellitus, excessive consumption of ethanol, and obesity) increase the risk of developing hepatic inflammation and advanced liver disease (8–10). Second, severe liver disease has been reported in humans with abetalipoproteinemia (11, 12). Although treatment with short-chain triglycerides might have contributed to the liver disease in those cases, it is also possible that the inability of those livers to secrete lipoproteins caused them to be susceptible to steatohepatitis and advanced liver disease.

Normal human livers are required to face toxic insults. For example, intermittent lapses in the intestinal mucosal barrier can allow bacterial products to reach the liver (13). Normal livers from healthy individuals can cope with these challenges effectively, without inflammation or tissue injury. The livers of susceptible individuals, however, cannot effectively deal with these challenges, either because of genetic differences or metabolic derangements (14). This failure of normal protective mechanisms can lead to hepatic inflammation and, in some cases, to advanced liver disease.

We hypothesized that the blockade of hepatic lipoprotein production and resultant hepatic steatosis might render the liver more susceptible to toxic liver injury. To test this hypothesis, we compared the susceptibility of liver-specific MTP knockout mice and littermate controls to hepatic injury following challenges with exogenous toxins.

EXPERIMENTAL PROCEDURES

$Mttp^{flox/flox}$Mx1-Cre Mice—A conditional *Mttp* allele, *$Mttp^{flox}$*, in which exon 1 of *Mttp* is flanked by *loxP* sites, has been described previously (6). *$Mttp^{flox/flox}$* mice were bred with Mx1-*Cre* transgenic mice (15) to generate *$Mttp^{flox/flox}$*Mx1-*Cre* mice. To excise exon 1 of *Mttp* and thus eliminate MTP expression in the liver, 21–28-day-old male *$Mttp^{flox/flox}$*Mx1-*Cre* mice (16) were injected with polyinosinic-polycytidylic ribonucleic acid (pI-pC; Sigma; 500 μg every other day for 8 days) (6). Littermate *$Mttp^{flox/flox}$* mice lacking the *Cre* transgene were also injected with pI-pC. Excision of exon 1 was assessed by Southern blot analysis of *Sac*I-digested genomic DNA using a 3′-flanking probe. The mice had a mixed genetic background (~50% 129/SvJae and ~50% C57BL/6). They were housed in a pathogen-free barrier facility with a 12-h light/12-h dark cycle and were fed rodent chow containing 4.5% fat (Ralston Purina, St. Louis, MO). Genotypes were determined by Southern blots or by PCR with genomic DNA from tail biopsies.

Measurement of Insulin and Glucose Levels—Plasma glucose levels were measured with a glucose (Trinder) 100 kit from Sigma. Plasma insulin levels were measured with a 1-2-3 ultra-sensitive rat insulin enzyme-linked immunosorbent assay from Alpco (Windham, NH).

DNA Microarray Experiments—Murine 11K GeneChips (Affymetrix, Santa Clara, CA) were used to assess hepatic gene expression patterns. Total RNA was isolated from liver biopsies with TRizol Reagent (Invitrogen) and purified further with a RNeasy Midi kit (Qiagen, Los Angeles, CA). cDNA was synthesized from the RNA with the Superscript Choice System (Invitrogen) and T7-$(dT)_{24}$ primers (Genset, La Jolla, CA). Biotin-labeled cRNA was transcribed from the cDNA in the presence of biotin-labeled nucleotides (RNA Transcript Labeling kit for nucleic acid arrays, Enzo Diagnostics, Farmingdale, NY). The integrity of the total RNA and the cRNA was assessed by electrophoresis on a 1% agarose/formaldehyde gel. Fragmented cRNA was mixed with control oligonucleotides Bio B, C, D, and *Cre* (American Type Culture Collection, Manassas, VA) and hybridized to the GeneChip at 45 °C for 16 h. The GeneChip Fluidics Station 400 (Affymetrix) was used to stain the GeneChips with R-phycoerythrin streptavidin (Molecular Probes, Eugene, OR), and the signal was amplified with a biotin-labeled anti-streptavidin antibody (Vector Laboratories, Burlingame, CA). The expression data were obtained by scanning the arrays in a GeneArray Scanner (Hewlett-Packard, Palo Alto, CA); data were analyzed with GeneChip 3.1 software (Affymetrix).

Northern Blot Analysis—Expression of the stearoyl-CoA desaturase 1 gene (*Scd1*) was assessed by Northern blotting with a probe described previously (17). Briefly, 25 μg of total liver RNA was denatured and separated on 1% agarose/formaldehyde gel electrophoresis. The integrity of the total RNA was confirmed on ethidium bromide-stained gels before transfer to a Nytran SuPerCharge membrane (Schleicher & Schuell). Prehybridization, hybridization, and washing procedures were performed as described previously (18). Membranes were probed with [α-^{32}P]dCTP-labeled cDNA fragments, and signals were visualized by autoradiography (Hyperfilm ECL, Amersham Biosciences). Band intensity was quantified by densitometry (Molecular Imager FX, Bio-Rad). An 18 S probe (Ambion, Austin, TX) was used to normalize *Scd1* expression levels.

Western Blots—Levels of Scd1 protein were determined by Western blotting of whole-liver homogenates. Levels of sterol regulatory element-binding protein (SREBP)-1 and SREBP-2 were determined by Western blotting of nuclear extracts (19). To prepare the nuclear extracts, livers from four mice were pooled (~1.5 g) and homogenized in 30 ml of buffer A (10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM sodium EDTA, 2 M sucrose, 10% (v/v) glycerol, 150 μM spermine, 2 μM spermidine) and protease inhibitors (Complete-Mini, Roche Molecular Biochemicals). The homogenate was subjected to several strokes with a Teflon pestle and filtered through three layers of cheesecloth. To isolate the nuclear pellet, a 25-ml portion of the homogenate was then layered over 10 ml of buffer A and spun in an SW28 Ti rotor (Beckman Instruments, Palo Alto, CA) at 24,000 rpm for 1 h at 4 °C. The pellet was resuspended in 1 ml of buffer (10 mM Hepes, pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM dithiothreitol, 10% glycerol), and protease inhibitors (Complete-Mini), 0.1 volume of 4 M $(NH_4)_2SO_4$, pH 7.9, were added. The resuspended pellet was gently mixed and then centrifuged at 85,000 rpm in a TLA-100.2 rotor (Beckman Instruments) for 45 min at 4 °C. Aliquots of the supernatant containing the nuclear extracts (150 μg) and the whole-liver homogenates (100 μg) were then size-fractionated on 8% polyacrylamide gels. Western blots were performed with rabbit antisera against mouse SREBP-1 (20) and mouse SREBP-2 (21) and a rabbit antiserum against rat Scd1 (22). The binding of the primary antibodies was assessed by a horseradish peroxidase-labeled donkey anti-rabbit antibody and ECL Western blotting detection reagents (Amersham Biosciences).

Lipid Analyses—Liver pieces (~100 mg) were homogenized with a Polytron, Ultra-Turbax T8 (VWR, San Francisco, CA), and lipids were extracted with chloroform/methanol, 2:1 (v/v). Plasma lipids were extracted by hexane/isopropyl alcohol, 3:2 (v/v). Before the lipid extraction, known amounts of tri- and pentadecanoic acid (Sigma) were added as internal standards (23). Triglycerides, phospholipids, and fatty acids were identified by thin-layer chromatography, transesterified with methanolic HCl (Aldrich), and quantified by gas chromatography (23).

Administration of Toxins—One week after the last pI-pC injection, *$Mttp^{\Delta/\Delta}$* mice and *$Mttp^{flox/flox}$* littermate controls were given an intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS, Sigma; 1.0 μg/g) or intravenous injections of concanavalin A (ConA; 400 μg) (Sigma) or *Pseudomonas aeruginosa* exotoxin A (PEA, Sigma, 600 μg/kg). Plasma triglycerides and liver injury-associated enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH)) were determined in the clinical chemistry laboratory of San Francisco General Hospital 12 h before and 4 (LPS, ConA, and PEA) and 24 h (LPS) after the injections of the toxins. Plasma tumor necrosis factor-α (TNF-α) was determined by a commercial immunoassay with antibodies against mouse TNF-α (R & D Systems, Minneapolis, MN). All procedures were approved by the Committee on Animal Research at the University of California, San Francisco.

RNase Protection Assay—Hepatic levels of mRNAs for a variety of cytokines were quantified by RNase protection assays (24) with a multiprobe cDNA template kit (mCK1b, mCK3, mCK5; PharMingen, San Diego, CA). Briefly, cRNA probes were transcribed with [α-^{32}P]UTP (>800 Ci/mmol, Amersham Biosciences). Radiolabeled cRNA (5×10^5 Cerenkov cpm) was combined with 20 μg of liver RNA in 10 μl of hybridization buffer. The mixture was incubated at 55 °C for 16 h, and unhybridized RNA was digested by adding ribonuclease A and T1. RNase digestion was terminated with proteinase K and SDS, and the RNA-RNA hybrids were purified by phenol/chloroform extraction and ethanol precipitation. The double-stranded RNA was resuspended in electrophoresis buffer, denatured at 100 °C, and separated through 5% polyacrylamide/urea gels. RNA bands were visualized by autoradiography, and band intensity was quantified by densitometry (Hoefer Scientific Instruments, San Francisco, CA). Signals for specific cytokines were normalized to control RNAs (L32 or glyceraldehyde-3-phosphate dehydrogenase).

Lipid Peroxidation Assay—Thiobarbituric acid-reactive substances (TBARS), frequently used to estimate levels of lipid peroxides (25, 26), were determined with 50-mg liver fragments. To prevent the peroxidation of lipids during the procedure, liver fragments were homogenized in a 1.15% KCl solution containing 50 mM desferroxamine (Sigma).

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TABLE I
Characteristics of Mttp$^{\Delta/\Delta}$ and littermate control Mttp$^{flox/flox}$ mice

Data represent means and S.D. p values calculated by two-tailed, unpaired t test. Liver weights and body weights determined in two separate groups of mice.

Mouse phenotype	Mttp$^{flox/flox}$ (n = 24)	Mttp$^{\Delta/\Delta}$ (n = 23)	p value
Age (days)	49 ± 8	49 ± 9	0.93
Body weight (g)	19.9 ± 4.3	22.7 ± 4.5	0.09
Liver weight (g)	1.24 ± 0.13[a]	1.56 ± 0.23[b]	<0.0001
Plasma triglycerides (mg/dl)	65.8 ± 18.2	37.2 ± 13.2	<0.0001
Plasma fatty acids (mmol/liter)	0.21 ± 0.09[a]	0.25 ± 0.04[b]	0.38
Liver triglycerides (μmol/g)	107 ± 87[c]	333 ± 165[a]	0.005

[a] n = 8.
[b] n = 12.
[c] n = 7.

RESULTS

Phenotypic Analyses of Liver-specific MTP Knockout Mice—To generate mice lacking MTP in the liver (*i.e.* Mttp$^{\Delta/\Delta}$ mice), *Cre* expression in *Mttp*$^{flox/flox}$Mx1-*Cre* mice was induced with pI-pC. Consistent with previous studies (6), the plasma triglyceride levels were lower in *Mttp*$^{\Delta/\Delta}$ mice than in *Mttp*$^{flox/flox}$ mice (Table I). The reduction in plasma triglyceride levels in *Mttp*$^{\Delta/\Delta}$ mice was accompanied by an increase in hepatic lipid stores, which was evident both from the gross appearance of the liver (Fig. 1, *A* and *B*) and from histology (Fig. 1, *C–F*). Biochemical studies revealed that the liver triglyceride stores were 3-fold higher in *Mttp*$^{\Delta/\Delta}$ mice than in littermate *Mttp*$^{flox/flox}$ mice (Table I). The amount of lipid accumulation in this model was modest in comparison to some other genetic models of lipid accumulation. For example, the livers of mice expressing a truncated SREBP-1a synthesize high levels of fatty acids and have a 21-fold increase in liver triglyceride stores (20).

We predicted that the microarray experiments might uncover many perturbations in the expression of genes affecting lipid metabolism. To address this issue, we compared hepatic gene expression in *Mttp*$^{\Delta/\Delta}$ and *Mttp*$^{flox/flox}$ mice with Affymetrix GeneChips. Remarkably, most genes involved in lipid metabolism were unchanged (*e.g.* acetyl-CoA carboxylase, acyl-coenzyme A:cholesterol acyltransferase, apoE, ATP-citrate lyase, cholesterol 7-α-hydroxylase, fatty-acid synthase, fatty acid transport protein, 3-hydroxy-3-methylglutaryl-coenzyme A lyase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, 3-hydroxy-3-methylglutaryl-coenzyme A synthase, low density lipoprotein receptor, lipoprotein lipase, and peroxisome proliferator-activated receptor-α) (definition of unchanged: fold change $<30\%$, $p > 0.15$). However, there were two noteworthy exceptions. First, *Mttp* expression was undetectable in the livers of *Mttp*$^{\Delta/\Delta}$ mice (n = 5), whereas *Mttp* expression in *Mttp*$^{flox/flox}$ mice was 6-fold higher than the threshold detection level (n = 7) (p = 0.00000002). Second, *Scd1* expression in the livers of *Mttp*$^{\Delta/\Delta}$ mice was reduced by 69% compared with the livers of *Mttp*$^{flox/flox}$ mice ($p < 0.0005$). Northern blots and Western blots confirmed the reduction in *Scd1* expression *Mttp*$^{\Delta/\Delta}$ livers (Fig. 2, *A* and *B*). *Scd1* expression is up-regulated by SREBP-1 (27), so we hypothesized that the levels of mature SREBP-1 would be reduced in livers of *Mttp*$^{\Delta/\Delta}$ mice. Indeed, this was the case. SREBP-1 (but not SREBP-2) levels were reduced by ~50% in the livers of *Mttp*$^{\Delta/\Delta}$ mice (Fig. 2*C*).

SREBP-1c expression is reduced by low levels of insulin and induced by insulin replacement (28, 29). To determine whether the inactivation of *Mttp* affected glucose or insulin levels, plasma triglycerides, glucose, and insulin levels were measured in *Mttp*$^{flox/flox}$ mice (n = 20), *Mttp*$^{flox/flox}$ mice treated with subcutaneous injections of water (n = 10), and *Mttp*$^{\Delta/\Delta}$ mice (*i.e.* *Mttp*$^{flox/flox}$ mice treated with subcutaneous injections of pI-pC; n = 10). Consistent with the results in Table I, plasma triglyceride levels were significantly reduced in *Mttp*$^{\Delta/\Delta}$ mice ($p < 0.001$). Plasma glucose levels were reduced by ~20% in *Mttp*$^{\Delta/\Delta}$ mice (14.06 ± 0.88 mmol/liter in *Mttp*$^{flox/flox}$ mice *versus* 11.44 ± 0.50 in *Mttp*$^{\Delta/\Delta}$ mice; $p < 0.05$). Plasma insulin levels were reduced by ~45% in *Mttp*$^{\Delta/\Delta}$ mice (0.39 ± 0.30 ng/ml in *Mttp*$^{flox/flox}$ mice *versus* 0.21 ± 0.06 in *Mttp*$^{\Delta/\Delta}$ mice; $p < 0.001$). Thus, the lower plasma insulin levels in *Mttp*$^{\Delta/\Delta}$ mice might well contribute to the lower SREBP-1 levels.

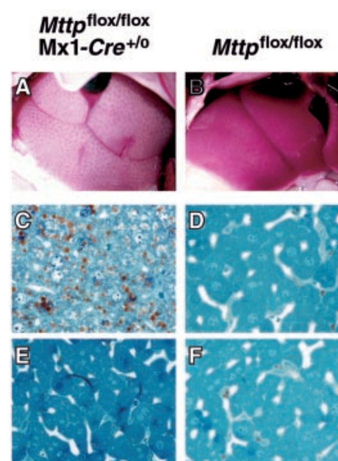

FIG. 1. **Gross and microscopic appearances of the livers in different groups of mice.** *A*, liver from an *Mttp*$^{flox/flox}$Mx1-*Cre* mouse 1 week after injections of pI-pC. *B*, liver from a littermate *Mttp*$^{flox/flox}$ mouse 1 week after injections of pI-pC. *C*, osmium tetroxide-stained section of the liver from an *Mttp*$^{flox/flox}$Mx1-*Cre* mouse 1 week after injections of pI-pC. *D*, liver from a littermate *Mttp*$^{flox/flox}$ mouse 1 week after injections of pI-pC. *E*, liver from an *Mttp*$^{flox/flox}$Mx1-*Cre* mouse 1 week after injections of normal saline. *F*, liver from an age-matched littermate *Mttp*$^{flox/flox}$ mouse 1 week after injections of normal saline.

Scd1 expression is also down-regulated by polyunsaturated fatty acids (30–32), so we sought to determine whether levels of polyunsaturated fatty acids were increased in *Mttp*$^{\Delta/\Delta}$ mice. Interestingly, the predominant polyunsaturated fatty acid, linoleic acid, was increased significantly in the livers of *Mttp*$^{\Delta/\Delta}$ mice. The amount of linoleic acid (as a percentage of the total fatty acids) in liver triglycerides was 34.2 ± 4.2 in *Mttp*$^{flox/flox}$ mice (n = 8) and 40.8 ± 2.7 in *Mttp*$^{\Delta/\Delta}$ mice (n = 7) (p = 0.0037); the percentage of linoleic acid in liver free fatty acids was 15.8 ± 2.7 in *Mttp*$^{flox/flox}$ mice and 22.7 ± 2.7 in *Mttp*$^{\Delta/\Delta}$ mice (p = 0.0003). These differences could not be accounted for by differences in the fatty acid composition of the plasma. The amount of linoleic acid (as a percentage of the total fatty acids) in plasma triglycerides was 27.2 ± 5.9 in *Mttp*$^{flox/flox}$ mice (n = 8) and 24.2 ± 16.3 in *Mttp*$^{\Delta/\Delta}$ mice (n = 7) (p = 0.62).

Expression of Inflammation-related Genes in Mttp$^{\Delta/\Delta}$ *Mice*—Because hepatic steatosis in some mouse models leads to hepatic inflammation (26), we suspected that the accumulation of lipids in *Mttp*$^{\Delta/\Delta}$ mice might affect the expression of many genes, including those involved in inflammatory responses. However, the microarray experiments did not uncover evidence for an active inflammatory response in *Mttp*$^{\Delta/\Delta}$ livers. Expression levels for inflammation-related genes (*e.g.* macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, interferon-α, interferon-β, interferon-γ; and TNF-α) and apoptosis-related genes (*bax*, *bcl-2*, caspases 1, 2, 3 and 7, c-*jun*, c-*myc,* cytochrome *c*, and *fas*) were either equally low in *Mttp*$^{\Delta/\Delta}$ and *Mttp*$^{flox/flox}$ livers or below the threshold of detection.

Susceptibility of Livers to Toxins—To determine whether *Mttp*$^{\Delta/\Delta}$ mice were particularly sensitive to hepatic injury, *Mttp*$^{\Delta/\Delta}$ and *Mttp*$^{flox/flox}$ mice were challenged with three toxins

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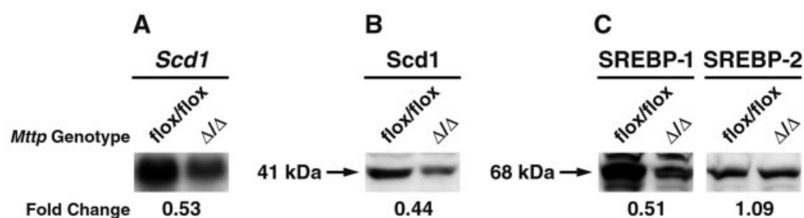

FIG. 2. **Changes in Scd1 and SREBP levels in livers of $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice.** *A*, Northern blot showing liver *Scd1* mRNA levels in $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice ($n = 5$ in each group examined). After normalization to 18 S expression, the ratio of *Scd1* mRNA levels in $Mttp^{\Delta/\Delta}$ livers divided by *Scd1* mRNA levels in $Mttp^{flox/flox}$ livers was 0.53. *B*, Western blot analyses of Scd1 protein levels in the livers of $Mttp^{\Delta/\Delta}$ and littermate $Mttp^{flox/flox}$ mice ($n = 5$ in each group examined). The results were quantified by densitometry. After normalization to actin expression, the ratio of Scd1 protein levels in $Mttp^{\Delta/\Delta}$ livers divided by Scd1 protein levels in $Mttp^{flox/flox}$ livers was 0.44. *C*, Western blot analyses of SREBP-1 and SREBP-2 protein levels in the livers of $Mttp^{\Delta/\Delta}$ and littermate $Mttp^{flox/flox}$ mice ($n = 4$ in each group examined). The results were quantified by densitometry. After normalization to actin expression, the ratios of SREBP-1 and SREBP-2 protein levels in $Mttp^{\Delta/\Delta}$ livers divided by levels in $Mttp^{flox/flox}$ livers were 0.51 and 1.09, respectively.

known to cause acute liver inflammation (LPS, ConA, and PEA). The inflammatory response triggered by these toxins is characterized by the release of pro-inflammatory cytokines (*e.g.* TNF-α, interferon-γ, IL-2, and IL-6), which leads to hepatocyte injury and increased plasma levels of AST, ALT, and LDH (33–35). LPS stimulates monocytes and macrophages (33), whereas ConA primarily stimulates T lymphocytes (34). PEA inhibits protein synthesis, particularly in the liver, and also is a weak T-cell mitogen (35).

Baseline plasma levels of ALT, AST, and LDH were normal in $Mttp^{\Delta/\Delta}$ mice and $Mttp^{flox/flox}$ mice (Fig. 3). At 4 and 24 h after intraperitoneal injections of *E. coli* LPS (1.0 μg/g), the plasma ALT, AST, and LDH levels were higher in $Mttp^{\Delta/\Delta}$ mice than in littermate $Mttp^{flox/flox}$ mice (Fig. 3). The increased transaminase levels in $Mttp^{\Delta/\Delta}$ mice were associated with an infiltration of polymorphonuclear leukocytes into the liver parenchyma and with occasional foci of hepatocellular necrosis (Fig. 4). The results were similar after challenges with ConA and PEA (Fig. 5). Again, AST, ALT, and LDH were significantly higher in $Mttp^{\Delta/\Delta}$ mice than in $Mttp^{flox/flox}$ controls (Fig. 5).

We considered the possibility that the increased susceptibility of $Mttp^{\Delta/\Delta}$ mice to toxin-mediated injury was not due to the blockade in lipoprotein secretion but instead was a spurious and unanticipated effect of the Mx1-*Cre* transgene (carried by the $Mttp^{\Delta/\Delta}$ mice but not the $Mttp^{flox/flox}$ controls). To test this possibility, groups of $Mttp^{flox/flox}$Mx1-*Cre* mice and littermate $Mttp^{flox/flox}$ mice were given injections of normal saline rather than pI-pC and then challenged with LPS (1.0 μg/g). The $Mttp^{flox/flox}$Mx1-*Cre* mice did not exhibit an increased susceptibility to liver injury (Fig. 6), indicating that the enhanced sensitivity of $Mttp^{\Delta/\Delta}$ mice to LPS was caused by the elimination of hepatic *Mttp* expression and the resultant blockade in lipoprotein secretion.

Expression of Cytokines in the Liver after the LPS Challenge—To determine the mechanism of enhanced liver injury in $Mttp^{\Delta/\Delta}$ mice, we compared the expression of several cytokines in the plasma and liver of both groups of mice following LPS challenge. We first investigated TNF-α because it is known to be induced by LPS and because it is a mediator of tissue injury and inflammation (36). TNF-α was not detectable in the plasma of either $Mttp^{\Delta/\Delta}$ mice or controls before the LPS challenge but was easily detectable within 4 h after LPS administration (Fig. 7). Of note, the post-challenge TNF-α levels were no different in $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice (Fig. 7). Consistent with the plasma data, hepatic TNF-α mRNA levels were similar in $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice (Fig. 8*A*).

To determine whether the increased susceptibility of $Mttp^{\Delta/\Delta}$ mice to toxins resulted from enhanced production of other inflammatory mediators within the liver, we examined the hepatic expression of multiple immunomodulatory cytokines 4 h after LPS challenge. The mRNA levels of the T-cell cytokine

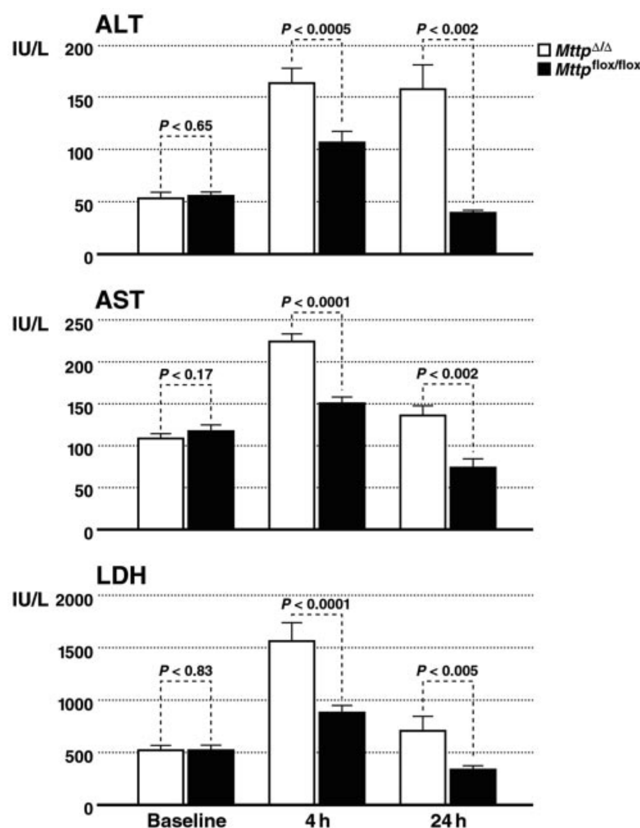

FIG. 3. **Plasma levels (mean ± S.E.) of ALT, AST, and LDH in $Mttp^{\Delta/\Delta}$ mice and littermate $Mttp^{flox/flox}$ mice after an LPS challenge.** Measurements were performed at base line and 4 and 24 h after an intraperitoneal injection of *E. coli* LPS (1.0 μg/g). *p* values were calculated with two-tailed, unpaired *t* tests. At base line, $n = 23$ $Mttp^{\Delta/\Delta}$ mice and $n = 24$ $Mttp^{flox/flox}$ mice; at 4 h, $n = 14$ $Mttp^{\Delta/\Delta}$ mice and $n = 18$ $Mttp^{flox/flox}$ mice; at 24 h, $n = 5$ for both $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice.

IL-2 were no different between $Mttp^{\Delta/\Delta}$ and $Mttp^{flox/flox}$ mice nor were the mRNA levels for the anti-inflammatory cytokine IL-10 (Fig. 8*B*). Interestingly, however, hepatic expression levels for several chemokines (macrophage inflammatory protein (MIP) 1α, MIP-1β, and MIP-2) were induced to a greater extent in the $Mttp^{\Delta/\Delta}$ mice than in $Mttp^{flox/flox}$ mice (Fig. 8*C*).

Lipid Peroxidation Products in $Mttp^{\Delta/\Delta}$ Livers—LPS induces oxidant stress in the liver (37–40), which promotes lipid peroxidation, particularly in the presence of polyunsaturated fatty acids (41, 42). We therefore predicted that lipid peroxides would increase significantly in the livers of $Mttp^{\Delta/\Delta}$ mice after an LPS challenge, due to their higher basal levels of polyunsaturated fatty acids. To test that possibility, we measured hepatic levels of TBARS as an indicator of tissue lipid peroxides

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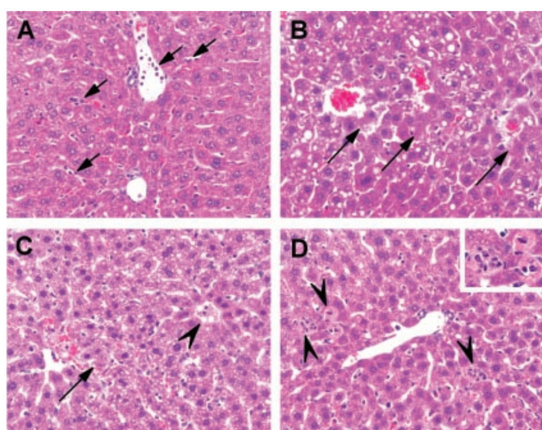

FIG. 4. **Liver histology following LPS treatment.** Photomicrographs of liver sections stained with hematoxylin and eosin 4 h after an intraperitoneal injection with LPS (1.0 μg/g). *A*, liver from an *Mttp*$^{flox/flox}$ mouse. *B–D*, livers from three separate *Mttp*$^{\Delta/\Delta}$ mice. In the *Mttp*$^{flox/flox}$ mouse, LPS causes neutrophil sequestration within portal veins and hepatic sinusoids (*short arrows*) but no significant hepatocellular injury. In *Mttp*$^{\Delta/\Delta}$ mice, LPS causes hepatocellular necrosis (*B* and *C*, *long arrows*) and intraparenchymal hemorrhage. Neutrophils in *Mttp*$^{\Delta/\Delta}$ mice are often observed in clusters (*C* and *D*, *arrowheads* and *D*, *inset*) in regions of hepatocellular destruction. Original magnification ×20.

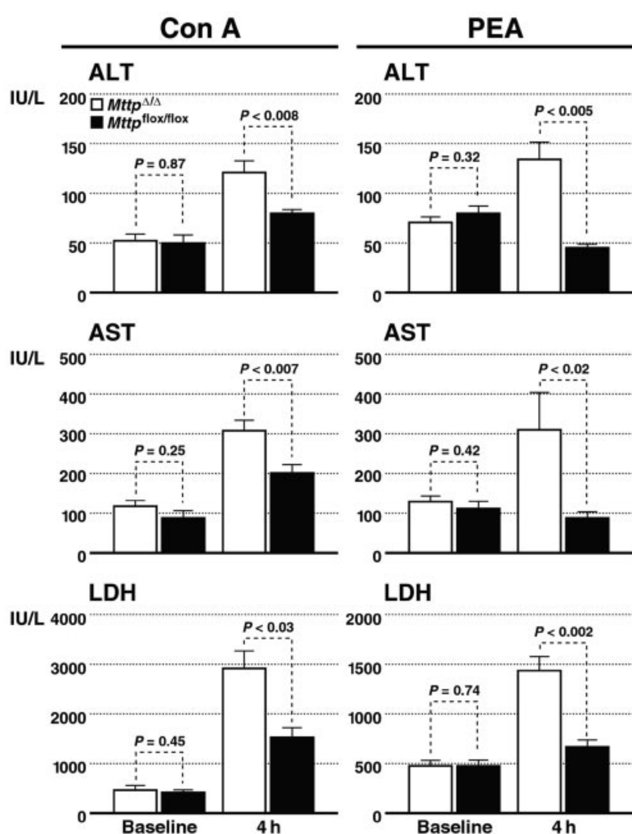

FIG. 5. **Plasma levels (mean ± S.E.) of ALT, AST, and LDH in *Mttp*$^{\Delta/\Delta}$ mice ($n = 10$) and *Mttp*$^{flox/flox}$ mice ($n = 9$) after ConA and PEA challenges.** Measurements were performed at baseline and 4 h after injections of ConA (400 μg) or PEA (600 μg/kg) ($n = 10$ for each group with each toxin). p values were calculated with two-tailed, unpaired t tests.

in *Mttp*$^{\Delta/\Delta}$ and *Mttp*$^{flox/flox}$ mice before and after an LPS challenge. Before the challenge, the livers from *Mttp*$^{\Delta/\Delta}$ mice had slightly higher TBARS than livers from *Mttp*$^{flox/flox}$ mice (Fig. 9). After the LPS challenge, however, the TBARS in livers from *Mttp*$^{\Delta/\Delta}$ mice increased quite significantly, 7-fold more than in *Mttp*$^{flox/flox}$ livers (Fig. 9).

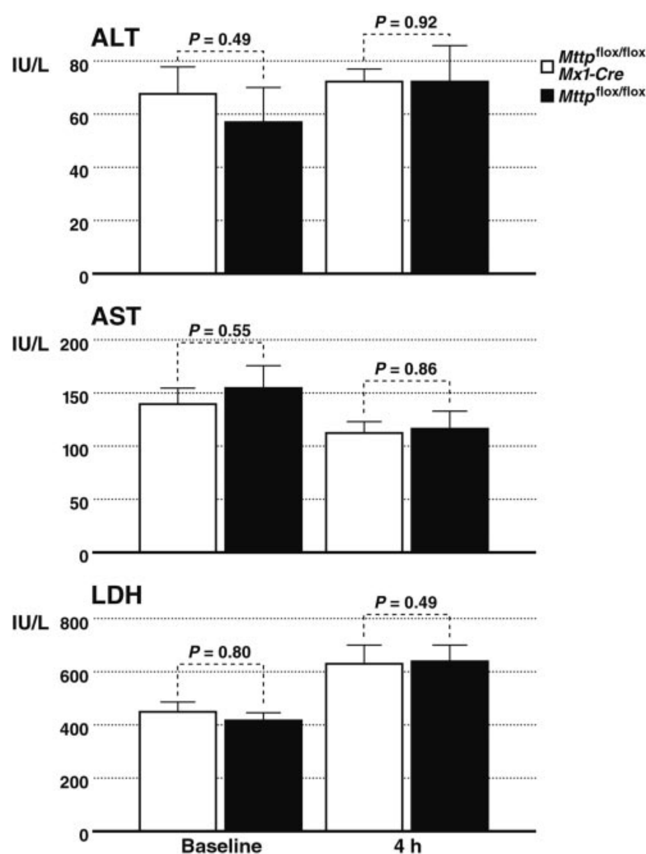

FIG. 6. **Plasma levels (mean ± S.E.) of ALT, AST, and LDH in *Mttp*$^{flox/flox}$Mx1-*Cre* mice ($n = 10$) and littermate control *Mttp*$^{flox/flox}$ mice ($n = 5$) after an LPS challenge.** Measurements were performed at baseline and 4 h after an intraperitoneal injection of LPS (1.0 μg/g). Both groups of mice had received injections of normal saline (rather than pI-pC) 1 week earlier. p values were calculated with two-tailed, unpaired t tests.

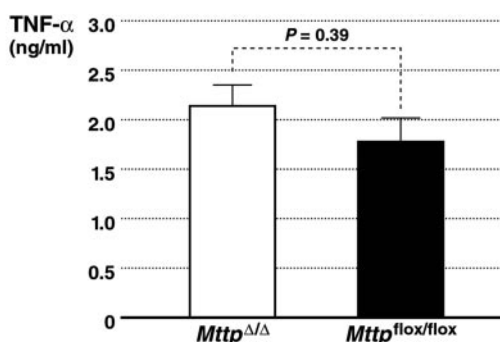

FIG. 7. **Plasma levels (mean ± S.E.) of TNF-α in *Mttp*$^{\Delta/\Delta}$ mice ($n = 14$) and littermate *Mttp*$^{flox/flox}$ mice ($n = 17$).** Measurements were performed 4 h after an intraperitoneal injection of LPS (1.0 μg/g). The p value was calculated with a two-tailed, unpaired t test.

DISCUSSION

The inability of livers from *Mttp*$^{\Delta/\Delta}$ mice to secrete VLDL reduced plasma triglyceride levels and was associated with an accumulation of triglycerides in the liver. Interestingly, the amount of hepatic steatosis in *Mttp*$^{\Delta/\Delta}$ mice was relatively modest, far less than that observed in SREBP-1a transgenic mice where the synthesis of fatty acids and cholesterol is activated (20). In *Mttp*$^{\Delta/\Delta}$ mice, the blockade in lipoprotein production did not cause widespread changes in the expression of the genes governing lipid metabolism. The serum transaminases in *Mttp*$^{\Delta/\Delta}$ mice were normal, and the animals exhibited normal vitality, growth, and fertility. Presumably, the ability of

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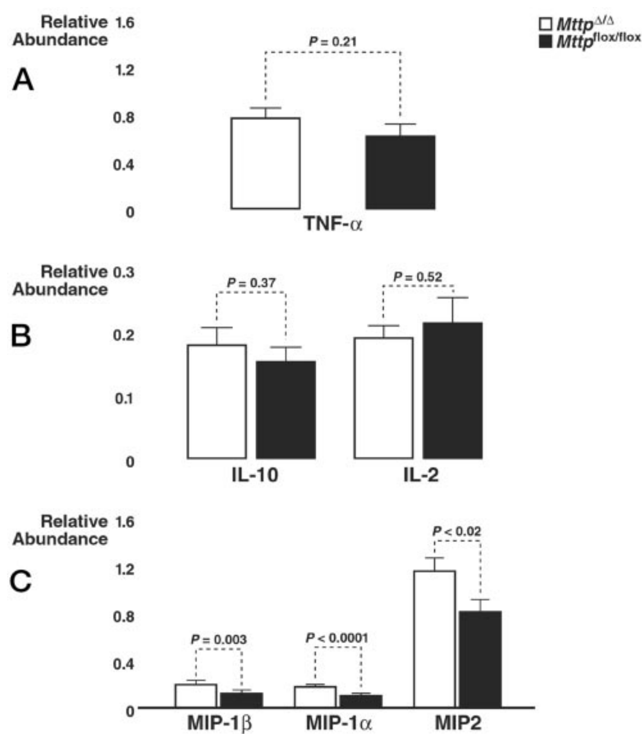

FIG. 8. **Hepatic expression of cytokine mRNAs (mean ± S.E.) in** ***Mttp*** $^{\Delta/\Delta}$ **(*n* = 14) and littermate *Mttp*** $^{flox/flox}$ **(*n* = 14) mice following LPS treatment.** Measurements were performed 4 h after an intraperitoneal injection of LPS (1.0 μg/g). Values represent steady-state mRNA expression by RNase protection assays, in relative absorbance units normalized to a control RNA signal (L32 or glyceraldehyde-3-phosphate dehydrogenase). *A*, mRNA levels of TNF-α. *B*, mRNA levels of IL-2 and IL-10. *C*, mRNA levels of three chemokines (MIP-1α, MIP-1β, MIP-2). *p* values were calculated with two-tailed, unpaired *t* tests.

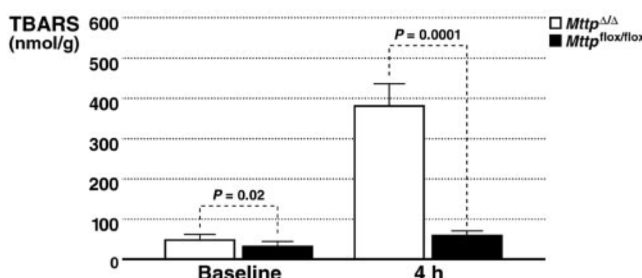

FIG. 9. **TBARS in the livers of *Mttp*** $^{\Delta/\Delta}$ **mice and littermate *Mttp*** $^{flox/flox}$ **mice.** Measurements were performed at base line and 4 h after an intraperitoneal injection of LPS (1.0 μg/g). TBARS are expressed as nanomoles of malondialdehyde/g wet liver weight. Base-line measurements, $n = 4$ mice in each group; 4-h measurements, $n = 10$ mice in each group. *p* values were calculated with two-tailed, unpaired *t* tests.

Mttp$^{\Delta/\Delta}$ mice to absorb dietary lipids and package them into chylomicrons prevented significant nutritional deficiencies and ill health. Given the vitality of *Mttp*$^{\Delta/\Delta}$ mice and the modest amount of hepatic lipid accumulation, one might have reasonably inferred that the blockade in hepatic lipoprotein secretion is entirely benign. The current study might be interpreted as raising doubts about this conclusion. Despite their vitality, the *Mttp*$^{\Delta/\Delta}$ mice had higher levels of transaminases than control mice following the administration of liver toxins.

The increased sensitivity of *Mttp*$^{\Delta/\Delta}$ mice to three toxins, *E. coli* LPS, ConA, and PEA, emphasizes the need for caution in the development of MTP inhibitors to treat hyperlipidemias in human beings. Specifically, it will be important to determine whether MTP inhibitor drugs render humans more susceptible to steatohepatitis and cirrhosis. At the same time, however, we believe that it is important to underscore several caveats regarding our experiments. First, our studies were conducted in *Mttp*$^{\Delta/\Delta}$ mice, and the precise relevance of that animal model to humans is not yet established. *Mttp*$^{\Delta/\Delta}$ mice lack MTP activity only in the liver. An MTP inhibitor drug would block MTP in both liver and intestine, and inhibiting intestinal lipoprotein production could actually limit the amount of lipid accumulation in the liver. Second, both hepatic MTP activity levels and apoB100 secretion rates were blocked by 95% in *Mttp*$^{\Delta/\Delta}$ mice (6). The blockade would almost certainly be less profound in humans treated with MTP inhibitor drugs, because the drugs have been shown to partially block lipoprotein secretion at doses that only partially block MTP activity (3). A more modest level of MTP inhibition might be associated with less lipid accumulation in the liver and, correspondingly, less toxicity.

One might argue that the increased susceptibility of the *Mttp*$^{\Delta/\Delta}$ mice to hepatic injury was due, at least in part, to their low plasma levels of triglyceride-rich lipoproteins. Triglyceride-rich lipoproteins bind LPS and direct it away from macrophages in the liver (*i.e.* Kupffer cells). This bypass of the Kupffer cell population reduces LPS-mediated TNF-α release (43–45) and limits LPS-mediated organ injury. Interestingly, low plasma lipoprotein levels have been reported to enhance LPS hepatotoxicity (46). The low levels of triglyceride-rich lipoproteins in *Mttp*$^{\Delta/\Delta}$ mice did not, however, appear to be the cause of their heightened sensitivity to LPS. In our experiments, LPS-induced increases in TNF-α and hepatic TNF-α transcripts were no different in *Mttp*$^{\Delta/\Delta}$ mice and controls. Those findings suggest that the plasma lipoprotein levels did not significantly affect the access of Kupffer cells to the toxin. Furthermore, *Mttp*$^{\Delta/\Delta}$ mice also exhibited exaggerated toxicity in response to ConA and PEA, neither of which bind lipoproteins.

Our findings with *Mttp*$^{\Delta/\Delta}$ mice are reminiscent of the increased sensitivity to LPS in obese mice and rats. For example, Yang *et al.* (47) demonstrated that *ob*/*ob* mice and Zucker diabetic fatty rats (both of which have increased liver lipid stores) are more susceptible than nonobese controls to the development of steatohepatitis after an LPS challenge. These rodent models of obesity are clearly different from the *Mttp*$^{\Delta/\Delta}$ mice in that they do not involve a blockade in VLDL secretion. They are also far more complex from a metabolic perspective. MTP deficiency in the liver simply prevents the assembly and secretion of VLDL, whereas a deficiency in leptin (as in the *ob*/*ob* mice) results in substantial changes in caloric intake, induces frank diabetes mellitus, and even changes the function of the immune system (48–51). Diabetes mellitus also produces complex metabolic changes in the liver as well as in multiple other tissues (52). Nevertheless, leptin deficiency, obesity, diabetes, and *Mttp* deficiency all share a common feature, hepatic steatosis. The current studies are important because they show that increased hepatic lipid stores from a blockade in lipoprotein secretion heighten the risk for toxin-mediated hepatic injury, and do so without the many metabolic derangements associated with leptin deficiency, obesity, and diabetes.

Although *Mttp*$^{\Delta/\Delta}$ and *ob*/*ob* mice both displayed exaggerated sensitivity to LPS compared with their respective controls, their responses to ConA and PEA were quite different. Faggioni *et al.* (53) reported that *ob*/*ob* mice are resistant to ConA- and PEA-mediated hepatotoxicity, whereas in the current study *Mttp*$^{\Delta/\Delta}$ mice were more sensitive to toxicity from these compounds. The toxicities of ConA and PEA are thought to be mediated by T cells (34, 35, 53). Faggioni *et al.* (53) speculated that the resistance of the *ob*/*ob* mice to hepatic injury from those agents might be related to the deficiency in T-cell-mediated immunity associated with leptin deficiency (50, 53). A

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deficiency in MTP, which is expressed largely in hepatocytes and intestinal enterocytes, would not be expected to cause immunodeficiency, and thus it is logical that that $Mttp^{\Delta/\Delta}$ mice would exhibit similar sensitivities to the three different toxic challenges.

The concept that hepatic steatosis can heighten susceptibility to the development of inflammation and more advanced liver disease is supported by more than data from experimental animals. Humans with diabetes mellitus, obesity, and chronic exposure to ethanol have increased hepatic lipid stores and are at increased risk for developing steatohepatitis and cirrhosis (8, 54, 55). Bacterial products *(e.g.* LPS or exotoxins) have been implicated as important cofactors in the pathogenesis of inflammation arising in fatty livers (8, 14). These compounds can cause liver injury not only by inducing cytokines such as TNF-α but also by causing release of reactive oxygen species from Kupffer cells (39).

The toxicity of LPS is mediated in part through the induction of TNF-α (33) and in part by stimulating macrophage production of reactive oxygen species (56). TNF-α also induces the production of reactive oxygen species within cells (57–59), which in turn can cause cellular injury. Studies have shown that in the setting of fatty liver, the severity of oxidative injury depends upon the degree of unsaturation of cellular lipids (60, 61). High levels of unsaturation amplify oxidative insults, leading to enhanced lipid peroxidation and downstream consequences such as chemokine production (62–64). In our experiments, the substantial LPS-induced rise in TBARS that occurred in $Mttp^{\Delta/\Delta}$ mice relative to control mice is likely due to their disproportionate stores of linoleic acid, an essential polyunsaturated fatty acid. Despite comparable induction of TNF-α in both groups of mice, the $Mttp^{\Delta/\Delta}$ mice, with their increased hepatic stores of linoleic acid, displayed more severe tissue damage. This manifested not only in a significant increase in lipid peroxidation but also in enhanced induction of inflammatory mediators (*e.g.* several chemokines).

We are at a loss to explain the enrichment of liver triglycerides in $Mttp^{\Delta/\Delta}$ mice with linoleic acid, particularly because there was no change in dietary lipids and no difference between $Mttp^{\Delta/\Delta}$ and control mice in the composition of fatty acids in the plasma. Perhaps the inability to secrete VLDL changes the spectrum of fatty acids that undergo β-oxidation, causing linoleic acid to accumulate. Alternatively, one could speculate that VLDL serves a special role in exporting essential polyunsaturated amino fatty acids to peripheral tissues. If so, a blockade in VLDL production might cause polyunsaturated fatty acids to accumulate. Whatever the mechanism, the enrichment in linoleic acid could render the liver more susceptible to oxidant damage (60, 61). Also, as noted under "Results," the accumulation of linoleic acid might explain, at least in part, the reduced levels of SREBP-1 (31, 32) and *Scd1* (30).

It would be interesting to determine whether the increased sensitivity of $Mttp^{\Delta/\Delta}$ mice to liver injury would be mitigated by additional manipulations that limit lipid accumulation. One potential approach was suggested in a recent paper by Matsuda and co-workers (65). They produced a conditional allele for SREBP cleavage-activating protein and then used the inducible Mx1-*Cre* transgene to produce mice lacking that protein in the liver. On a chow diet, those mice manifested reduced expression of genes driven by SREBP-1 and SREBP-2, an 80% reduction in hepatic lipid biosynthesis, and a 65% reduction in liver triglyceride stores (65). It would be interesting to determine whether a deficiency in hepatic SREBP cleavage-activating protein would completely block the hepatic lipid accumulation in $Mttp^{\Delta/\Delta}$ mice, and if so, whether those mice would be protected from hepatic injury in response to exogenous toxins.

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