Hepatic Expression of Microsomal Triglyceride Transfer Protein and In Vivo Secretion of Triglyceride-Rich Lipoproteins Are Increased in Obese Diabetic Mice

Emil D. Bartels, Morten Lauritsen, and Lars B. Nielsen

Secondary hyperlipidemia is a major cardiovascular risk factor in individuals with type 2 diabetes. Increased hepatic production of apolipoprotein B (apoB)-containing lipoproteins contributes to the elevated plasma levels, but the mechanism is poorly understood. Recent results have established that microsomal triglyceride transfer protein (MTP) is rate limiting for the assembly and secretion of apoB-containing lipoproteins. To better understand the mechanism of type 2 diabetes–associated hyperlipidemia, we quantified hepatic MTP mRNA levels, hepatic microsomal triglyceride transfer activity, and in vivo triglyceride secretion from the liver in two diabetic mouse models. Obese diabetic (*ob/ob*) mice had 45% higher ($P = 0.006$) hepatic MTP mRNA levels, 54% higher ($P < 0.0001$) microsomal triglyceride transfer activity, and 70% higher ($P < 0.0001$) in vivo triglyceride secretion rates compared with ob/+ control mice. In contrast, in lean streptozotocin-treated diabetic mice, hepatic MTP mRNA levels were unchanged, whereas microsomal triglyceride transfer activity and in vivo triglyceride secretion rates were marginally decreased. These studies suggest that obesity-induced type 2 diabetes in mice confers increases in hepatic MTP expression and secretion of triglyceride-rich lipoproteins. High blood glucose and altered hepatic expression of sterol regulatory element binding protein genes play a minor role in this diabetic response. *Diabetes* 51: 1233–1239, 2002

The incidence and prevalence of type 2 diabetes are rapidly rising (1). Individuals with type 2 diabetes have an increased risk of cardiovascular disease, which is closely associated with elevation of the plasma levels of apolipoprotein B (apoB)-containing VLDL and LDL (2,3). Metabolic labeling studies have indicated that the increased plasma levels of VLDL and LDL in individuals with diabetes are caused, at least in part, by increased hepatic secretion of apoB-containing lipoproteins (4,5). Hence, a better understanding of the factors in patients with type 2 diabetes that mediate increased lipoprotein secretion from the liver could potentially lead to rational therapeutic strategies to prevent cardiovascular disease in this patient group.

During the past 5 years, it has become evident that microsomal triglyceride transfer protein (MTP) is rate limiting for the production of apoB-containing VLDL (6–10). MTP is an exclusively intracellular protein (6). Its principal role is to transfer lipids onto the apoB polypeptide in the endoplasmic reticulum of lipoprotein-secreting cells (6). Reduction of the MTP activity in animals by MTP inhibitor drugs (7) or genetic manipulations (11,12) lowers plasma lipoprotein levels, whereas adenoviral overexpression of an MTP cDNA in mouse liver in vivo increases hepatic secretion of triglyceride-rich apoB–containing lipoproteins (13,14).

Studies of cultured liver cells suggest that insulin and glucose reduce the expression of the MTP gene (15); the MTP gene promoter region contains a putative insulin-responsive element (16). Also, reporter gene studies in HepG2 cells suggest that MTP gene expression is negatively regulated by the sterol regulatory element binding protein (SREBP) family of transcription factors (17). Insulin increases SREBP-1 mRNA levels as well as levels of the active transcription factors (18,19). Accordingly, hepatic SREBP-1 mRNA and protein levels are increased in the liver of a mouse with type 2 diabetes (20). These previous observations all would support the prediction that type 2 diabetes is associated with decreased MTP gene expression in the liver. This seems to contrast with the findings of increased hepatic lipoprotein secretion in individuals with type 2 diabetes who are both hyperinsulinemic and hyperglycemic (21–23). These observations could also reflect that other factors, e.g., lipid availability in hepatocytes, are more important than MTP levels in determining lipoprotein secretion rates from the liver of individuals with diabetes or that MTP gene expression might be differently regulated in vivo than in cultured liver cells. A recent study indicated that diabetic hamsters have increased hepatic MTP protein levels (24). Because MTP activity, protein, and mRNA levels are closely associated (12,14,25,26), these results could suggest that MTP gene expression is actually increased in type 2 diabetes in vivo. However, brown adipose tissue–deficient mice that are hyperinsulinemic and obese display increased secretion of triglyc-

From the Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark.

Address correspondence and reprint requests to Lars B. Nielsen, MD, DMSc, Department of Clinical Biochemistry KB 3011, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, 2100 Copenhagen, Denmark. E-mail: larsbo@rh.dk.

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apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; SREBP, sterol regulatory element binding protein; STZ, streptozotocin; TLC, thin-layer chromatography.

eride-rich lipoproteins from the liver without any change in hepatic MTP mRNA expression (27).

To improve the understanding of increased lipoprotein secretion from the liver in type 2 diabetes, we examined hepatic MTP and apoB mRNA levels, microsomal triglyceride transfer activity, and in vivo triglyceride secretion rates in obese (*ob/ob*) diabetic mice and in lean streptozotocin (STZ)-treated diabetic mice. In comparison with control mice, hepatic MTP gene expression, microsomal triglyceride transfer activity, and in vivo triglyceride secretion all were increased in the obese diabetic mice but not in the lean diabetic mice. A high blood glucose concentration or altered expression of SREBP genes seems to play a minor role in this diabetic response.

RESEARCH DESIGN AND METHODS

Animals and diets. Fifty-four male and 34 female C57BL/6 mice from 5 to 7 weeks of age were obtained from M&B (Ry, Denmark) and were fed a standard laboratory diet (Altromin no. 1314; Altromin, Rugarden, Denmark). The mice were housed at the Panum Institute, University of Copenhagen, in a temperature-controlled (21–23°C) facility with a 12-h light/dark cycle. Female leptin-deficient Lep^{ob}/Lep^{ob} (*ob/ob*) mice (n = 20) and heterozygote $Lep^{ob}/+$ (ob/+) controls (n = 20) of the Umea strain were obtained from M&B, maintained under similar conditions at the Panum Institute or at Novo Nordisk A/S (Bagsvaerd, Denmark), and killed at 10 weeks of age. The Danish Government body that supervises animal experiments (Dyreforsoegstilsynet) approved the study protocols.

Streptozotocin induction of diabetes. To induce diabetes in C57BL/6 mice, we injected STZ (40 μg/g per injection; Sigma, Vallensbaek Strand, Denmark) intraperitoneally seven times (once a day for 4 or 5 consecutive days and once a day for an additional 2–3 consecutive days after 2–3 days of recovery). STZ was dissolved in citrate buffer (0.05 mol/l [pH 4.5], 4°C) to a final concentration of 4 μg/μl. The STZ solution was kept on ice and used within 20 min of preparation. Control mice received the citrate-buffer vehicle (200 μl/mouse per day). To maintain hyperglycemia in the 9-week STZ treatment group, STZ was injected for an additional 5 days (40 μg/g per injection) during week 7. Mice that were used to study hepatic gene expression and lipid content were killed by cervical dislocation after 3 (n = 20), 6 (n = 20), or 9 weeks (n = 28). Equal numbers of males and females and STZ-treated and vehicle-treated control mice were studied at each time point. Liver biopsies were placed in liquid nitrogen immediately upon removal and stored at −141°C. Because differences in gene expression, plasma, and tissue lipid levels between STZ-treated mice and controls were similar at 3, 6, and 9 weeks, data from the three time points were pooled and are presented together. Hepatic triglyceride transfer activity and in vivo triglyceride secretion were studied in male mice 3 weeks after STZ induction of diabetes.

Hepatic triglyceride transfer activity. All procedures were performed on ice. Microsomal proteins were prepared by homogenization of ~100-mg liver samples in 1 ml buffer (50 mmol/l Tris-HCl, 50 mmol/l KCl, 5 mmol/l EDTA, and freshly added Protease Inhibitor [Roche A/S]) with a PT 1200 Polytron. The protein concentration in homogenates was determined with the BCA-protein assay (Pierce, Copenhagen, Denmark) using BSA as a standard. After the protein concentration was adjusted to 1.75 mg/ml, liver homogenates were subjected to ultracentrifugation in a Beckman Optima LE-80K ultracentrifuge with the 50.4 Ti rotor head at 100,000*g* for 60 min. The supernatant containing the microsomal fraction was added to 1/10 volume of 0.54% sodiumdeoxycholate (pH 7.5) and incubated on ice for 30 min followed by overnight dialysis at 4°C against buffer (15 mmol/l Tris [pH 7.4], 40 mmol/l NaCl, 10 mmol/l EDTA, and 0.02% NaN_3). Triglyceride transfer activity in the microsomal protein fraction was measured as transfer of ^{14}C-triglycerides from labeled donor vesicles to acceptor vesicle that contained only unlabeled triglycerides, exactly as described by Wetterau et al. (28,29). Initial evaluations of the assay showed that triglyceride transfer activity in normal mouse liver microsomal extracts increased linearly with an increasing amount of protein up to ~25 μg protein. Consequently, we chose to analyze triglyceride transfer activity in microsomal extracts corresponding to 10 μg total protein. In protein purification and triglyceride transfer procedures, samples from *ob/ob* and ob/+-control mice were alternated and analyzed together. Samples from STZ- and vehicle-treated mice were likewise analyzed together by alternating samples from the two groups of mice.

In vivo triglyceride secretion. Ob/ob, ob/+, STZ-treated, and vehicle-treated mice (n = 10 in each group) were fasted from 10:00 P.M. The next day, the mice were anesthetized with hypnorm:diazepam:saline (1:1:2, 6 ml/kg s.c.), and Triton WR-1339 (500 mg/kg in 200 μl sterile 0.9% NaCl; Sigma) was injected trough a tail vein; intravenous injection was successful in nine mice from each group. Blood samples (~75 μl) were taken 1, 30, 60, and 120 min after injection from the retro-orbital plexus. Triglyceride secretion rate (in μmol triglycerides per hour per gram body weight) was calculated as the increase in plasma triglyceride concentration per hour multiplied by the plasma volume (i.e., 3.5% of the body weight) and divided by the body weight. Because triglyceride secretion rates were similar in the ob/+ and vehicle-treated control mice (0.068 ± 0.005 vs. 0.065 ± 0.007 $\mu mol \cdot h^{-1} \cdot g^{-1}$), data for control mice are presented together.

Glucose, insulin, and plasma lipid analyses. The glucose concentration in tail blood was determined with the HUMACHECK^{plus} glucose meter (HUMAN, Wiesbaden, Germany). Blood samples for plasma insulin and lipid analyses were drawn into tubes that contained Na_2EDTA. Plasma aliquots were stored at −80°C until analyses. Plasma insulin was determined with enzyme-linked immunosorbent assay by Dr. B. Rolin (Novo Nordisk A/S) (30). Enzymatic kits were used to determine plasma concentrations of free fatty acids (WAKO NEFA C kit; TriChem Aps, Frederikssund, Denmark), triglycerides (GPO-TRINDER; Sigma), total cholesterol (CHOD-PAP; Roche, Mannheim, Germany), and HDL cholesterol (Roche). HDL cholesterol was measured after precipitation of apoB-containing lipoprotein with phosphotungstic acid and $MgCl_2$.

Tissue lipid analysis. Liver biopsies (~150 mg) were minced with scissors in 1 ml methanol before adding 1.5 ml methanol and 5 ml chloroform. After thorough mixing, 2.5 ml methanol was added and the mixture was vortexed and centrifuged for 15 min at 4°C at 2,000*g*. The supernatant containing the lipid extract was transferred to a new vial. The remaining tissue extract was washed in 1 + 1 ml chloroform-methanol and centrifuged. The supernatant of the wash was transferred to the vial with the lipid extract, and the wash was repeated. After evaporation of solvents under N_2, the lipids were redissolved in toluol (1 μl/mg wet weight of tissue) and quantified by thin-layer chromatography (TLC) (31). Briefly, TLC plates (DC-Fertigplatten SIL G-25, Macherey-Nagel, 20 × 20 cm) were impregnated with Na_2EDTA (1 mmol/l, pH 5.5) by ascending development, dried, and washed in chloroform:methanol:water (60:40:10). After activation of the TLC plates at 110°C for 30 min, 1-μl tissue extracts and a dilution series of a standard were applied. The standard contained triglyceride, free cholesterol, cholesterol esters, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin (all from Sigma). The plates were developed in a six-step procedure using chloroform:methanol:water (60:40:10), chloroform:methanol:water (65:40:5), chloroform:methanol:ethylacetat:2-propanol:ethanol:0.25% KCl (22:15:35:5:20:9), ethanol:toluol:diethylether (3:60:40), diethylether:n-heptan (8:94), and pure n-heptan. After development, the plates were placed in 10% cupric sulfate (wt/vol) in 8% phosphoric acid (vol/vol) for 10 s, dried with a hair dryer, and baked for 2 min at 200°C. For quantification, the TLC plates were scanned at 300 × 300 dpi with an HP flatbed 4c/T scanner (Hewlett Packard, Copenhagen, Denmark). The digitalized images were analyzed with the MULTI-ANALYST software (Bio-Rad, Copenhagen, Denmark). All samples were analyzed at least twice on different TLC plates. The interassay coefficients of variation of lipid determination were 11% for triglyceride and ~14% for the other lipid fractions (n = 205–228 duplicate analyses).

RNA purification. Frozen liver samples (20–100 mg) were homogenized with a Polytron PT1200CL (Buch & Holm, Herlev, Denmark) in TRIzol reagent (Life Technologies, Taastrup, Denmark). Total RNA was extracted according to the manufacturer's manual and suspended in RNase-free H_2O. The RNA concentration was calculated from the absorbance at 260 nm (A_{260}). The integrity of the RNA was always ensured by electrophoresis on a 1% agarose gel.

Synthesis and amplification of cDNA. First-strand cDNA was synthesized at 37°C for 60 min: 1 μg RNA, M-MULV reverse transcriptase (40 units; Roche A/S, Copenhagen, Denmark), and random hexamer primers (0.02 A_{260}) were mixed in 10-μl reactions. The primers used for PCR were as follows: apoB, 5′-ATGGGAAGAAACAGGCTTGA-3′ and 5′-TTCTGTCCCACGAATTGACA-3′; MTP, 5′-TGAGCGGCTATACAAGCTCAC-3′ and 5′-CTGGAAGATGCT-CTTCTCGC-3′ (12); β-actin, 5′-ACTGGGACGACATGGAGAAG-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′; SREBP-1a, 5′-GCCGGCGCCATGGACGAG-3′ and 5′-ATGTCGTTCAAAACCGCTGTGTC-3′; SREBP-1c, 5′-CGCGGAAGCTGT-CGGGGTAG-3′ and 5′-AAATGTGCAATCCATGGCTCCGTGGTC-3′; and SREBP-2, 5′-CACAATATCATTGAAAAGCGCTACCGGTCC-3′ and 5′-TTTTTCTGATTGG-CCAGCTTCAGCACCATG-3′ (32) (all from SIGMA-GENOSYS, Pampisford, U.K.).

The expected length of each PCR product was confirmed by electrophoresis on 2% agarose gels. Primer pairs for apoB, MTP, β-actin, glyceraldehyde-3-phosphate dehydrogenase, and SREBP-2 spanned introns. We did not observe amplification of genomic DNA in any of the cDNA preparations. The specificity of each RT-PCR reaction was also ensured by DNA sequencing the

TABLE 1
Basic metabolic variables in *ob/ob*, STZ-treated, and control mice

	ob/ob	ob/+ controls	STZ-treated (blood glucose ≥13.1 mmol/l)	STZ-treated (blood glucose <13.1 mmol/l)	Vehicle-treated controls
Number of mice	10	10	17	17	34
Body weight (g)	43.3 ± 1.4‡	21.0 ± 0.4	24.4 ± 1.0	22.8 ± 0.9†	26.7 ± 0.8
Fasting blood glucose (mmol/l)	15.1 ± 0.9‡	8.1 ± 0.3	17.2 ± 0.7‡	8.5 ± 0.4‡	6.6 ± 0.1
Plasma insulin (pmol/l)§	2400 ± 208†	121 ± 15	69 ± 5.5†		106 ± 10.6
Plasma total cholesterol (mmol/l)	3.83 ± 0.19‡	1.66 ± 0.09	2.46 ± 0.10†	2.17 ± 0.06	2.11 ± 0.08
Plasma HDL cholesterol (mmol/l)	1.61 ± 0.08*	1.39 ± 0.06	1.88 ± 0.11	1.82 ± 0.07	1.66 ± 0.06
Plasma VLDL+LDL cholesterol (mmol/l)	2.23 ± 0.16‡	0.28 ± 0.13	0.59 ± 0.09	0.35 ± 0.06	0.48 ± 0.07
Plasma triglycerides (mmol/l)	1.05 ± 0.19	0.94 ± 0.05	1.42 ± 0.11‡	0.82 ± 0.07	0.81 ± 0.04
Plasma free fatty acids (mmol/l)	0.27 ± 0.02	0.23 ± 0.02	0.30 ± 0.02	0.29 ± 0.02	0.30 ± 0.01

Data are means ± SD. Blood samples for glucose and plasma lipid analysis were taken at the end of the experiment after fasting the mice for 4 h. Values are mean ± SE. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.0005$ compared with controls. §Determined on pooled plasma aliquots from two to four mice.

RT-PCR products in both directions with the ABI-Prism 310 sequencer and terminator ready reaction mix (PE Applied Biosystems, Naerum, Denmark). In some cases, the PCR products were cloned into a plasmid before sequencing; in other cases, PCR products were sequenced directly after purification on JET quick-spin columns (Genomed, Bad Oeynhausen, Germany).

mRNA quantification with real-time PCR. We used the LightCycler instrument (software version 3.39) and DNAmaster SYBR GREEN kit (Roche A/S, Hvidovre, Denmark) to perform quantitative real-time PCR analysis of hepatic cDNA preparations. The PCR reactions (20 μl) contained 2 μl SYBR GREEN I mix, 2–3 mmol/l $MgCl_2$, 10 pmol of each primer, cDNA synthesized from 20 ng total RNA, and PCR-grade H_2O. The melting point of the PCR products, the optimal $MgCl_2$ concentration, and annealing temperature of each PCR were carefully established. The amount of a specific mRNA transcript in each liver sample was determined from the time point of the log-linear increase in amplified DNA during the PCR. The fit-point option of the LightCycler software was always used in the quantifications. The relation between the time point of the log-linear increase in amplified DNA and the relative concentration of an mRNA transcript were determined in each run by analyzing dilution series (liver cDNA synthesized from 100 ng, 20 ng, 2 ng, and 0.2 ng total RNA) of a liver cDNA pool. This reference cDNA pool was made by mixing liver cDNA from three normal C57BL/6 male mice. We were initially concerned that the effectiveness of the PCR could be lower in standards with the highest amount of cDNA. Such an effect could potentially introduce an error in the mRNA quantifications. Therefore, in control experiments, we generated dilution series of mouse liver cDNA in which yeast cDNA had been included to obtain the same amount of cDNA in each sample (i.e., cDNA synthesized from 200 ng total RNA). However, the addition of yeast cDNA did not change the effectiveness of three different PCRs (data not shown). Consequently, all standard curves were generated by diluting the cDNA pool with H_2O. The real-time PCR assays were sufficiently sensitive to quantify MTP, apoB, and β-actin mRNA in <20 pg total liver RNA and SREBP-1a, -1c, and -2 mRNA in ~200 pg total liver RNA. To assess the imprecision of the mRNA quantifications, we quantified MTP, apoB, and β-actin mRNAs on five different occasions using the same liver cDNA preparation made from 20 ng total RNA. The interassay coefficients of variation (i.e., SDs divided by the mean) were 5.0%, 3.5%, and 4.8% for MTP, apoB, and β-actin mRNA, respectively. In each sample, mRNA levels were normalized by the β-actin mRNA content.

Statistics. Differences between groups were analyzed with Student's *t* test with correction for different variance whenever appropriate. The analyses were done with the GraphPad Prism software (San Diego, CA).

RESULTS

ob/ob mice were hyperglycemic and had markedly elevated plasma insulin levels compared with ob/+ control mice (Table 1). The total plasma cholesterol concentration was 2.3-fold higher in the *ob/ob* mice than in the ob/+ control mice. This difference almost exclusively reflected an increased concentration of VLDL+LDL cholesterol (Table 1). The hepatic triglyceride content was increased ~13-fold in the *ob/ob* mice (Table 2). This triglyceride accumulation was accompanied by an ~twofold increase in cholesterol ester and a relative reduction in other lipid fractions (Table 2).

The hepatic MTP mRNA level was increased by 45% in the *ob/ob* mice compared with the ob/+ control mice ($P = 0.006$), whereas hepatic apoB mRNA levels were similar in the two groups (Fig. 1*A*). Analysis of triglyceride transfer activity in microsomal liver proteins revealed a 54% ($P < 0.0001$) increased activity in *ob/ob* mice compared with ob/+ control mice (Fig. 2). Furthermore, the in vivo

TABLE 2
Hepatic lipid content in *ob/ob*, ob/+, STZ-treated, and vehicle-treated control mice

	ob/ob	ob/+§	STZ-treated (blood glucose ≥13.1 mmol/l)	STZ-treated (blood glucose <13.1 mmol/l)	Controls
Number of mice	10	10	12	12	24
Triglycerides (nmol/mg wet wt)	136.37 ± 1.16‡	10.61 ± 1.08	7.21 ± 0.49‡	8.73 ± 0.61‡	16.01 ± 1.00
Free cholesterol (nmol/mg wet wt)	2.81 ± 0.18‡	4.49 ± 0.24	3.36 ± 0.11*	3.40 ± 0.17*	3.82 ± 0.11
Cholesterol esters (nmol/mg wet wt)	3.06 ± 0.28†	1.42 ± 0.30	0.99 ± 0.12†	1.55 ± 0.22	1.72 ± 16
Sphingomyelin (μg/mg wet wt)	0.32 ± 0.01*	0.57 ± 0.07	0.55 ± 0.02	0.53 ± 0.05	0.55 ± 0.03
Phosphatidylcholine (μg/mg wet wt)	15.68 ± 0.41*	17.93 ± 0.52	19.17 ± 0.76	18.72 ± 0.94	19.69 ± 0.70
Phosphatidylserine (μg/mg wet wt)	0.99 ± 0.05*	1.53 ± 0.22	1.59 ± 0.06	1.62 ± 0.08	1.77 ± 0.06
Phosphatidylinositol (μg/mg wet wt)	2.82 ± 0.08	3.39 ± 0.29	3.42 ± 0.12	3.41 ± 0.20	3.71 ± 0.13
Phosphatidylethanolamine (μg/mg wet wt)	5.64 ± 0.24‡	7.87 ± 0.33	11.15 ± 0.60	10.68 ± 0.66*	12.56 ± 0.53
Cardiolipin (μg/mg wet wt)	1.62 ± 0.12†	2.27 ± 0.09	1.98 ± 0.08	1.97 ± 0.10	1.98 ± 0.07

Data are means ± SE. *$P < 0.05$, †$P < 0.005$, ‡$P < 0.0005$ compared with controls. §Controls for *ob/ob* mice; vehicle-treated controls for STZ-treated mice.

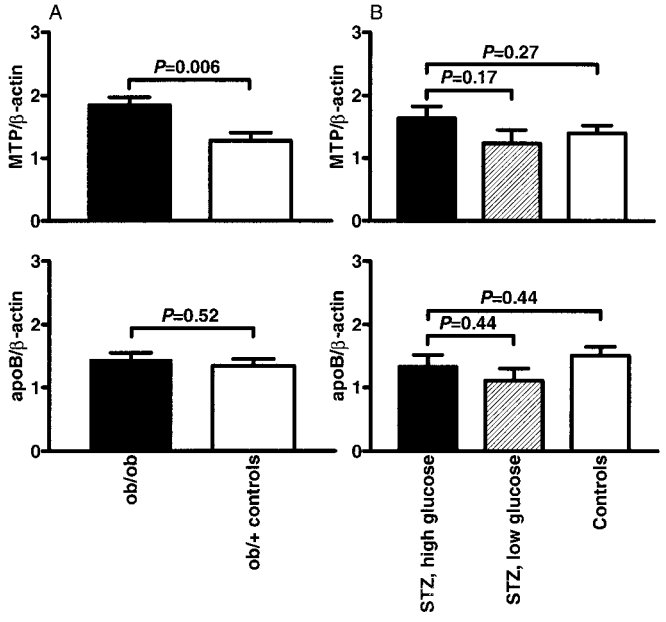

FIG. 1. MTP (top panels) and apoB (bottom panels) mRNA levels in the liver of diabetic *ob/ob* (*A*) and STZ-treated mice (*B*). The MTP and apoB mRNA levels were determined with real-time PCR using cDNA made from 20 ng total liver RNA as template. MTP and apoB mRNA levels were normalized by the β-actin mRNA level in each sample. Similar results were obtained without this normalization (data not shown). *A*: Closed bars represent *ob/ob* mice, $n = 10$. Open bars represent control ob/+ mice ($n = 10$). *B*: Closed bars represent STZ-treated mice with fasting blood glucose ≥13.1 mmol/l M ($n = 17$). Hatched bars represent STZ-treated mice with fasting blood glucose <13.1 mmol/l M ($n = 17$). Open bars represent vehicle-treated control mice ($n = 34$). *P* values for two-group comparisons are indicated above brackets. Values are mean ± SE.

triglyceride secretion rate from the liver was increased 70% in *ob/ob* mice compared with controls (0.113 ± 0.008 vs. 0.066 ± 0.004 μmol/h per g body wt; $P < 0.0001$; Fig. 3). Because plasma triglyceride levels were not increased in *ob/ob* mice (Table 1 and Fig. 3), these data suggest that the

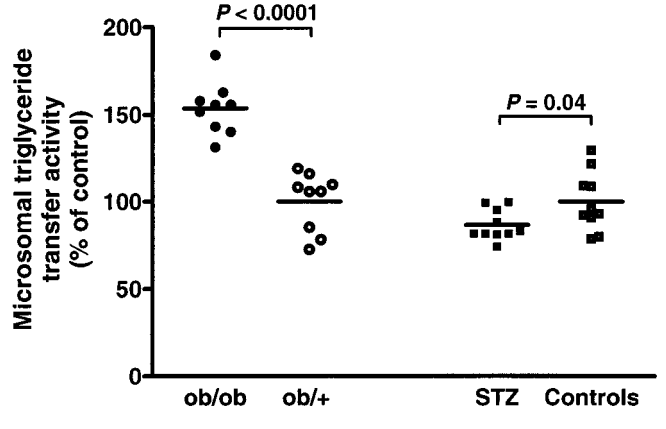

FIG. 2. Hepatic microsomal triglyceride transfer activity in diabetic *ob/ob* and STZ-treated mice. Microsomal proteins were isolated from mouse livers and used for determination of triglyceride transfer activity as described in RESEARCH DESIGN AND METHODS. Each data point represents the value of an individual mouse. The data are derived from double (*ob/ob* and ob/+ mice) or triple (STZ- and vehicle-treated mice) determinations of triglyceride transfer activity. The indicated differences between diabetic and control groups were confirmed in each assay. Because the level of triglyceride transfer activity could vary between experiments, the data are expressed as percentage of mean value in the respective control groups. *P* values for two-group comparisons are indicated above brackets. Mean values are indicated as lines.

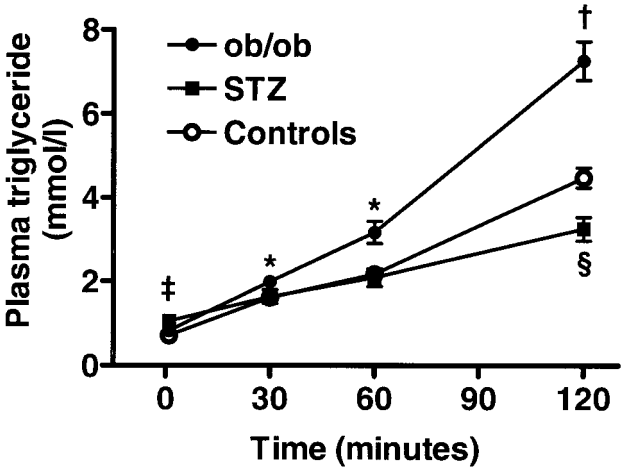

FIG. 3. In vivo triglyceride secretion in diabetic *ob/ob* and STZ-treated mice. Fasted *ob/ob*, ob/+, STZ-treated, and vehicle-treated control mice ($n = 9$ in each group) received an intravenous injection of triton WR-1339 (500 mg/kg). The plasma triglyceride concentration was determined after 1, 30, 60, and 120 min. Plasma triglyceride concentration versus time curves for ob/+ and vehicle-treated control mice were superimposable. *$P < 0.01$, †$P < 0.0001$ for *ob/ob* mice versus control mice; ‡$P < 0.05$, §$P < 0.005$ for STZ-treated mice versus control mice. Values are mean ± SE.

clearance of triglycerides from the blood is increased in *ob/ob* mice.

To explore further the putative effects of diabetes on hepatic MTP expression, we induced diabetes in lean C57BL/6 mice by repeated injections of STZ. The plasma insulin level was lower in the STZ-treated mice than in the vehicle-treated control mice (Table 1). However, the diabetic response varied between STZ-treated mice. Therefore, the STZ-treated mice were arbitrarily divided at the median blood glucose concentration into mice with fasting blood glucose ≥13.1 mmol/l and mice with fasting blood glucose <13.1 mmol/l. STZ-treated mice with blood glucose ≥13.1 mmol/l had 75% higher plasma triglycerides ($P < 0.0005$) and 17% higher total plasma cholesterol ($P < 0.01$) levels compared with vehicle-treated control mice (Table 1). Plasma lipid levels were similar in STZ-treated mice with blood glucose <13.1 mmol/l and vehicle-treated control mice. Consistent with previous studies of STZ-treated C57BL/6 mice that were fed a standard diet (33), the concentration of hepatic triglycerides and cholesterol esters were lower in both groups of STZ-treated mice when compared with the vehicle-treated control mice (Table 2).

There was no difference in hepatic MTP and apoB mRNA levels between STZ-treated diabetic and vehicle-treated control mice, irrespective of the blood glucose concentration (Fig. 1*B*). To assess hepatic microsomal triglyceride transfer activity and in vivo triglyceride secretion rates in STZ-treated mice, we studied another group of STZ-treated or vehicle-treated male mice 3 weeks after induction of diabetes. The mean blood glucose concentration was 22.0 ± 1.0 mmol/l in STZ-treated mice and 8.0 ± 0.2 mmol/l in vehicle-treated control mice. Hepatic microsomal triglyceride transfer activity was 14% lower ($P = 0.04$) and in vivo triglyceride secretion rates were 42% lower (0.039 ± 0.003 vs. 0.066 ± 0.004 $\mu mol \cdot h^{-1} \cdot g^{-1}$; $P = 0.0002$) in STZ-treated diabetic mice compared with control mice (Figs. 2 and 3). The small difference in MTP

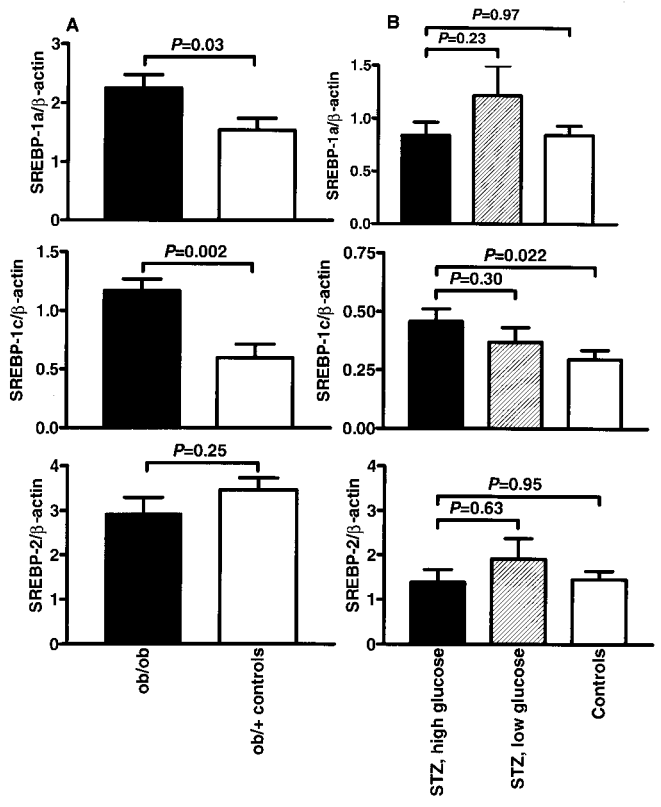

FIG. 4. SREBP-1a (top panels), SREBP-1c (middle panels), and SREBP-2 (bottom panels) mRNA levels in the liver of diabetic *ob/ob* (*A*) and STZ-treated mice (*B*). The SREBP-1a, -1c, and -2 mRNA levels were determined with real-time PCR using cDNA made from 20 ng total liver RNA as template. SREBP mRNA levels were normalized by the β-actin mRNA level in each sample. Similar results were obtained without this normalization (data not shown). *A*: Closed bars represent *ob/ob* mice (n = 10). Open bars represent control ob/+ mice (n = 10). *B*: Closed bars represent STZ-treated mice with fasting blood glucose ≥13.1 mmol/l M (n = 17). Hatched bars represent STZ-treated mice with fasting blood glucose <13.1 mmol/l M (n = 17). Open bars represent vehicle-treated control mice (n = 34). *P* values for two-group comparisons are indicated above brackets. Values are mean ± SE.

activity between STZ-treated and vehicle-treated control mice (P = 0.04) should be interpreted with caution because of the multiple two-group comparisons made in this study. Because STZ-treated mice had increased plasma triglycerides (Table 1 and Fig. 3), an unchanged (or perhaps slightly decreased) triglyceride secretion rate suggests that triglyceride clearance from the blood is attenuated in STZ-treated mice.

Reporter-gene studies suggest that MTP gene expression can be regulated by SREBP transcription factors (17). To assess whether the observed changes in MTP expression in *ob/ob* mice might be paralleled by changes in SREBP gene expression, we measured the hepatic mRNA levels of SREBP genes. In accordance with a previous report (20), mRNA levels of SREBP-1a and -1c were increased 1.5- and 2.0-fold, respectively, in the *ob/ob* mice (Fig. 4*A*; P = 0.03 for SREBP-1a and P = 0.002 for SREBP-1c). SREBP-2 mRNA levels were similar in *ob/ob* and ob/+ control mice (Fig. 4*A*). In STZ-treated mice with blood glucose ≥13.1 mmol/l, SREBP-1c mRNA was increased by 54% (P = 0.02), whereas the STZ mice with blood glucose <13.1 mmol/l had intermediate SREBP-1c mRNA levels (Fig. 4*B*). SREBP-1a and -2 mRNA levels were similar in STZ-treated and vehicle-treated control mice (Fig. 4*B*).

DISCUSSION

Cell culture studies suggest that insulin and glucose negatively regulate MTP gene expression in liver cells (15). These in vitro observations suggest that hepatic MTP gene expression might be decreased in individuals with hyperinsulinemic (type 2) diabetes. However, several metabolic studies have shown that individuals with type 2 diabetes display increased hepatic production of VLDL (21,24,34). The principal aim of the present study was to determine the effect of obesity-associated diabetes in mice on hepatic MTP gene expression, microsomal triglyceride transfer activity, and secretion of triglyceride-rich lipoproteins. Previous studies of mice with genetically altered lipoprotein secretion from the liver (i.e., apoB and MTP knockout mice [12,35,36]) have suggested that an ~50% reduction in the expression levels of the apoB or MTP gene is accompanied by a significant lowering of the plasma concentration of apoB-containing lipoproteins. Therefore, secondary hyperlipidemia in diabetes could be caused by relatively small changes (e.g., 25–50%) in hepatic gene expression levels. Although such small changes in mRNA expression can be of definite physiological importance, they are difficult to detect with standard techniques. Northern blot and RNase protection assays are often only semiquantitative and cannot efficiently be applied to a large number of tissue samples. The present study demonstrates that the real-time PCR technology is a sensitive and reproducible technology for quantification of mRNA in multiple tissue samples. The studies revealed that hepatic MTP mRNA expression is increased by 45% in hyperinsulinemic diabetic *ob/ob* mouse and that this difference in gene expression is paralleled by increases in microsomal triglyceride transfer activity and secretion of triglycerides from the liver. These findings support previous observations in vitro and in vivo of tight correlations between MTP gene expression and MTP protein content in liver cells (12,14,25,26). Thus, the results suggest that increased MTP gene expression confers accelerated production of triglyceride-rich apoB-containing lipoproteins in the liver of obese diabetic mice.

It is unknown what causes increased MTP expression in the liver of obese diabetic mice. A lipid enrichment of the diet stimulates the hepatic expression of MTP mRNA in hamsters in a dose-dependent manner (37), and the genetically obese OLEFT rat, which has increased fatty acid levels in the portal vein, also displays increased hepatic MTP activity and lipoprotein secretion (38). This has led to the suggestion that prolonged increases in flux of fatty acid to the liver may stimulate MTP expression (38). The present data are in accordance with this idea because an elevation of MTP expression in *ob/ob* mice occurred in the setting of increased triglyceride clearance from the blood as well as increased triglyceride stores in the liver. However, in cultured liver cells, addition of oleic acid did not affect MTP expression (15). Thus, additional studies are needed to resolve how MTP gene expression might be regulated in the mouse liver in vivo.

We quantified hepatic SREBP mRNA levels because a recent study proposed that SREBPs might regulate MTP

gene expression in liver cells (17). Because SREBP-1c gene expression was increased and SREBP-2 gene expression was unchanged in both obese and lean diabetic mice, the selective increase in MTP expression in obese diabetic mice seems to be unrelated, at least in a direct manner, to changes in SREBP-1c gene expression. Whereas an increase in SREBP-1 gene expression was previously reported in *ob/ob* mice (20), we were surprised to find that SREBP-1c mRNA expression was also increased in STZ-treated mice with blood glucose >13.1 mmol/l. In a study of STZ-treated diabetic rats, Shimomura et al. (18) found that hepatic triglyceride levels increased by 60% and SREBP-1c mRNA levels were reduced by 80% shortly (42 h) after a single high-dose STZ injection into fasted animals; these acute effects of STZ were completely preventable by insulin treatment. We suspect that differences in study designs might explain the divergent results. In the present study, diabetes was induced in mice by repeated low-dose injections of STZ. After 3–9 weeks, this treatment caused a mild decrease in plasma insulin levels, a reduction in liver triglyceride stores, and a 54% increase in SREBP-1c mRNA levels in mice with severe hyperglycemia, whereas mice with mild hyperglycemia had an intermediate phenotype. The results are compatible with the idea that prolonged exposure to a high blood glucose level stimulates SREBP-1c gene expression in the mouse liver in vivo, even in the presence of slightly decreased plasma insulin levels. In accordance with this idea, a recent study described a glucose-responsive element in the SREBP-1 gene promoter region that confers concentration-dependent increases in SREBP-1 gene expression after prolonged exposure of mouse liver cells to glucose (39). SREBP-1 encodes membrane-bound precursors that need to undergo proteolytic cleavage (40) as well as phosphorylation (41) to induce transcriptional activation of target genes. Thus, it remains to be tested how an increased SREBP-1c mRNA expression in STZ-treated diabetic mice might affect the level of the active NH_2 fragment of SREBP-1c.

Studies of rabbits and hamsters treated with MTP inhibitor drugs have shown that an ~50% reduction in MTP activity effectively lowered plasma lipoprotein levels and had only minor (but definitive) effects on triglyceride deposition in liver cells (7,9). Even though simple mild hepatic steatosis often has a benign course in nondiabetic individuals, ~20% of individuals with type 2 diabetes develop nonalcoholic steatohepatitis that ultimately results in cirrhosis in 20–30% of the cases (42). Recent observations propose a link between hepatic MTP gene expression levels and development of nonalcoholic steatohepatitis: indexes of nonalcoholic steatohepatitis in individuals with type 2 diabetes are more frequent in carriers of the G allele of the −493 G/T polymorphism in the MTP promoter than in carriers of the T allele (43). The G allele confers a 50% lower promoter activity compared with the T allele (44). Physiologically, the present finding of increased MTP gene transcription in the fatty livers of type 2 diabetic *ob/ob* mice may reflect an attempt of the liver to limit triglyceride accumulation. Thus, even though the present studies suggest that MTP inhibition may be a rational approach to pharmacological treatment of hyperlipidemia in individuals with type 2 diabetes, it is possible that reducing hepatic MTP activity might accelerate development of nonalcoholic steatohepatitis in this patient group.

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REFERENCES

1. Seidell JC: Obesity, insulin resistance and diabetes—a worldwide epidemic. *Br J Nutr* 83 (Suppl. 1):S5–S8, 2000
2. Howard BV: Insulin resistance and lipid metabolism. *Am J Cardiol* 84:28J–32J, 1999
3. Wagner AM, Perez A, Calvo F, Bonet R, Castellvi A, Ordonez J: Apolipoprotein(B) identifies dyslipidemic phenotypes associated with cardiovascular risk in normocholesterolemic type 2 diabetic patients. *Diabetes Care* 22:812–817, 1999
4. Duvillard L, Pont F, Florentin E, Galland-Jos C, Gambert P, Verges B: Metabolic abnormalities of apolipoprotein B-containing lipoproteins in non-insulin-dependent diabetes: a stable isotope kinetic study. *Eur J Clin Invest* 30:685–694, 2000
5. Ginsberg HN: Very low density lipoprotein metabolism in diabetes mellitus. *Diabetes Metab Rev* 3:571–589, 1987
6. Gordon DA, Jamil H: Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. *Biochim Biophys Acta* 1486:72–83, 2000
7. Wetterau JR, Gregg RE, Harrity TW, Arbeeny C, Cap M, Connolly F, Chu CH, George RJ, Gordon DA, Jamil H, Jolibois KG, Kunselman LK, Lan SJ, Maccagnan TJ, Ricci B, Yan M, Young D, Chen Y, Fryszman OM, Logan JV, Musial CL, Poss MA, Robl JA, Simpkins LM, Biller SA: An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. *Science* 282:751–754, 1998
8. Gordon DA, Jamil H, Gregg RE, Olofsson SO, Boren J: Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J Biol Chem* 271:33047–33053, 1996
9. Leung GK, Veniant MM, Kim SK, Zlot CH, Raabe M, Bjorkegren J, Neese RA, Hellerstein MK, Young SG: A deficiency of microsomal triglyceride transfer protein reduces apolipoprotein B secretion. *J Biol Chem* 275: 7515–7520, 2000
10. Raabe M, Kim E, Veniant M, Nielsen LB, Young SG: Using genetically engineered mice to understand apolipoprotein-B deficiency syndromes in humans. *Proc Assoc Am Physicians* 110:521–530, 1998
11. Raabe M, Veniant MM, Sullivan MA, Zlot CH, Bjorkegren J, Nielsen LB, Wong JS, Hamilton RL, Young SG: Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J Clin Invest* 103:1287–1298, 1999
12. Raabe M, Flynn LM, Zlot CH, Wong JS, Veniant MM, Hamilton RL, Young SG: Knockout of the abetalipoproteinemia gene in mice: reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes. *Proc Natl Acad Sci U S A* 95:8686–8691, 1998
13. Tietge UJ, Bakillah A, Maugeais C, Tsukamoto K, Hussain M, Rader DJ: Hepatic overexpression of microsomal triglyceride transfer protein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B. *J Lipid Res* 40:2134–2139, 1999
14. Liao W, Kobayashi K, Chan L: Adenovirus-mediated overexpression of microsomal triglyceride transfer protein (MTP): mechanistic studies on the role of MTP in apolipoprotein B-100 biogenesis. *Biochemistry* 38:7532–7544, 1999
15. Lin MC, Gordon D, Wetterau JR: Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression. *J Lipid Res* 36:1073–1081, 1995
16. Hagan DL, Kienzle B, Jamil H, Hariharan N: Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. Cell type-specific expression and response to metabolic regulators. *J Biol Chem* 269:28737–28744, 1994
17. Sato R, Miyamoto W, Inoue J, Terada T, Imanaka T, Maeda M: Sterol

regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J Biol Chem* 274:24714–24720, 1999
18. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL: Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:13656–13661, 1999
19. Foretz M, Pacot C, Dugail I, Lemarchand P, Guichard C, Le Liepvre X, Berthelier-Lubrano C, Spiegelman B, Kim JB, Ferre P, Foufelle F: ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol Cell Biol* 19:3760–3768, 1999
20. Shimomura I, Bashmakov Y, Horton JD: Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* 274:30028–30032, 1999
21. Cummings MH, Watts GF, Umpleby AM, Hennessy TR, Naoumova R, Slavin BM, Thompson GR, Sonksen PH: Increased hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 in NIDDM. *Diabetologia* 38:959–967, 1995
22. Ginsberg HN: Diabetic dyslipidemia: basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. *Diabetes* 45 (Suppl. 3):S27–S30, 1996
23. Reaven GM, Greenfield MS: Diabetic hypertriglyceridemia: evidence for three clinical syndromes. *Diabetes* 30:66–75, 1981
24. Taghibiglou C, Carpentier A, Van Iderstine SC, Chen B, Rudy D, Aiton A, Lewis GF, Adeli K: Mechanisms of hepatic very low density lipoprotein overproduction in insulin resistance: evidence for enhanced lipoprotein assembly, reduced intracellular apoB degradation, and increased microsomal triglyceride transfer protein in a fructose-fed hamster model. *J Biol Chem* 275:8416–8425, 2000
25. Mathur SN, Born E, Murthy S, Field FJ: Microsomal triglyceride transfer protein in CaCo-2 cells: characterization and regulation. *J Lipid Res* 38:61–67, 1997
26. Navasa M, Gordon DA, Hariharan N, Jamil H, Shigenaga JK, Moser A, Fiers W, Pollock A, Grunfeld C, Feingold KR: Regulation of microsomal triglyceride transfer protein mRNA expression by endotoxin and cytokines. *J Lipid Res* 39:1220–1230, 1998
27. Siri P, Candela N, Zhang Y, Ko C, Eusufzai S, Ginsberg HN, Huang LS: Post-transcriptional stimulation of the assembly and secretion of triglyceride-rich apolipoprotein B-lipoproteins in a mouse with selective deficiency of brown adipose tissue, obesity, and insulin resistance. *J Biol Chem* 276:46064–46072, 2001
28. Wetterau JR, Aggerbeck LP, Bouma ME, Eisenberg C, Munck A, Hermier M, Schmitz J, Gay G, Rader DJ, Gregg RE: Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science* 258:999–1001, 1992
29. Wetterau JR, Zilversmit DB: Quantitation of lipid transfer activity. *Methods Biochem Anal* 30:199–226, 1984
30. Fuhlendorff J, Rorsman P, Kofod H, Brand CL, Rolin B, MacKay P, Shymko R, Carr RD: Stimulation of insulin release by repaglinide and glibenclamide involves both common and distinct processes. *Diabetes* 47:345–351, 1998
31. Ruiz JI, Ochoa B: Quantification in the subnanomolar range of phospholipids and neutral lipids by monodimensional thin-layer chromatography and image analysis. *J Lipid Res* 38:1482–1489, 1997
32. Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS: Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J Clin Invest* 99:838–845, 1997
33. Kunjathoor VV, Wilson DL, LeBoeuf RC: Increased atherosclerosis in streptozotocin-induced diabetic mice. *J Clin Invest* 97:1767–1773, 1996
34. Coppack SW: Postprandial lipoproteins in non-insulin-dependent diabetes mellitus. *Diabet Med* 14 (Suppl. 3):S67–S74, 1997
35. Veniant MM, Kim E, McCormick S, Boren J, Nielsen LB, Raabe M, Young SG: Insights into apolipoprotein B biology from transgenic and gene-targeted mice. *J Nutr* 129:451S–455S, 1999
36. Farese RV Jr, Ruland SL, Flynn LM, Stokowski RP, Young SG: Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc Natl Acad Sci U S A* 92:1774–1778, 1995
37. Bennett AJ, Billett MA, Salter AM, White DA: Regulation of hamster hepatic microsomal triglyceride transfer protein mRNA levels by dietary fats. *Biochem Biophys Res Commun* 212:473–478, 1995
38. Kuriyama H, Yamashita S, Shimomura I, Funahashi T, Ishigami M, Aragane K, Miyaoka K, Nakamura T, Takemura K, Man Z, Toide K, Nakayama N, Fukuda Y, Lin MC, Wetterau JR, Matsuzawa Y: Enhanced expression of hepatic acyl-coenzyme A synthetase and microsomal triglyceride transfer protein messenger RNAs in the obese and hypertriglyceridemic rat with visceral fat accumulation. *Hepatology* 27:557–562, 1998
39. Hasty AH, Shimano H, Yahagi N, Amemiya-Kudo M, Perrey S, Yoshikawa T, Osuga J, Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, Harada K, Gotoda T, Nagai R, Ishibashi S, Yamada N: Sterol regulatory element-binding protein-1 is regulated by glucose at the transcriptional level. *J Biol Chem* 275:31069–31077, 2000
40. Brown MS, Goldstein JL: A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci USA* 96:11041–11048, 1999
41. Roth G, Kotzka J, Kremer L, Lehr S, Lohaus C, Meyer HE, Krone W, Muller-Wieland D: MAP kinases Erk1/2 phosphorylate sterol regulatory element-binding protein (SREBP)-1a at serine 117 in vitro. *J Biol Chem* 275:33302–33307, 2000
42. Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA: Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 107:1103–1109, 1994
43. Bernard S, Touzet S, Personne I, Lapras V, Bondon PJ, Berthezene F, Moulin P: Association between microsomal triglyceride transfer protein gene polymorphism and the biological features of liver steatosis in patients with type II diabetes. *Diabetologia* 43:995–999, 2000
44. Karpe F, Lundahl B, Ehrenborg E, Eriksson P, Hamsten A: A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Arterioscler Thromb Vasc Biol* 18:756–761, 1998