Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice

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Abstract Apolipoprotein B (apoB)-containing lipoproteins play a critical role in whole body lipid homeostasis and the pathogenesis of atherosclerosis. The assembly of hepatic apoB-containing lipoproteins, VLDL, is governed by the availability of lipids, including triacylglycerol (TG). The majority of TG associated with VLDL is derived from the hepatic cytoplasmic lipid stores by a process involving lipolysis followed by reesterification. Microsomal triacylglycerol hydrolase (TGH) has been demonstrated to play a role in the lipolysis/reesterification process. To evaluate the potential regulatory role of TGH in hepatic VLDL assembly, we developed inducible transgenic mice expressing a human TGH minigene under the control of the mouse metallothionein promoter. Induction of human TGH by zinc resulted in liver-specific expression of the enzyme associated with 3- to 4-fold increases in lipolytic activity that could be attenuated with a TGH-specific inhibitor. Augmented TGH activity led to increased secretion of newly synthesized apoB and plasma TG levels. These results suggest that increased hepatic expression of TGH leads to a more proatherogenic plasma lipid and apoB profile.—Wei, E., M. Alam, F. Sun, L. B. Agellon, D. E. Vance, and R. Lehner. **Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice.** ***J. Lipid Res.*** **2007.** 48: **2597–2606.**

Supplementary key words very low density lipoprotein • lipolysis • metallothionein • liver

Atherosclerosis is the principal cause of heart attack and stroke in the Western world. The relationship between high levels of plasma LDL and atherosclerosis has been known for several decades (1). LDL is derived from triacylglycerol (TG)-rich VLDL in the circulatory system via a complex series of reactions involving hydrolases and the transfer of lipids and apolipoproteins between lipoproteins (2). VLDL assembly in the liver and the secretion of these particles into the blood stream is dependent upon the availability of lipids (3–8). The majority (60–70%) of VLDL-TG secreted from rat hepatocytes is derived from the lipolysis of stored TG to partial acylglycerols and fatty acids followed by reesterification of the lipolytic products by endoplasmic reticulum (ER)-localized acyltransferases (9–14). HepG2 cells, a human hepatoma cell line, secrete few VLDL-sized particles; rather, they secrete apolipoprotein B (apoB) in the LDL density range (15–17). The impaired secretion of VLDL in HepG2 cells is likely attributable to a defect in the mobilization of TG from storage pools (15, 17).

Similarly, we have shown that McArdle RH7777 cells, a rat hepatoma cell line, are also deficient in the mobilization of stored TG for VLDL assembly and secretion (18), although these cells (unlike HepG2) are capable of secreting a significant amount of VLDL-sized particles when an exogenous supply of fatty acids is available. Lipolysis of stored TG for VLDL assembly is not catalyzed by lysosomal (acidic) lipase (9). Additionally, cytosolic hormone-sensitive lipase is not found in appreciable quantities in the liver. Ectopic expression of hormone-sensitive lipase in HepG2 cells leads to the diversion of released fatty acids into the oxidative, and not the secretory, pathway, suggesting that the subcellular localization of the lipase(s) may be important in the channeling of substrates (19). Combined, these findings suggest that an ER-localized lipase participates in the provision of lipid for VLDL assembly. Specifically, lipolysis of an ER-associated lipid droplet followed by the resynthesis of TG by acyltransferases would provide TG for loading onto nascent apoB-containing particles.

Studies of a microsomal triacylglycerol hydrolase (TGH) suggested that this enzyme may participate in the provision of lipids for VLDL assembly (18, 20–22). TGH is absent from liver-derived HepG2 and McArdle RH7777 hepatoma cells (18, 20, 23). Expression of TGH cDNA in

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Abbreviations: apoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; P-407, poloxamer-407; TG, triacylglycerol; TGH, triacylglycerol hydrolase.

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McArdle RH7777 cells increased the lipolysis of prelabeled TG stores, TG secretion, and levels of apoB in the VLDL density range (18). Inhibition of TGH activity in intact primary rat hepatocytes by a cell-permeable TGH-specific inhibitor resulted in decreased secretion of apoB and TG (21). These data support an active role for TGH in intracellular TG mobilization for VLDL assembly.

We wished to address the contribution of TGH to plasma lipid levels in vivo. Using transgenic mice that express human TGH specifically in the liver, we now demonstrate that increased hepatic expression of TGH leads to more proatherogenic plasma lipid and apoB profiles. These observations provide further evidence that TGH may be a suitable target for pharmacological inhibition to reduce circulating apoB and neutral lipid levels.

MATERIALS AND METHODS

Materials

Polyclonal goat anti-human apoB antibodies were purchased from Chemicon International (Temecula, CA). Mouse anti-goat and goat anti-rabbit secondary antibodies conjugated to HRP were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Anti-protein disulfide isomerase antibodies were from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Polyclonal antibodies against human TGH and rat albumin were raised in rabbits and affinity-purified in our laboratory. Poloxamer-407 (P-407) was gift from BASF Customer Care. ECL® immunoblotting reagents were purchased from Amersham-Pharmacia Canada (Oakville, Ontario, Canada). The TGH-specific inhibitor 4,4,4-trifluoro-2-[2-(3-methylphenyl)hydrazono]1-(2-thienyl)butane-1,3-dione (GR148672X or GSKi) was provided by GlaxoSmithKline (Les Ulis, France). [^{35}S]Promix was purchased from Amersham-GE Healthcare (Buckinghamshire, UK). All other reagents were of analytical grade or higher.

Generation of human TGH transgenic mice

A 12.5 kb synthetic human TGH (*hTGH*) minigene was assembled by combining genomic and cDNA sequences. The genomic fragments of hTGH were amplified from a human genomic BAC library. The 5′ genomic region (including exons 1 and 2, introns I and II, and part of exon 3) and the 3′ genomic region (including part of exon 12, introns XII and XIII, exons 13 and 14, and 657 bp of 3′ flanking sequence) were linked together using a fragment from the human TGH cDNA. This fusion resulted in the complete removal of introns III–XI and the generation of one synthetic exon. No alterations were made to the remaining *TGH* sequence, and all of the coding sequences were in-frame. The ∼13.5 kb fully assembled *TGH* transgene was obtained by linking the human TGH minigene to the mouse metallothionein-I promoter (0.94 kb). The structure of the construct was verified by mapping with restriction endonucleases, by sequencing, and by expression of the minigene as protein in McArdle RH7777 and NIH-3T3 cells. The gene construct was subcloned in pSTBlue-1 vector (Novagen), was released from the vector using *Not*I and *Xho*I, and was purified for microinjection into the pronuclei of fertilized mouse eggs taken from superovulated C57BL/6J females. Injected embryos were implanted into the oviducts of surrogate C57BL/6J females. Transgenic mice were identified by screening tail DNA by PCR using a forward primer from the promoter region and a reverse primer from intron I. The presence of transgene was further characterized by Southern blotting. The founder male mouse was mated with female C57BL/6J mice to establish a line.

RNA isolation and RT-PCR analysis

Total RNA was isolated from mouse liver using Trizol® reagent (Life Technologies, Inc.) according to the manufacturer's instructions. First-strand cDNA synthesis from 2 μg of total RNA was performed using SuperScript™ II reverse transcriptase (Invitrogen) primed by oligo(dT)$_{12-18}$ primers. PCR was performed using the human TGH primers 5′-CACAGCCGGGGGAACTGG-3′ and 5′-CTGGTGAAGAAAGGTGATGTC-3′. Primers for cyclophilin were 5′-TCCAAAGACAGCAGAAAACTTTCG-3′ and 5′-TCTTCTTGCTGGTCTTGCCATTCC-3′. Primers for GAPDH were 5′-GAGCCAAACGGGTCATCATC-3′ and 5′-CATCACGCCACAGCTTTCCA-3′. The PCR program included denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 40 s (25 cycles). The PCR product was separated by electrophoresis on 1% (w/v) agarose gels.

Stable transfection of NIH-3T3 cells

NIH-3T3 cells were cotransfected with the *hTGH* transgene and pCI-neo vector. Stably transfected clones were selected by resistance to G418 (1.6 mg/ml) and maintained in a growth medium supplemented with 0.4 mg/ml G418.

Induction of the human *TGH* transgene in mice

All procedures were approved by University of Alberta's Animal Welfare Committee and were in accordance with guidelines of the Canadian Council on Animal Care. Mice, housed four to five per cage, were exposed to a 12 h light/dark cycle beginning with light at 8:00 AM. Adult male and female mice, 12–24 weeks old, were fed ad libitum a chow diet from LabDiet (PICO Rodent Diet 20) and had free access to distilled water. To induce *hTGH* expression in the liver, homozygote transgenic mice from the F_4–F_6 generation and control C57BL/6J mice were supplied distilled water containing 25 mM $ZnSO_4$ for 6 days; on day 6, a concentrated dose of $ZnSO_4$ (20 mg/kg body weight) was provided by intraperitoneal injection. Experiments were done on day 7 of postzinc treatment. For all experiments, mice were fasted overnight (16 h) before euthanasia. Transgenic mice and corresponding controls were age-matched for all studies.

Lipase assay

Tissues were homogenized using a glass/Teflon homogenizer in 2 ml of buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 1 mM EDTA) followed by sonication for 20 s. Protein concentration was determined using the Bio-Rad protein assay protocol (Bio-Rad Laboratories, Mississauga, Ontario, Canada), with BSA as a standard. Lipase activity was assayed using two methods. A fluorescence-based assay using 4-methylumbelliferyl heptanoate as a substrate was performed essentially as described previously (24). Alternatively, lipase activity was monitored spectrophotometrically at 405 nm via the liberation of *p*-nitrophenol from *p*-nitrophenyl laurate as described previously (25, 26), except that the assays were performed on 96-well clear microtiter plates and read on a Molecular Devices SpectraMax 250 (Sunnyvale, CA) (24). The specific method used is indicated in the figure legends.

Immunoblot analyses

Liver homogenates (50 μg of protein) were heated for 5 min at 95°C in 62.5 mM Tris-HCl, pH 8.3, 10% (v/v) glycerol, 5% (v/v)

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2-mercaptoethanol, 1% SDS, and 0.004% Bromophenol Blue. The protein samples were electrophoresed on an SDS/10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with 5% (w/v) skim milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20, then incubated for 1 h with antibody raised against the specified protein followed by a 1 h wash and a 1 h incubation with secondary goat anti-rabbit (detection of TGH, albumin, and protein disulfide isomerase) or mouse anti-goat (detection of apoB) antibody conjugated to HRP (18, 21, 24). Immunoreactivity was detected by the ECL™ system according to the manufacturer's instructions. Where indicated, primary and secondary antibodies were stripped from membranes by incubation with 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C, and the blot was reprobed with another antibody. For analysis of plasma apoB-48 and apoB-100 levels, blood was collected from each mouse via cardiac puncture in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Plasma (2 μl) was resolved on a 4–15% SDS-polyacrylamide gradient gel. Proteins were transferred to nitrocellulose membranes and probed with anti-apoB and anti-albumin antibodies.

Determination of liver and plasma cholesterol, cholesteryl ester, phospholipid, and TG

The amounts of indicated lipids were determined in liver homogenates (0.5 mg of protein) or 50 μl of plasma. After digestion (2 h, 30°C) of the phospholipids with phospholipase C, tridecanoin (20 ng) was added as an internal standard and lipids were extracted. The mass of TG, cholesterol, and cholesteryl ester was determined by gas-liquid chromatography as described previously (27).

Analysis of hepatic TG production rate

To measure hepatic TG production rate, mice were fasted for 16 h and injected with P-407 (1 g/kg body weight) in saline. Immediately before P-407 injection, and at 6 h after injection, blood samples were drawn in heparin capillary tubes, plasma was prepared, and TG concentrations were determined by gas-liquid chromatography. The TG production rate was calculated from the difference in plasma TG levels over 6 h after detergent injection and was expressed as micrograms of lipid per deciliter of plasma per gram of body weight per hour.

Analysis of hepatic apoB production

Mice were fasted for 4 h and injected via tail vein with 200 μl of PBS containing 250 μCi of [^{35}S]Promix. Thirty minutes later, P-407 (1 g/kg body weight) in saline was injected intraperitoneally. Two hours after the injection of P-407, which was shown to be linear for hepatic TG and apoB production (28), blood samples were collected and plasma was isolated. To 100 μl of plasma were added 800 μl of PBS, 100 μl of 10× immunoprecipitation buffer [1.5 M NaCl, 0.5 M Tris-Cl, pH 7.4, 50 mM EDTA, 5% (v/v) Triton X-100, and 1% (w/v) SDS], and 10 μl of goat anti-human apoB IgG. The mixture was incubated on a rotating rack overnight at 4°C, after which 50 μl of protein A-Sepharose was added, and the mixture was further incubated for 3 h. The beads were pelleted by brief centrifugation and washed three times with excess immunoprecipitation buffer, followed by denaturing electrophoresis sample buffer. Samples were boiled and underwent electrophoresis on SDS-5% polyacrylamide gels. Gels were stained by Coomassie Blue, dried, and exposed to X-ray films.

Fast-protein liquid chromatography analysis of lipoproteins

To determine plasma lipoprotein levels, size-exclusion fast-protein liquid chromatography was used. Fasting plasma was pooled from each set of mice and applied onto a Sepharose 6 fast-protein liquid chromatography column (Pharmacia, Uppsala, Sweden). Eluted lipoprotein fractions were mixed in-line with the Infinity Cholesterol Reagent or Infinity Triglyceride Reagent using a postcolumn T-connector/Solvent Delivery Module (model 110B; Beckman Coulter, Mississauga, Ontario, Canada) and passed through a CH-30 Column Heater (Eppendorf, Mississauga, Ontario, Canada) set at 37°C. Reaction products were monitored at 500 nm (cholesterol) or 525 nm (TG) in real time using a Programmable Detector Module (model 166; Beckman Coulter).

Statistical analysis

Data are presented as means ± SEM using Student's unpaired *t*-test. Five to 11 animals were used in each experimental group. $P < 0.05$ was interpreted as a significant difference.

RESULTS

Transfection of the *hTGH* minigene results in the expression of active human TGH

The human *TGH* gene spans ~30 kb and contains 14 exons (29, 30). Because the gene is large and transgenes based on cDNAs are often poorly expressed, we constructed a minigene containing both genomic and cDNA sequences (**Fig. 1A**) to facilitate the production of transgenic mice. To test whether expression of the *hTGH* minigene yields an active TGH, stably transfected NIH-3T3 cells were analyzed for TGH protein levels and lipase activity. Figure 1B shows that increased lipolytic activity in the homogenates of NIH-3T3 cells correlates with increased levels of human TGH (Fig. 1C). Therefore, the *hTGH* minigene is structurally functional and capable of encoding active recombinant human TGH in transfected cells.

Mice express the *hTGH* minigene specifically in the liver

A C57Bl/6J transgenic line was established from the male founder. Southern blotting and real-time PCR of the DNA isolated from the founder and from the subsequent generations indicated integration of the transgene (data not shown). To determine whether hTGH transgenic mice expressed human TGH mRNA and produced hTGH protein, the presence of hTGH mRNA and protein in the livers was analyzed. Expression of the *hTGH* minigene and the production of hTGH were detected only in the transgenic line by measurement of mRNA (**Fig. 2A**) and immunoblotting (Fig. 2B). Analysis of RNA from various tissues for hTGH mRNA by PCR showed that the *hTGH* transgene was expressed only in the liver (data not shown). The liver-specific expression of hTGH was confirmed by functional analysis measuring lipase activity in the various tissues after zinc treatment. Significant 3- to 4-fold increases of lipolytic activity were observed only in the livers of transgenic mice (**Fig. 3A**). The increased lipolytic ac-

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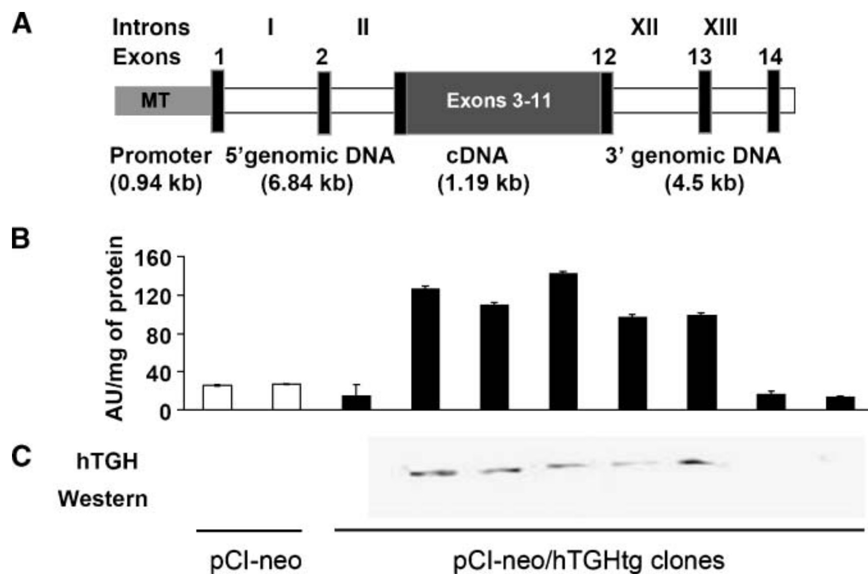

Fig. 1. Structure and expression of the human triacylglycerol hydrolase (*hTGH*) transgene. A: Organization of the *hTGH* transgene. MT, metallothionein. B: Lipase activity in homogenates of NIH-3T3 cells stably transfected with the pCI-neo vector or cotransfected with the *hTGH* transgene and the pCI-neo vector. Lipase activity was determined with *p*-nitrophenyl laurate as described in Materials and Methods. Data are presented as means ± SEM. AU, arbitrary units. C: Immunoblotting of hTGH in homogenates of NIH-3T3 cells stably transfected with the pCI-neo vector or cotransfected with the hTGH transgene and the pCI-neo vector. Immunoblotting was determined using polyclonal anti-human TGH antibodies as described in Materials and Methods.

tivity in the liver was sensitive to a TGH-specific inhibitor (Fig. 3B), indicating that the augmented lipolysis was solely attributable to the expression of the *hTGH* transgene. Addition of zinc to the drinking water of the mice caused an increase of hTGH mRNA in the liver (Fig. 3C) and hepatic lipolytic activity in the transgenic animals of both genders (Fig. 3D, E).

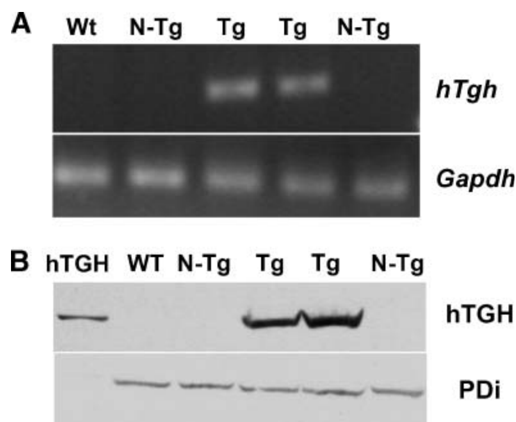

Fig. 2. Expression of the *hTGH* transgene in mice. A: RT-PCR analysis of *hTGH* transgene expression in the livers of wild-type (Wt), nontransgenic littermates (N-Tg), and transgenic (Tg) C57BL/6J male mice. GAPDH expression was analyzed as a control for RNA levels used in the reaction. B: Immunoblotting of hTGH in liver homogenates. Purified hTGH was loaded in the left lane. Immunoblotting of protein disulfide isomerase (PDI) was used as a loading control.

Induction of hTGH expression leads to increased levels of plasma lipid and apoB

An increase of TG in male mice was seen in the VLDL fraction (**Fig. 4B**), whereas in female mice increased TG levels were found in LDL-sized lipoproteins (Fig. 4D). In basal (uninduced) conditions, *hTGH* transgenic mice had a similar plasma lipid profile as control mice (**Fig. 5A, C**). Zinc supplementation resulted in increased free cholesterol plasma levels in both male and female transgenic mice (Fig. 5B, D). This increase was mainly attributable to LDL-cholesterol levels, and no significant changes in HDL-cholesterol profiles were observed in either male or female transgenic mice compared with controls (data not shown). Although supplementation with zinc led to higher plasma TG levels, statistical significance was reached only in the male transgenic mice (Fig. 5B). Uninduced transgenic mice had similar apoB profiles as control mice (**Fig. 6A, C**). Induction of *hTGH* expression increased plasma apoB levels in males (Fig. 6B) and females (Fig. 6D).

Consistent with studies addressing the effects of zinc deficiency on lipid metabolism in rats (31, 32), zinc treatment reduced plasma TG levels in both controls and transgenic mice (Fig. 5). Zinc supplementation decreased fasting plasma TG by 32.4% and 56.8% in male and female wild-type mice, respectively, whereas in mice expressing the *hTGH* transgene, plasma TG levels were decreased by only 3.7% in males and 29% in females. The overexpression of human TGH thus counteracted the reduction of plasma TG by zinc; as a result, the zinc-induced transgenic mice presented with increased TG secretion compared with their nontransgenic controls. The changes in apoB

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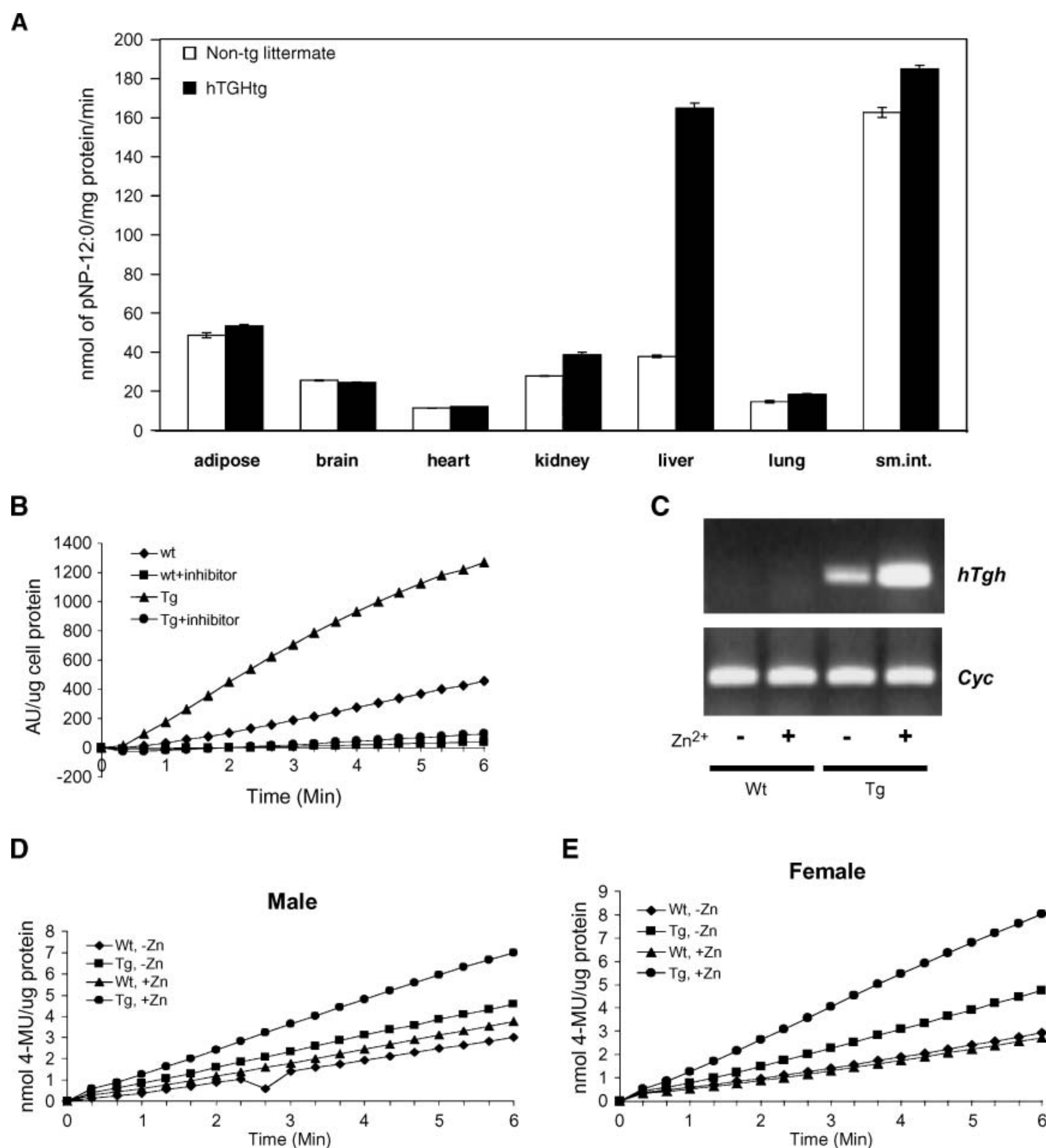

Fig. 3. Tissue specificity of *hTGH* transgene expression. A: Mouse adipose tissue, brain, heart, kidney, liver, lung, and small intestine (sm.int.) were harvested from both nontransgenic littermates and hTGH transgenic mice, and lipolytic activities in different tissues were determined using *p*-nitrophenyl laurate (pNP). Data are presented as means ± SEM. B: Lipase activities in liver homogenates of wild-type (wt) and hTGH transgenic (Tg) mice were analyzed using 4-methylumbelliferyl heptanoate. A TGH-specific inhibitor was included in the assay to assess the level of the increase in lipase activity attributable to hTGH expression. AU, arbitrary units. C: Mice were treated with or without $ZnSO_4$ for 6 days, livers were collected, and RT-PCR analyses of hTGH and cyclophilin (Cyc) mRNA levels were performed. Lipase activities in male (D) and female (E) liver homogenates of mice treated with or without $ZnSO_4$ were analyzed using 4-methylumbelliferyl heptanoate.

(Fig. 6) were consistent with the lipid profiles (Fig. 4). Both male and female wild-type mice showed decreased apoB levels in response to zinc, whereas both male and female *TGH* transgenic mice maintained apoB levels in zinc-supplemented conditions.

Induction of hTGH increases TG and apoB secretion rates in vivo

To measure hepatic TG secretion rate, mice were injected with P-407, a detergent known to inhibit plasma lipoprotein lipase activity (28). The accumulation of plasma TG was monitored in zinc-supplemented male and female wild-type and *hTGH* transgenic mice (**Fig. 7A**). The secretion of TG was similar between wild-type male and female mice. In zinc-induced *hTGH* transgenic mice, the TG secretion rate appeared to be somewhat augmented in both sexes compared with zinc-treated control mice, but the increase did not reach statistical significance (Fig. 7A). In concert with increased plasma apoB levels in *hTGH* transgenic mice, zinc induction resulted

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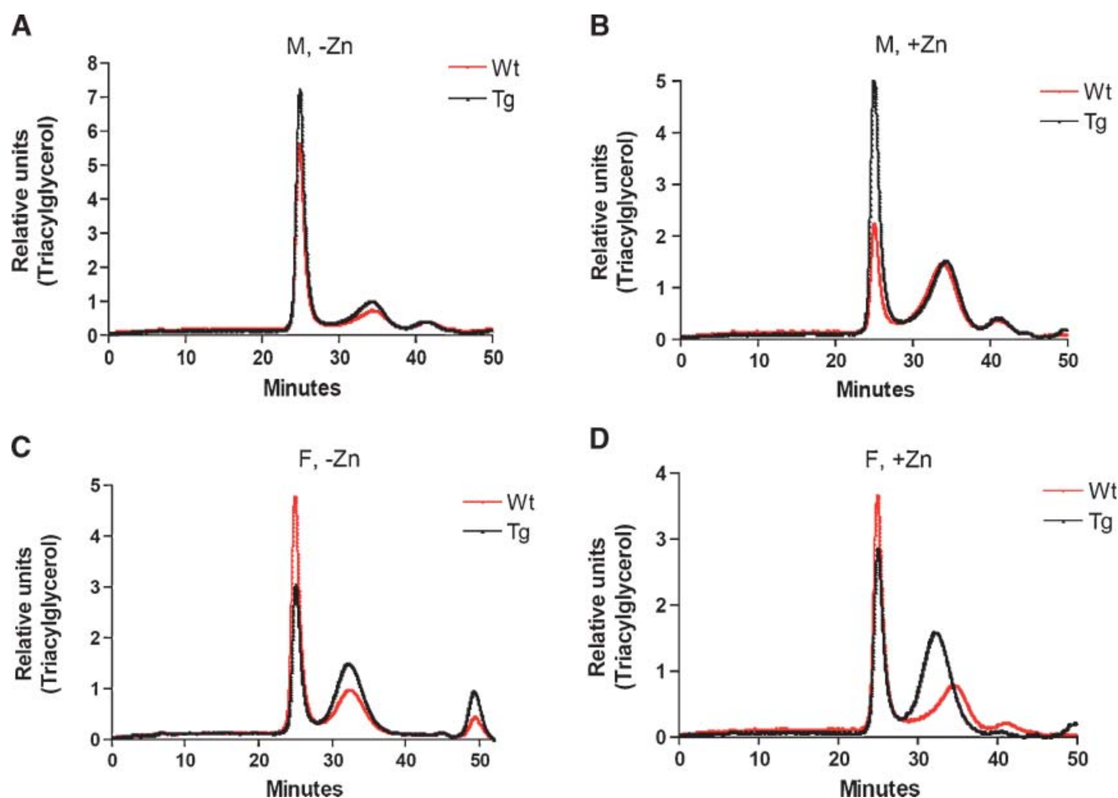

Fig. 4. Fast-protein liquid chromatography profiles of total plasma triacylglycerol (TG) in fasted mice. Wild-type (Wt) and *hTGH* transgenic (Tg) mice were supplied with or without $ZnSO_4$ in drinking water for 6 days. Mice were fasted for 16 h before blood collection. Fasting plasma from 7–11 mice per group was pooled, and analyses of TG content in lipoprotein fractions by fast-protein liquid chromatography were performed. Red lines, plasma from wild-type mice; black lines, plasma from *hTGH* transgenic mice. Fractions were as follows: VLDL, 24–28 min; LDL, 29–38 min; HDL, 39–45 min; free glycerol, 48–52 min. A: Male mice without $ZnSO_4$ in water. B: Male mice with $ZnSO_4$ in water. C: Female mice without $ZnSO_4$ in water. D: Female mice with $ZnSO_4$ in water.

in a 60% increase in the secretion of newly synthesized apoB-100 (Fig. 7B).

DISCUSSION

The synthesis and secretion of hepatic VLDL is a complex process requiring both cotranslational and post-translational addition of lipids to the apoB polypeptide (14, 33, 34). The availability of TG, the major lipid present in the core of VLDL, is thought to play a major role in VLDL production. The majority of VLDL-TG is believed to be derived from cytoplasmic TG stores through a process that involves lipolysis followed by reesterification of the released fatty acids and partial acylglycerols (9–13). TGH has been demonstrated to play a role in the lipolysis/re-esterification cycle in in vitro studies (18, 21, 22, 35); however, the potential regulatory role of this enzyme on hepatic VLDL assembly and secretion in vivo was unknown. We hypothesized that increased TGH activity would provide substrates for apoB lipidation and therefore would lead to increased secretion of TG, similar to what we previously observed in McArdle RH7777 cells transfected with TGH cDNA (18).

Our data suggest that the expression of human TGH in livers of C57Bl/6J mice leads to increased fasting plasma VLDL-TG levels in males, whereas this was not the case for *hTGH* transgenic females, which had increased TG in LDL-sized lipoproteins. The reason for the male/female differences is not known. It is unlikely to be related to the secretion of smaller (less lipidated) lipoprotein particles by the females, as similar rates of TG secretion were observed in both sexes. Both male and female *hTGH* transgenic mice contained higher levels of apoB in plasma, and female but not male transgenic mice also appeared to have substantially increased circulating apoB-100 levels, whereas males presented with increased apoB-48 levels. This difference could be related to increases in the LDL-sized particles in females and VLDL-sized particles in males.

Although hTGH expression leads to an ~30–80% increase in plasma neutral lipid levels, suggesting a regulatory role of the enzyme in VLDL assembly, the observed increases in lipids are relatively modest. Interestingly, unchanged plasma lipid levels were observed in transgenic mice overexpressing the nuclear form of sterol-regulatory element binding protein 1a. Increased hepatic lipogenesis and increased plasma lipids were seen only after the mice were crossed into the LDL receptor null background (36). A widely used method to assess VLDL secretion in vivo involves injections of nonionic detergents into mice, because this treatment inhibits plasma lipases and VLDL catabolism and results in the accumulation of lipoprotein

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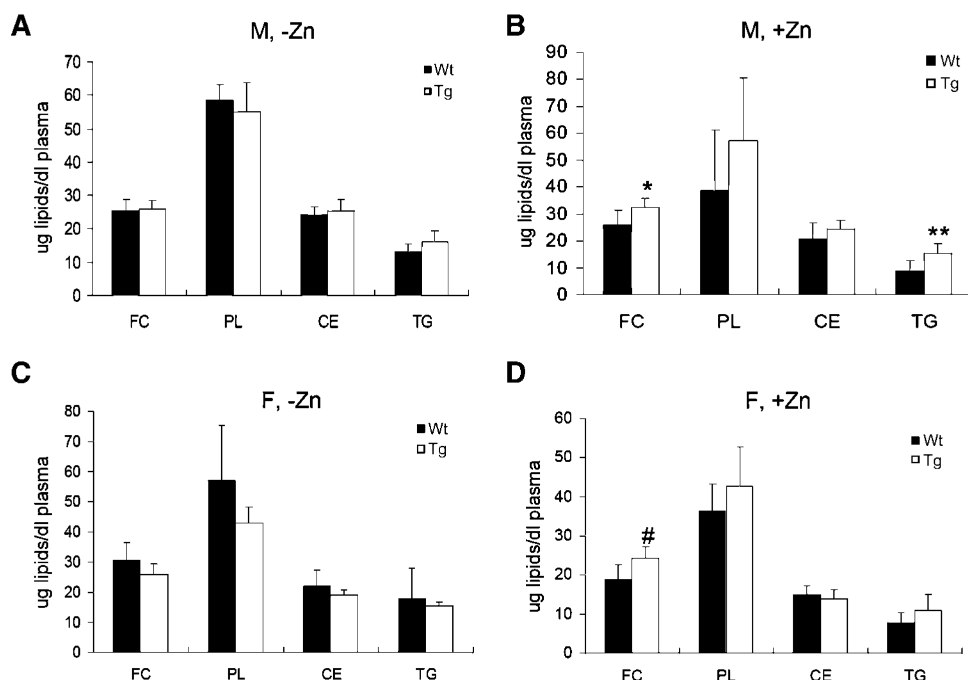

Fig. 5. Analysis of plasma lipids in fasted mice. Wild-type (Wt) and *hTGH* transgenic (Tg) mice were supplied with or without $ZnSO_4$ in drinking water for 6 days. Mice were fasted for 16 h before blood collection. Lipids were extracted and analyzed by gas chromatography. A: Male mice without $ZnSO_4$ in water. B: Male mice with $ZnSO_4$ in water. C: Female mice without $ZnSO_4$ in water. D: Female mice with $ZnSO_4$ in water. FC, free (unesterified) cholesterol; PL, phospholipid; CE, cholesteryl ester. Data are from a minimum of seven animals per genotype and treatment and are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$, # $P < 0.03$.

particles (28). We used P-407 in our study rather than the more commonly used Triton WR-1339 because the latter detergent has been demonstrated to be rapidly taken up by the liver, where it affects metabolic processes (37–39). TGH activity is sensitive to nonionic detergents (25), and the uptake of the detergents into the liver could result in decreased TGH activity. The effect of Triton WR-1339 or P-407 on the intracellular mobilization of stored TG

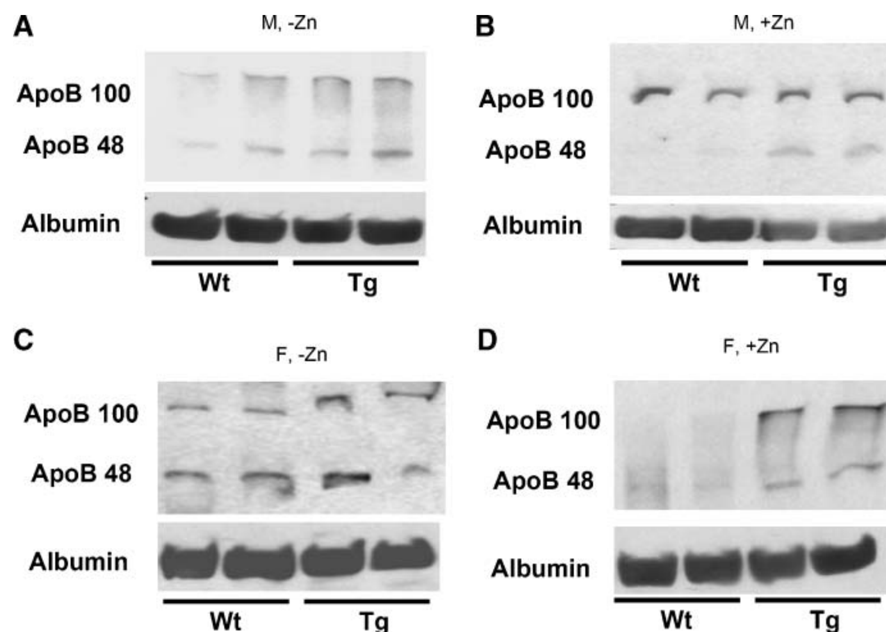

Fig. 6. Analysis of plasma apolipoprotein B (apoB) in fasted mice. Wild-type (Wt) and *hTGH* transgenic (Tg) mice were supplied with or without $ZnSO_4$ in drinking water for 6 days. Mice were fasted for 16 h before blood collection. Fasted plasma samples were electrophoresed on 4–15% polyacrylamide gels in SDS, proteins were transferred to nitrocellulose membranes, and apoB and albumin levels were determined by immunoblotting. A: Male mice without $ZnSO_4$ in water. B: Male mice with $ZnSO_4$ in water. C: Female mice without $ZnSO_4$ in water. D: Female mice with $ZnSO_4$ in water.

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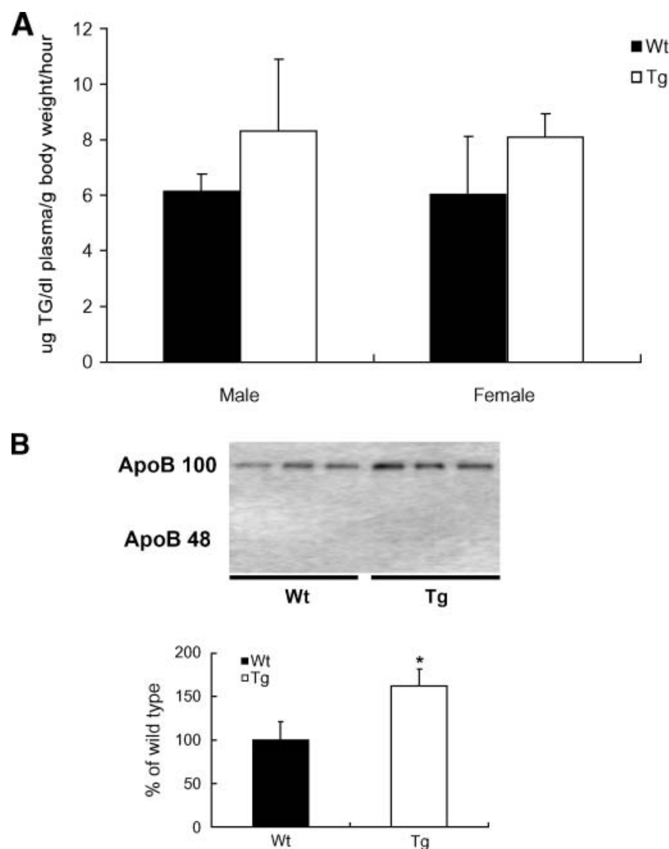

Fig. 7. TG and apoB secretion in mice treated with poloxamer-407 (P-407). A: Wild-type (Wt) and *hTGH* transgenic (Tg) mice fasted for 16 h were injected intraperitoneally with P-407 in saline (1 g/kg body weight). Immediately before injection and 6 h after injection, blood samples were collected and TG concentrations were determined by GC. The TG production rate was calculated from the difference in plasma TG levels over 6 h after P-407 injection and was expressed as indicated. Data are averages ± SD (n = 5). B: Male mice were fasted for 4 h, after which 200 μl of PBS containing 250 μCi of [^{35}S]Promix was injected into a tail vein.Thirty minutes later, P-407 (1 g/kg body weight) in saline was injected intraperitoneally. The animals were euthanized after 2 h, apoB was immunoprecipitated from 100 μl of plasma and electrophoresed on SDS-5% polyacrylamide gels, and the gels were dried and exposed to X-ray films. Data are averages ± SD (n = 3). * $P < 0.05$.

for VLDL assembly in vivo is not known, but studies using P-407 suggested that, contrary to Triton WR-1339, this treatment does not lead to the accumulation of hepatic TG (28). Our in vitro studies in isolated hepatocytes also suggested that P-407 does not enter the cellular compartment where TGH resides, as incubations of hepatocytes with increasing concentrations of the detergent (up to 50 μM) did not result in the accumulation of intracellular TG or the inhibition of apoB secretion, an effect that is observed upon the inhibition of hepatic TGH activity (21).

Other studies addressing whether the overexpression of enzymes involved in VLDL core lipid synthesis increases apoB lipidation and secretion in vivo are either limited or controversial. Overexpression of diacylglycerol acyltransferase 1 (DGAT1), ACAT1, or ACAT2 via transfection of McArdle RH7777 cells resulted in increased VLDL secretion (40). Adenovirus-mediated hepatic overexpression of microsomal triglyceride transfer protein (41) and ACAT1 (42) in mice also augmented VLDL secretion. However, adenoviral delivery of DGAT1 or DGAT2 to mice has been reported to either increase (43) or not change (44) hepatic VLDL secretion. Therefore, studies investigating increased activities of individual gene products involved in the provision of lipids for VLDL assembly in vivo have in general failed to unequivocally demonstrate a stimulatory role for any particular enzyme.

On the other hand, several studies in which the expression of genes coding for enzymes catalyzing the synthesis or transfer of lipids that have been ablated have shown aberrant assembly of VLDL. Mice deficient in phosphatidylethanolamine *N*-methyltransferase, an enzyme responsible for 30% of the hepatic production of phosphatidylcholine, secrete less VLDL (8). Similarly, mice lacking the expression of hepatic cytidylyltransferase-α, a rate-limiting enzyme responsible for providing substrate for 70% of hepatic phosphatidylcholine production, have reduced VLDL secretion (45). Inhibition of cholesterol synthesis also decreases VLDL secretion (46–50), as does inhibition or genetic ablation of microsomal triglyceride transfer protein (51). Interestingly, although the availability of neutral lipids that constitute the core of VLDL (TG and cholesteryl ester) has been considered to be the major regulator of VLDL assembly, studies using mice lacking ACAT2, the enzyme responsible for hepatic cholesteryl ester synthesis, or DGAT1, one of the two enzymes responsible for hepatic TG synthesis, failed to find a regulatory role of either enzyme in the process. Although ablation of ACAT2 resulted in the nearly complete absence of hepatic cholesteryl esters, secretion of apoB containing large amounts of TG persisted (52). Similarly, ablation of DGAT1 expression did not change plasma TG levels (53), suggesting that TG synthesized by the other DGAT (DGAT2) could be the primary source for the lipidation of apoB. DGAT2-deficient mice are lipopenic and die soon after birth; therefore, the evaluation of the role of this enzyme in VLDL assembly has not yet been possible (54).

In summary, our data suggest that increased hepatic TGH activity couples the provision of substrates for the synthesis of TG with apoB-containing lipoprotein secretion. Therefore, TGH may catalyze one of the important steps in the mobilization of lipids for lipoprotein assembly and secretion.

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