Short-term overexpression of DGAT1 or DGAT2 increases hepatic triglyceride but not VLDL triglyceride or apoB production

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Abstract **Increased triglyceride synthesis resulting from enhanced flux of fatty acids into liver is frequently associated with VLDL overproduction. This has led to the common belief that hepatic triglyceride synthesis can directly modulate VLDL production. We used adenoviral vectors containing either murine acyl-coenzyme A:diacylglycerol transferase 1 (DGAT1) or DGAT2 cDNA to determine the effect of a short-term increase in hepatic triglyceride synthesis on VLDL triglyceride and apolipoprotein B (apoB) production in female wild-type mice. Hepatic DGAT1 and DGAT2 overexpression resulted in 2.0-fold and 2.4-fold increases in the triglyceride content of liver, respectively. However, the increase in hepatic triglyceride content had no effect on the production rate of VLDL triglyceride or apoB in either case. Liver subfractionation showed that DGAT1 and DGAT2 overexpression significantly increased the content of triglyceride within the cytoplasmic lipid fraction, with no change in the triglyceride content of the microsomal membrane or microsomal VLDL. The increased cytoplasmic triglyceride content was observed in electron micrographs of liver sections from mice overexpressing DGAT1 or DGAT2. Overexpression of DGAT1 or DGAT2 resulted in enhanced [^{3}H]glycerol tracer incorporation into triglyceride within cytoplasmic lipids. These results suggest that increasing the cytoplasmic triglyceride pool in hepatocytes does not directly influence VLDL triglyceride or apoB production. In the presence of adequate cytoplasmic lipid stores, factors other than triglyceride synthesis are rate-limiting for VLDL production.**—Millar, J. S., S. J. Stone, U. J. F. Tietge, B. Tow, J. T. Billheimer, J. S. Wong, R. L. Hamilton, R. V. Farese, Jr., and D. J. Rader. **Short-term overexpression of DGAT1 or DGAT2 increases hepatic triglyceride but not VLDL triglyceride or apoB production.** ***J. Lipid Res.*** **2006.** 47: **2297–2305.**

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The assembly of VLDL within the endoplasmic reticulum (ER) is dependent on an adequate supply of neutral lipids, triglyceride and cholesteryl ester, which occupy the core of nascent VLDL. Studies in cultured cells have shown that low availability of neutral lipids for lipoprotein assembly, attributable either to reduced rates of triglyceride and cholesteryl ester synthesis or to a lack of the lipid transfer protein microsomal triglyceride transfer protein (MTP), results in a large proportion of translated apolipoprotein B (apoB) being misfolded and targeted for degradation within the ER (1). Consequently, there is decreased secretion of VLDL and apoB from the hepatocyte under these conditions. When the supply of neutral lipids for lipoprotein synthesis is abundant, a greater proportion of translated apoB is correctly folded and secreted as VLDL (1–3).

Whereas a relatively low availability of neutral lipids, such as triglycerides, clearly decreases the secretion of apoB from liver, an overabundance of triglycerides in liver is conventionally thought to increase hepatic apoB secretion. Although the type of fat consumed can influence VLDL secretion after nascent VLDL formation in the ER (4), in general, conditions in which there is an increased availability of triglyceride or triglyceride precursors to the hepatocyte have resulted in enhanced VLDL triglyceride and apoB secretion (5, 6). Similarly, an overproduction of VLDL triglyceride and apoB is seen in humans and animals consuming high-fat or lipogenic diets and under conditions, such as insulin resistance, in which triglyceride precursors are abundant in plasma (7, 8).

Acyl-coenzyme A:diacylglycerol transferases (DGATs) are enzymes that mediate the final and only committed

Abbreviations: AdmDGAT1 or AdmDGAT2, recombinant adenovirus containing the murine DGAT1 or DGAT2 cDNA; AdNull, recombinant adenovirus containing no transgene; ALT, alanine aminotransferase; apoB, apolipoprotein B; DGAT, acyl-coenzyme A:diacylglycerol transferase; ER, endoplasmic reticulum; GFP, green fluorescent protein; MTP, microsomal triglyceride transfer protein.

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step in triglyceride synthesis from acyl-CoA and diacylglycerol within the ER (9). To date, two mammalian DGATs (DGAT1 and DGAT2) have been cloned (10, 11). DGAT1 and DGAT2 are structurally unrelated and have different but overlapping tissue expression patterns (10, 11). The DGAT enzymes are membrane-associated enzymes whose activity has been detected on the cytosolic and luminal sides of the ER (12, 13). The presence of DGAT activity within the ER, where VLDL assembly occurs, suggests that one or both DGATs may play a direct role in synthesizing triglyceride for use in the assembly of apoB-containing lipoproteins. Both DGAT1 and DGAT2 are expressed in mouse liver (10, 11). DGAT1 knockout mice have normal plasma triglyceride levels, suggesting that this enzyme may not play a major role in modulating hepatic lipoprotein production (14). However, lipoprotein synthesis and secretion in DGAT1 knockout mice have not been studied extensively. Liang et al. (15) have shown that overexpression of DGAT1 and DGAT2 in a rat hepatoma cell line results in increased secretion of triglyceride and apoB. DGAT2 knockout mice, although not viable for more than 8 h after birth, have significantly lower plasma triglyceride levels than heterozygous knockout or wild-type littermates, suggesting that triglyceride synthesis by DGAT2 may modulate the production of apoB-containing lipoproteins (16).

In this study, we created recombinant adenoviruses containing the cDNA of either murine DGAT1 (AdmDGAT1) or DGAT2 (AdmDGAT2) and used these to overexpress DGAT1 and DGAT2 in mouse liver. Our goal was to determine the effect of increased hepatic triglyceride synthesis by these enzymes on triglyceride and apoB production rates. Our results show that DGAT1 or DGAT2 overexpression in wild-type mouse liver results in increased hepatic triglyceride content, with no effect on the VLDL triglyceride or apoB production rate.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Female human apoB transgenic mice on a C57BL/6 background (17), bred at the University of Pennsylvania, were also used in some experiments to measure triglyceride and the production VLDL apoB involving DGAT1. All mice were maintained on a chow diet (No. 5001; LabDiet, St. Louis, MO) containing 4.5% fat by weight (1.5% saturated, 1.6% monounsaturated, and 1.4% polyunsaturated). Blood samples were collected from mice fasted for 4 h and bled from the retro-orbital plexus using heparinized capillary tubes. Unless indicated otherwise, data presented are from experiments that included control [injected with recombinant adenovirus containing no transgene (AdNull)], DGAT1 (injected with AdmDGAT1), and DGAT2 (injected with AdmDGAT2) groups. Mice were injected intravenously (tail vein) with adenoviral preparations at a dose of 1×10^{11} viral particles in 100 μl of PBS per mouse. Blood samples were collected at baseline (day 0) and at day 4 after adenoviral injection. Serum was assayed for total cholesterol, triglycerides, and HDL as described previously (18). All experiments were terminated on day 4 after adenoviral injection. All procedures conducted in mice were in accordance with University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Construction of recombinant DGAT1 and DGAT2 adenoviral vectors

Plasmids containing the full-length cDNA of either murine DGAT1 (10) or murine DGAT2 (11), both of which include a FLAG epitope, or containing green fluorescent protein (GFP) were subcloned into the shuttle plasmid vector pAdCMVlink (18), generating the plasmids pAdCMVmDGAT1, pAdCMVmDGAT2, and pAdCMVGFP. Recombinant adenoviruses were produced with established methods (18) using adenoviral DNA containing a temperature-sensitive mutation (ts125) in the E2A region that renders the adenovirus incapable of replicating at 39°C. In this way, four recombinant adenoviruses containing mDGAT1 (AdmDGAT1), mDGAT2 (AdmDGAT2), GFP (AdGFP), or no transgene (AdNull), the latter two being controls, were generated. Recombinant adenovirus was grown, screened by PCR, subjected to two subsequent rounds of plaque purification, purified, and stored as described (18).

DGAT activity assays

DGAT activity assays were performed by measuring activity under apparent V_{max} conditions in tissue membranes (10–50 μg of protein) as described (11). In brief, these assays measured the incorporation of [^{14}C]oleoyl-CoA (specific activity, ~20,000 dpm/nmol) into triglycerides in a 5 min assay using 0.4 mM diacylglycerol and 25 μM oleoyl-CoA as substrates. Lipids were extracted with chloroform-methanol (2:1, v:v), dried under N_2, and separated by TLC in hexane-ethyl ether-acetic acid (80:20:1, v:v:v). Radioactivity in the triglyceride band was measured by scintillation counting.

Western blotting

Livers were perfused with ice-cold PBS, dissected, patted dry, weighed, and sectioned. A section was immediately homogenized in the presence of protease inhibitors and frozen in liquid nitrogen. Western blotting for DGAT1 and DGAT2 in mouse liver was performed on mouse liver homogenates using anti-FLAG M2 antibody (Sigma-Aldrich). Western blotting of liver subfractions was done using anti-adipophilin (American Research Products, Inc.), which cross-reacts with the mouse homolog adipose differentiation-related protein, anti-calnexin (Sigma-Aldrich), and anti-mouse apoB (Biodesign International).

Determination of hepatic triglyceride and apoB production rates

Hepatic triglyceride production rates were determined using the detergent P-407 as described previously (19). To determine apoB production rates, mice were injected via tail vein with 500 μCi of [^{35}S]methionine (Perkin-Elmer, Wellesley, MA) in 100 μl of PBS 5 min after the intraperitoneal injection of P-407. Blood was sampled before injection (0 h) and at 1 h and 2 h after injection. Similar results were obtained using the detergent Triton WR1339 (20).

Triglycerides were measured enzymatically using Wako reagents (Wako Chemicals USA, Inc., Richmond, VA). Total and VLDL triglyceride concentrations were expressed as mg/kg, assuming a plasma volume of 3.5% of body weight. Triglyceride production rates, in mg/kg/h, were calculated by subtracting the baseline value from the 2 h value and then expressed per hour (19).

Plasma for the baseline, 1 h, and 2 h samples was subjected to ultracentrifugation to isolate the VLDL fraction for the calcula-

tion of VLDL triglyceride production rate and VLDL apoB production rate.

VLDL (d < 1.006 g/ml) was separated from plasma by ultracentrifugation at 90,000 rpm for 3 h in a TL 100 ultracentrifuge (Beckman) using a TLA 100.2 rotor (Beckman). VLDL was subjected to SDS-PAGE, and protein bands were visualized with Coomassie blue staining. ApoB was cut from the gel, eluted with Solvable (Packard, Meriden, CT) according to the manufacturer's instructions, and subjected to scintillation counting. ApoB production rates, expressed as cpm/μl plasma/h, were calculated as the background-corrected counts at the 2 h time point, adjusted for plasma volume, and expressed per hour.

Determination of lipolysis/reesterification rates

Primary hepatocytes were isolated after liver perfusion with Liver Perfusion Medium (Invitrogen, Carlsbad, CA) followed by Liver Digest Medium (Invitrogen). Digested livers were minced, strained through a 40 μm filter, washed twice in DMEM, and purified in a Percoll density gradient. Cells were resuspended in DMEM containing 10% FBS supplemented with insulin (20 mU/ml), dexamethasone (25 nM), and antibiotics and were plated at a density of 400,000 cells/ml onto type I collagen-coated plates and left to attach for 4 h in a 5% CO_2 incubator at 37°C. After 4 h, medium was replaced with adenovirus-containing medium at a multiplicity of infection of 3,000 and incubated for 18 h. After 18 h, medium was replaced with DMEM containing 10% FBS supplemented with antibiotics containing 10 μCi/ml [^{3}H]glycerol (38.9 μCi/nmol) and 1 μCi/ml of [^{14}C]oleic acid (54 μCi/μmol). Cells were labeled for 4 h and washed three times, and a portion from each group was harvested for the determination of initial cellular triglyceride radioactivity. The remaining cells were incubated in DMEM containing 10% FBS supplemented with antibiotics for 18 h, at the end of which they were washed three times and harvested for the determination of final radioactivity. Lipids were extracted with chloroform-methanol (2:1, v/v), dried under N_2, and separated by TLC in hexane-diethyl ether-acetic acid (170:30:1, v/v/v). Radioactivity in the triglyceride band was measured by scintillation counting.

Gel filtration analysis

Pooled plasma samples from six mice of the same experimental group were subjected to fast-protein liquid chromatography gel filtration using two Superose 6 columns (Pharmacia LKB Biotechnology) as described (21). Samples were chromatographed at a flow rate of 0.5 ml/min, and fractions of 500 μl each were collected. Individual fractions were assayed for cholesterol concentration using a commercially available assay kit (Wako Pure Chemical Industries, Ltd.).

Subcellular fractionation

Livers were subfractionated into cytoplasmic, microsomal membrane, and microsomal VLDL fractions according to Chao, Stiers, and Ontko (22). Briefly, freshly perfused liver sections were weighed and minced with a scalpel. Buffer (0.25 M sucrose and 10 mM HEPES, pH 7.4) was then added, and livers were homogenized using a PowerGen 125 homogenizer (Fisher Scientific) for 30 s. Homogenates were spun at low speed (9,000 *g*) to remove cellular debris followed by high-speed centrifugation (105,000 *g*) to separate the cytoplasmic lipid fraction from the intact microsomal membrane fraction. The pelleted microsomal membranes were then ruptured by sonication followed by separation of the microsomal membrane and microsomal VLDL by high-speed centrifugation.

Liver ultrastructure analysis

Mice were perfused through the left ventricle with a flush of 0.1 M sodium cacodylate buffer, pH 7.4, followed by a fixative of 1.5% glutaraldehyde, 4% polyvinylpyrrolidone, 0.05% calcium chloride, and 0.1 M sodium cacodylate, pH 7.4. Tissues were stained for lipid by the imidazole-buffered osmium tetroxide procedure (23), en bloc stained in 2% aqueous uranyl acetate for 1 h at 4°C, processed, and embedded in Epon 812. Ultrathin sections were stained for 5 min with 0.8% lead citrate and photographed with the Siemens Elmiskop 101 (Siemens/CTI Corp., Knoxville, TN).

Statistics

Values are presented as means ± SD. Results were analyzed by Student's *t*-test for independent samples (two-tailed). Data for alanine aminotransferase (ALT), triglyceride mass, and radioactivity within the cytoplasmic lipid fraction were log-transformed before statistical analysis. Statistical significance for comparisons was assigned at $P < 0.05$.

RESULTS

Mice injected with AdmDGAT1 expressed a protein with a molecular mass of ~47 kDa in liver homogenates that reacted with anti-FLAG (**Fig. 1**). Expression of DGAT1 was associated with a 3.4-fold increase in total DGAT activity in liver compared with mice injected with control adenovirus (Fig. 1). Biochemical analysis showed significantly higher triglyceride content in livers from mice overexpressing DGAT1, the hepatic triglyceride being ~2-fold that found in control mice (**Table 1**). The content of total cholesterol or phospholipid (data not shown) in liver was unchanged as a result of DGAT1 overexpression. Although control mice had normal ALT levels (Table 1), indicating no hepatotoxic effect of the viral load, ALT levels were increased in plasma of mice overexpressing DGAT1, likely in response to increased hepatic lipid content.

Although overexpression of DGAT1 in liver increased hepatic triglyceride content, there was no effect on plasma lipid levels (Table 1) or lipoprotein profile (**Fig. 2**). This was consistent with the results from metabolic studies in which there were no differences in the hepatic production rate of the triglyceride or VLDL apoB DGAT1 overexpression (**Fig. 3**). Similar results were obtained when studies were conducted in female human apoB transgenic mice overexpressing DGAT1 compared with GFP-expressing controls (data not shown).

We next studied mice overexpressing DGAT2 after injection with adenovirus containing the cDNA for DGAT2. Mice injected with AdmDGAT2 expressed a protein with a molecular mass of ~44.5 kDa that reacted with anti-FLAG (Fig. 1). The levels of DGAT2 protein overexpression were similar to those for DGAT1. This was associated with a 1.4-fold increase in total DGAT activity in liver compared with mice injected with control adenovirus (Fig. 3). There was a significantly greater triglyceride content of livers from mice overexpressing DGAT2, the hepatic triglyceride content being ~2.5-fold higher than that found in control mice (Table 1). There were no significant changes in the

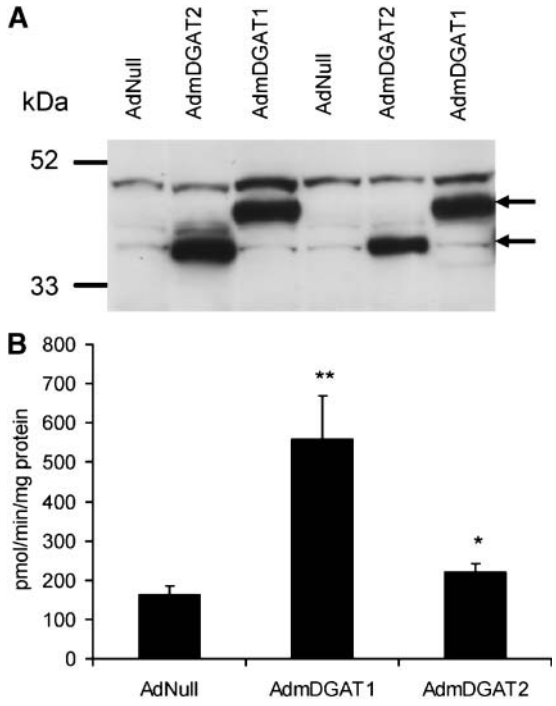

Fig. 1. Expression of transfected acyl-coenzyme A:diacylglycerol transferase 1 (DGAT1) and DGAT2 in mouse liver. A: Mice infected with recombinant adenovirus containing the murine DGAT1 cDNA (AdmDGAT1) expressed a protein with an estimated molecular mass of 47 kDa (upper arrow) that was detectable using anti-FLAG antiserum and that was not detected in mice infected with control adenovirus. Mice infected with AdmDGAT2 expressed a protein with an estimated molecular mass of 45 kDa (lower arrow) using anti-FLAG antiserum. There is a minor, nonspecific, anti-FLAG-reactive band visible in the recombinant adenovirus containing no transgene (AdNull)- and AdmDGAT1-expressing mice that comigrates with the DGAT2 band. B: Liver homogenates from AdmDGAT1-infected mice had 3.4-fold higher total DGAT activity than liver homogenates from mice infected with control adenovirus. Liver homogenates from AdmDGAT2-infected mice had 1.4-fold higher total DGAT activity than liver homogenates from mice infected with control adenovirus (n = 4 per group). Values are presented as means ± SD. * $P = 0.01$, ** $P = 0.0001$.

total cholesterol or phospholipid (data not shown) contents in mice overexpressing DGAT2 in liver. Compared with control mice, ALT levels were also increased in plasma of mice overexpressing DGAT2 (Table 1), likely in response to increased hepatic lipid content.

Similar to what was observed with DGAT1, the increased hepatic triglyceride content attributable to DGAT2 overexpression had no effect on plasma lipid levels (Table 1) or lipoprotein profile (Fig. 2). Lipoprotein production studies showed no changes in the hepatic production rate of triglyceride or VLDL apoB (Fig. 3).

To determine the subcellular location of the increased triglyceride mass measured in livers of mice overexpressing DGAT1 or DGAT2, we subfractionated livers into cytoplasmic, microsomal membrane, and microsomal VLDL fractions. The cytoplasmic fraction from livers of mice overexpressing DGAT1 and DGAT2 had a triglyceride mass that was significantly greater than that of control mice. The triglyceride masses of the microsomal membrane and microsomal VLDL were similar among the three groups of mice (**Fig. 4A**). We next examined the incorporation of [^{3}H]glycerol into triglyceride from each fraction as a measure of newly synthesized triglyceride in each subfraction (Fig. 4B). The synthesis of cytoplasmic triglyceride was greatest for all groups and was increased in mice overexpressing DGAT1 and DGAT2 compared with control mice. There were no differences in the amount of newly synthesized triglyceride in the microsomal membrane or microsomal VLDL fraction. The specific activity of the triglyceride was also greatest in the cytoplasmic fraction of mice overexpressing either DGAT1 or DGAT2 compared with control mice (Fig. 4C), although not statistically different. There was no difference in the triglyceride specific activity in VLDL within plasma among the three groups of mice (data not shown). Marker proteins for each subfraction are shown in Fig. 4D.

To confirm the lack of effect of DGAT1 or DGAT2 overexpression on the triglyceride and apoB production rates, the presence of VLDL within the secretory pathway was examined in electron micrographs of liver sections from mice overexpressing either DGAT1 or DGAT2 compared with those from mice infected with control adenovirus on day 4 after adenoviral injection (**Fig. 5**). Compared with control mice, mice overexpressing DGAT1 and DGAT2 had modestly increased numbers of lipid droplets in hepatocytes. Also, more and larger lipid droplets were observed within stellate (Ito cells) cells that store vitamin A esters in both DGAT-overexpressing livers (data not shown). Nascent VLDL particles within Golgi secretory vesicles were uniformly ~50 nm in diameter in control hepatocytes. Although increased numbers of lipoprotein

TABLE 1. Plasma and liver lipid levels in female control mice and those overexpressing murine DGAT1 and DGAT2 4 days after adenoviral infection

Adenovirus	TC (n = 8)	TG (n = 8)	HDL (n = 8)	Alanine Aminotransferase (n = 4)	Liver TG (n = 6)	Liver TC (n = 6)
	mg/dl			*U/l*	*mg/g liver*	
AdNull	58 ± 10	90 ± 12	44 ± 7	44 ± 4	18.8 ± 5.5	5.4 ± 0.7
DGAT1	57 ± 9	88 ± 21	45 ± 6	102 ± 57[a]	35.2 ± 12.2[a]	5.5 ± 0.9
DGAT2	64 ± 9	84 ± 13	47 ± 5	132 ± 60[b]	42.6 ± 10.1[a]	5.5 ± 0.8

AdNull, recombinant adenovirus containing no transgene; DGAT, acyl-coenzyme A:diacylglycerol transferase; TC, total cholesterol; TG, triglyceride.

[a] $P < 0.05$ versus AdNull.

[b] $P < 0.01$ versus AdNull.

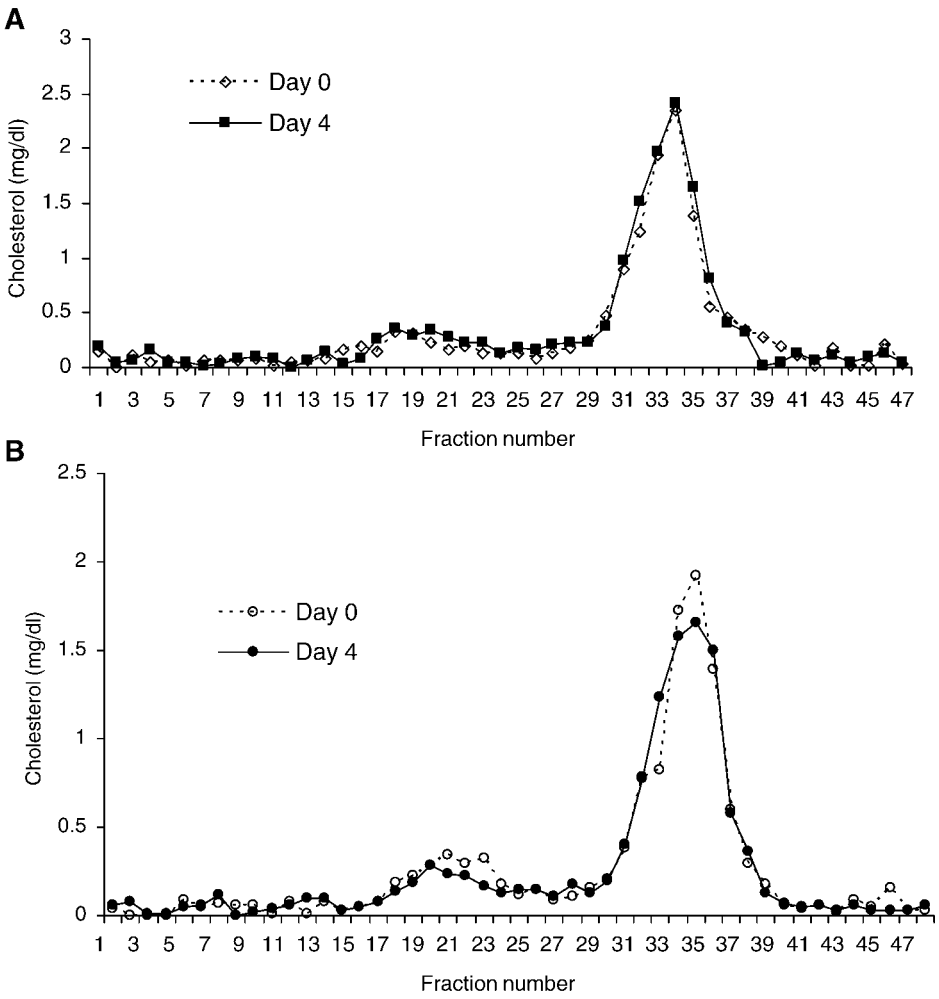

Fig. 2. Lipoprotein profiles of mice overexpressing DGAT1 or DGAT2. Pooled plasma (n = 6 mice per pool) from mice obtained on day 4 after adenoviral infection showed that overexpressing DGAT1 (A) or DGAT2 (B) resulted in no differences in lipoprotein compared with the day 0 profile.

particles were not observed in secretory vesicles of livers overexpressing either DGAT enzyme, in some hepatocytes of both DGAT-overexpressor livers, many nascent VLDL particles within Golgi secretory vesicles were larger and more variable in diameter, some as large as 150 nm.

Because larger VLDL particles were identified in the Golgi of some hepatocytes of mice overexpressing DGAT1 and DGAT2 but no increase in plasma triglyceride production rate was found for either group, it is possible that there was enhanced lipolysis/reesterification of VLDL triglyceride within the Golgi. We measured the rate of lipolysis/reesterification of triglyceride in primary hepatocytes infected with DGAT1, DGAT2, or control adenovirus (AdNull) by labeling cells with [^{3}H]glycerol or [^{14}C]oleic acid and measuring the change in the ratio of these labels in total cellular triglyceride after 18 h. As shown in **Fig. 6A**, cells expressing DGAT1 and DGAT2 had a higher percentage of tracer remaining, indicating reduced turnover of the glycerol backbone of triglyceride compared with control cells. A similar result was obtained for triglyceride [^{14}C]oleic acid, indicating that turnover was reduced significantly in cells expressing DGAT2 (Fig. 6B). The net effect of this was that the rate of lipolysis/reesterification of triglyceride was similar between cells expressing DGAT2 and control adenovirus, whereas the rate was reduced in cells expressing DGAT1 (Fig. 6C).

DISCUSSION

Conditions in which there are increased plasma free fatty acid levels are frequently associated with increased hepatic triglyceride content and an overproduction of VLDL. The availability of neutral lipid for VLDL synthesis is thought to be a major factor in the control of VLDL production. An abundance of hepatic triglyceride is believed to provide excess substrate for VLDL synthesis, leading to VLDL overproduction, possibly by rescuing apoB from degradation (24, 25). We sought to test the hypothesis that enhanced hepatic triglyceride synthesis influences the VLDL production rate. We used overexpression of two enzymes resident in the ER of the liver,

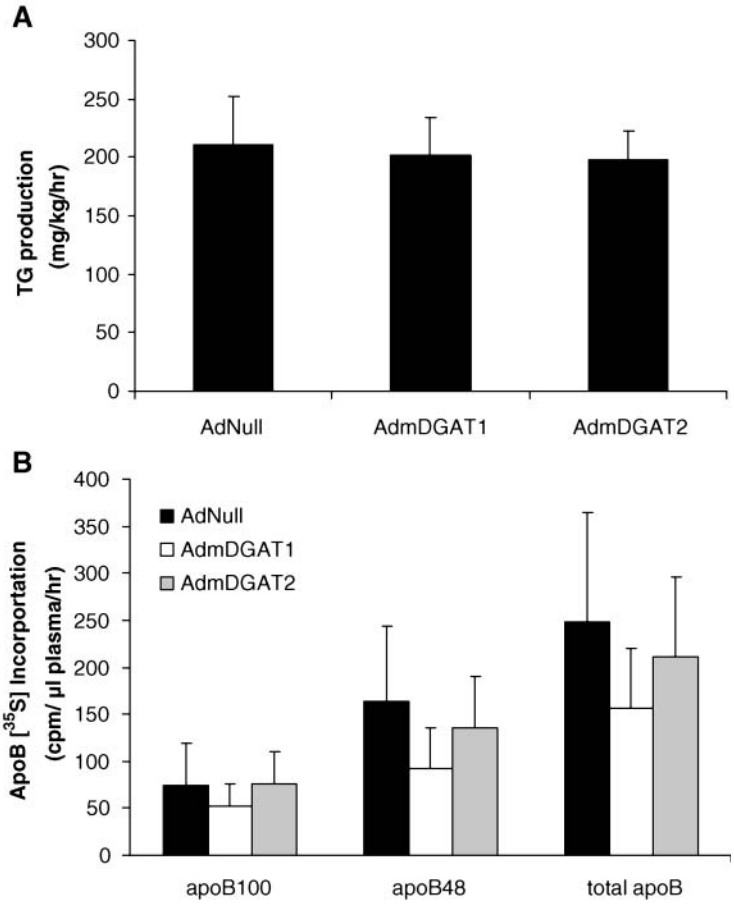

Fig. 3. Triglyceride and apolipoprotein B (apoB) production rates in mice overexpressing DGAT1 or DGAT2. Mice overexpressing DGAT1 or DGAT2 showed no differences in plasma triglyceride (TG) production rate (n = 11 per group) (A) or in production rates of apoB-48, apoB-100, or total apoB (B). Results shown are from production studies conducted using P-407 (n = 11 for the AdNull group; n = 10 for the DGAT1 and DGAT2 groups). Values are presented as means ± SD.

DGAT1 and DGAT2, that catalyze the final step in triglyceride synthesis. We found that overexpression of either of these enzymes in liver led to increased hepatic triglyceride synthesis without changing VLDL production.

The lack of an effect of DGAT1 on VLDL production with short-term overexpression in livers of female mice is consistent with the phenotype of the DGAT1 knockout mouse (14). The DGAT1 knockout mouse has a normal fasting plasma triglyceride level despite having an 82% lower hepatic triglyceride content than control mice. In this study, mice overexpressing DGAT1 in liver accumulated triglyceride in the cytoplasmic lipid fraction while maintaining normal triglyceride levels within the microsomal lumen. Thus, it appears that increased triglyceride synthesis by overexpression of DGAT1 is, in itself, insufficient to increase VLDL production.

Because short-term DGAT1 overexpression did not modulate the VLDL production rate in wild-type mice, we next addressed the hypothesis that DGAT2 overexpression would increase triglyceride synthesis and lead to enhanced VLDL production. This role would be consistent with the model proposed by Owen, Corstorphine, and Zammit (12), in which there are two independent sources of DGAT activity in the ER: an overt activity that functions in cytoplasmic triglyceride synthesis, and a latent activity that functions to produce triglyceride for lipoprotein synthesis. We found that, contrary to our hypothesis, DGAT2 overexpression also increased the cytoplasmic triglyceride content similar to DGAT1 (i.e., both DGAT1 and DGAT2 exhibited overt activity in vivo) and similarly had no effect on VLDL production. The increase in cytoplasmic triglyceride levels is in agreement with the observation that the DGAT2 knockout mouse has a near absence of triglyceride in all tissues, including adipose tissue (16). Thus, although DGAT1 and DGAT2 are both capable of synthesizing cytoplasmic triglyceride, under normal conditions DGAT2 mediates the synthesis of the majority of cytoplasmic triglyceride stores in murine tissues.

Our data, when viewed in the light of current studies in the literature, suggest that increasing triglyceride synthesis alone in liver influences hepatic triglyceride and apoB production only under conditions in which triglyceride is rate-limiting for VLDL synthesis. Liang et al. (15) showed that DGAT1 overexpression increased triglyceride and apoB secretion from the rat hepatoma cell line, McArdle 7777. These cells have relatively low levels of triglyceride, ~5% of those found in livers of mice in the current study (26). As proposed by Wang et al. (27), this would explain why McArdle 7777 cells respond to oleate stimulation of lipogenesis but primary hepatocytes do not. Under conditions in which hepatic triglyceride stores are sufficient to maintain basal levels of lipoprotein production, increasing hepatic triglyceride synthesis has no influence on VLDL production; therefore, other factors must be rate-limiting under these conditions. The DGAT2 knockout mouse, a model displaying reduced plasma lipid levels, may be an

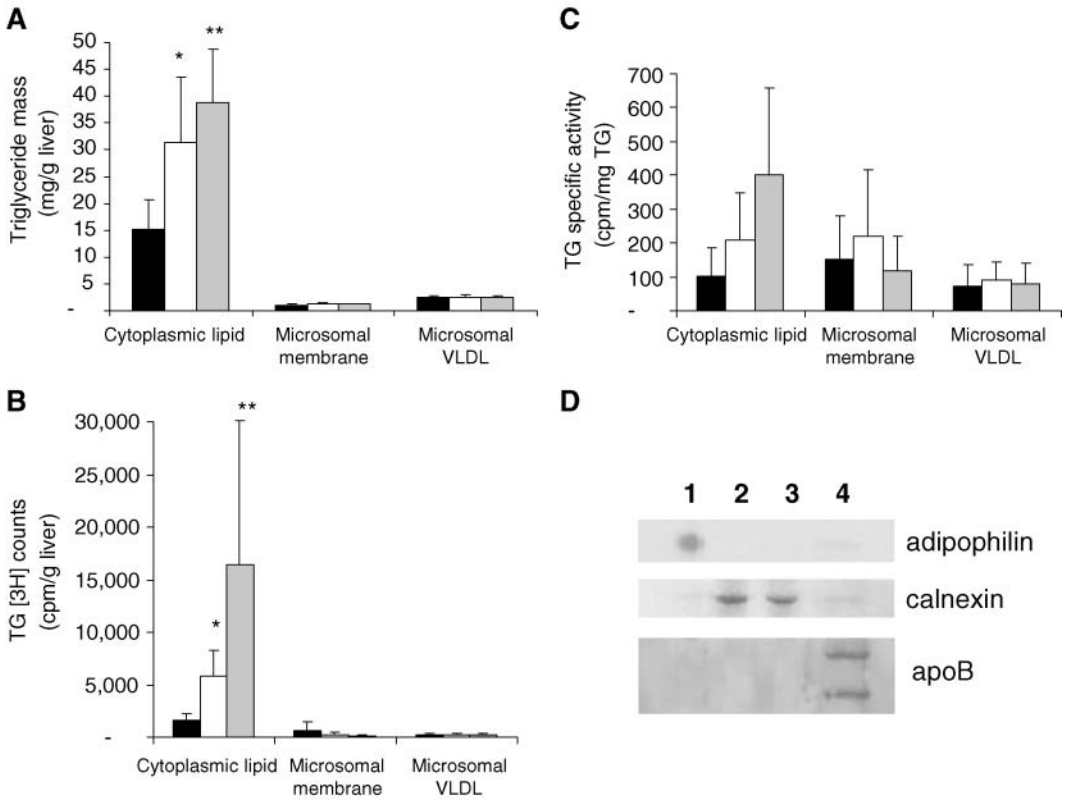

Fig. 4. Triglyceride mass and triglyceride specific activity of liver subfractions of mice overexpressing DGAT1 or DGAT2 (n = 4 per group). Mice injected with 20 μCi of [^{3}H]glycerol were euthanized at 2 h. Perfused livers were homogenized, and liver subfractions were isolated by ultracentrifugation. A: The triglyceride mass of the subfractions showed that DGAT1 and DGAT2 overexpression increased the triglyceride content in cytoplasm but not in the microsomal membrane or lumen. B: Triglyceride (TG) incorporation of [^{3}H]glycerol at 2 h showed the greatest incorporation of label into cytoplasmic triglyceride. There were significant differences between groups in cytoplasmic and microsomal membrane triglyceride content, whereas there were no differences found for triglyceride within the microsomal lumen. Closed bars, AdNull; open bars, AdmDGAT1; gray bars, AdmDGAT2. Values are presented as means ± SD. * $P < 0.05$, ** $P < 0.01$. C: Triglyceride specific activity at 2 h showed differences between groups in cytoplasmic and microsomal membranes, whereas no differences were found for triglyceride within the microsomal lumen. Values are presented as means ± SD. D: Protein markers for each lipid subfraction. Each lane was loaded with 30 μg of protein. Lane 1, cytoplasmic lipid; lane 2, total (undisrupted) microsomes; lane 3, sedimented membranes after microsomal disruption; lane 4, microsomal VLDL containing both apoB-100 (upper band) and apoB-48 (lower band). The lack of apoB reactivity in lane 2 reflects the minor amount of apoB present relative to total protein in the total microsomal fraction.

in vivo model in which triglyceride synthesis is rate-limiting for VLDL assembly and secretion. However, the DGAT1 knockout mouse, despite having mildly impaired triglyceride synthesis, maintains sufficient hepatic triglyceride stores to sustain a normal triglyceride production rate in short-term studies of lipoprotein production (J. T. Billheimer, unpublished data). Consistent with our data, wild-type mice fasted for 16–18 h have markedly increased hepatic triglyceride levels (28, 29) but no changes in the rate of VLDL production (29).

One possibility explaining the lack of effect of short-term DGAT1 and DGAT2 overexpression on triglyceride production is that the mobilization of cytoplasmic triglyceride stores at or near the ER membrane is rate-limiting for VLDL assembly and secretion. The movement of triglyceride across the ER membrane for lipoprotein synthesis has been proposed to occur by two pathways. One involves the transport of intact triglyceride across the membrane using MTP (3). Thus, MTP could be limiting. The second pathway, known as the lipolysis/reesterification pathway (30), initially involves the hydrolysis of cytoplasmic triglyceride into diacylglycerol and fatty acid, possibly mediated by triglyceride hydrolase (26). This is followed by movement of these products across the ER membrane, where they are reconstituted into triglyceride and added to the growing lipoprotein (11, 12). Although the results of the studies measuring cellular triglyceride turnover and lipolysis/reesterification are consistent with enhanced reesterification of lipolyzed cellular triglyceride in hepatocytes expressing DGAT1 and DGAT2, it should be noted that because the vast majority of cellular triglyceride in liver is within the cytoplasm, this method tends to measure the turnover of cytoplasmic triglyceride rather than triglyceride on nascent VLDL in primary hepatocytes. Our

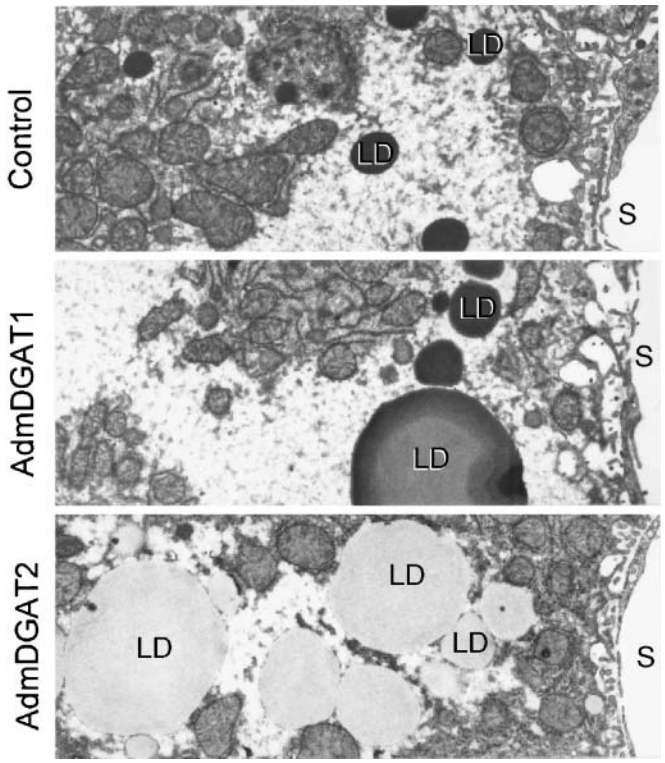

Fig. 5. Electron micrographs of hepatic cytoplasm near sinusoids (S) representative of control (top), DGAT1-overexpressing (middle), and DGAT2-overexpressing (bottom) mouse livers. Tissue was stained for lipids using imidazole-buffered osmium tetroxide (22). Both DGAT1- and DGAT2-overexpressing livers show an increase in the number and size of intracellular cytosolic lipid droplets (LD), with the DGAT2-overexpressing hepatocytes showing the most lipid deposition. The lipid droplets from livers of mice overexpressing DGAT2 are lighter than those from control and DGAT1-overexpressing mice, as a result of differences in the penetration of osmium into the numerous triglyceride-rich lipid droplet structures. Magnification, 12,000×.

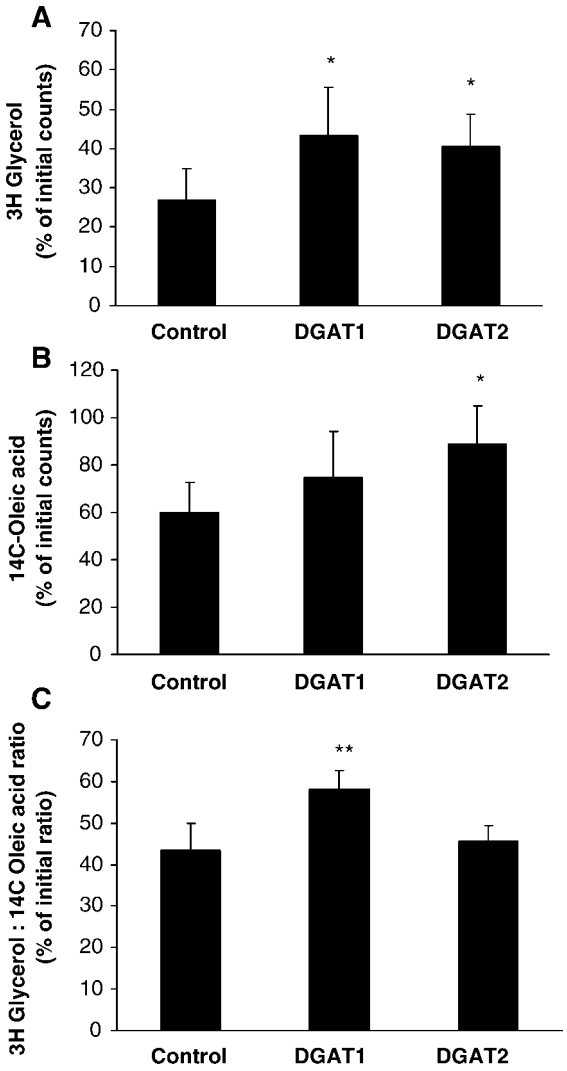

Fig. 6. Triglyceride turnover in primary hepatocytes overexpressing DGAT1 and DGAT2. A: Change from initial [^{3}H]glycerol radioactivity in total cellular triglyceride after 18 h of incubation. B: Change from initial [^{14}C]oleic acid radioactivity in total cellular triglyceride after 18 h of incubation. C: Lipolysis/reesterification of total cellular triglyceride as measured by the change in the initial ^{3}H/^{14}C ratio after 18 h of incubation. Values are presented as means ± SD. * $P < 0.05$, ** $P < 0.01$.

results suggest that neither DGAT1 nor DGAT2 overexpression increases reesterification of these triglyceride precursors in a process that is coupled with lipoprotein secretion. Our results may suggest, however, that the turnover of cytoplasmic triglyceride is reduced with DGAT1 and DGAT2 overexpression, as reflected in the increased specific activity of the triglyceride in these groups after in vivo and in vitro labeling of cellular triglyceride. Although it is possible that DGAT1 or DGAT2 overexpression could directly or indirectly interfere with the transfer of triglyceride across the ER membrane, we consider this unlikely because there was no reduction in the triglyceride production rate after overexpression of these enzymes.

Larger nascent VLDLs were observed in electron micrographs in the Golgi of some hepatocytes of DGAT1- and DGAT2-expressing mice. Yet, there was no increase in VLDL triglyceride production in these animals. One possible explanation for this discrepancy is that these particles are susceptible to oxidation and are removed from the secretory pathway before exiting the Golgi (4). If this is true, then supplementation of these animals with antioxidant-rich diets would be expected to result in increased triglyceride production in mice expressing both DGAT1 and DGAT2. This discrepancy could also be attributable to partial lipolysis of these particles before exiting the hepatocyte, a possibility we are currently investigating.

Yamazaki et al. (31) performed similar adenoviral overexpression studies in male mice and reported that DGAT1 but not DGAT2 stimulated hepatic triglyceride secretion. They also reported that DGAT1 overexpression increased "latent" triglyceride synthesis in the lumen of the ER. These results differ from ours in that we found that DGAT1 overexpression increased cytoplasmic triglyceride levels but did not change the triglyceride content of the microsomal VLDL fraction or increase triglyceride production. We believe that the reasons for these discrepancies include secondary effects of triglyceride accumulation

that may have occurred in the 12 day study conducted by Yamazaki et al. (31) but not in the 4 days of our study. We also observed increased triglyceride levels compared with control animals in male, but not female, mice 12 days after adenoviral overexpression of DGAT1 that were not apparent at day 4 (J. S. Millar, unpublished data).

In summary, we found that increasing hepatic triglyceride synthesis through overexpression of DGAT1 or DGAT2 in wild-type mouse livers had no direct effect on the hepatic triglyceride or apoB production rate. These results demonstrate conditions in which increasing hepatic triglyceride synthesis does not lead directly to an increase in the triglyceride or apoB production rate. Under these conditions, it appears that factors responsible for the mobilization of triglyceride for lipoprotein assembly and secretion are rate-limiting for hepatic VLDL production.

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