Specific Role for Acyl CoA:Diacylglycerol Acyltransferase 1 (Dgat1) in Hepatic Steatosis Due to Exogenous Fatty Acids

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Nonalcoholic fatty liver disease, characterized by the accumulation of triacylglycerols (TGs) and other lipids in the liver, often accompanies obesity and is a risk factor for nonalcoholic steatohepatitis and fibrosis. To treat or prevent fatty liver, a thorough understanding of hepatic fatty acid and TG metabolism is crucial. To investigate the role of acyl CoA:diacylglycerol acyltransferase 1 (DGAT1), a key enzyme of TG synthesis, in fatty liver development, we studied mice with global and liver-specific knockout of Dgat1. DGAT1 were required for hepatic steatosis induced by a high-fat diet and prolonged fasting, which are both characterized by delivery of exogenous fatty acids to the liver. Studies in primary hepatocytes showed that DGAT1 deficiency protected against hepatic steatosis by reducing synthesis and increasing the oxidation of fatty acids. In contrast, lipodystrophy (aP2-SREBP-1c436) and liver X receptor activation (T0901317), which increase de novo fatty acid synthesis in liver, caused steatosis independently of DGAT1. Pharmacologic inhibition of Dgat1 with antisense oligonucleotides protected against fatty liver induced by a high-fat diet. Conclusion: Our findings identify a specific role for hepatic DGAT1 in esterification of exogenous fatty acids and indicate that DGAT1 contributes to hepatic steatosis induced by this mechanism. (HEPATOLOGY 2009;50:434-442.)

Nonalcoholic fatty liver disease (NAFLD) is characterized by lipid accumulation in hepatocytes of people who consume little to no alcohol.1-3 Chronic lipid accumulation in the liver (hepatic steatosis) is a risk factor for nonalcoholic steatohepatitis (NASH), an inflammatory condition in some patients with fatty liver.4 NAFLD is the most common cause of abnormal liver enzyme tests,5 is associated with obesity and insulin resistance, and is increasing in prevalence, affecting >30 million adults in the U.S., making it the most common liver disorder.2,6 NAFLD portends epidemic problems for public health, and a better understanding of the pathways that regulate lipid accumulation in the liver is crucial for developing effective therapies for hepatic steatosis.

Lipids that accumulate in hepatic steatosis are mainly triacylglycerols (TGs). After adipose tissue, the liver has perhaps the largest capacity to synthesize and store TGs.7 TGs are products of the glycerol phosphate synthesis pathway, in which fatty acyl moieties are joined to glycerol by way of ester bonds.8,9 Fatty acids (FAs) in the liver may come from exogenous sources (e.g., dietary fat or...
mobilization from white adipose tissue [WAT] during fasting) or from endogenous de novo synthesis promoted by leptin deficiency or high levels of circulating insulin and glucose.1,10-12

The final step in TG synthesis is catalyzed by acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes. Mammals have two DGAT enzymes that are members of distinct gene families.13,14 Both are expressed widely in tissues and in the livers of mice and humans.13,15 Increased levels of DGAT1 messenger RNA (mRNA), in particular, occur in human livers with NAFLD,16 underscoring the importance of defining the role of DGAT1 in this tissue. Mice lacking Dgat1 (Dgat1−/−) are viable, have reduced tissue TG levels, exhibit increased sensitivity to insulin and leptin, and are protected against diet-induced obesity through increased energy expenditure.17,18 However, DGAT1’s function in hepatic steatosis has not been fully explored.

To investigate the role of DGAT1 in hepatic steatosis, we studied mice with global17 and liver-specific knockout of Dgat1 under conditions that promote hepatic steatosis. These included a high-fat diet, fasting (in which lipids are mobilized from the WAT to the liver), and two conditions in which endogenous FA synthesis is greatly increased—genetically induced lipodystrophy19 and treatment with the liver X receptor (LXR) agonist T0901317.20 Relevant to clinical therapies, we also determined whether knockdown of Dgat1 expression with antisense oligonucleotides (ASO) protects against diet-induced hepatic steatosis.

Materials and Methods

Mice. Dgat1−/− and wild-type mice (C57BL/6J background) were genotyped as described.17 Mice were housed in a pathogen-free barrier facility (12/12-hour light/dark cycle) and fed chow (5053 PicoLab Diet; Purina) or a high-fat diet (20% milk fat, 0.2% cholesterol by weight; TD 01064 Harlan-Teklad). Dgat1flox/flox mice were generated as described (Supporting Methods). During fasting, mice had access to water. Dgat1−/− mice were crossed with aP2-SREBP-1c Δ36 transgenic mice (Jackson Laboratory; 50% C57BL/6J, 50% SJL). Dgat1+/− aP2-SREBP-1c Δ36 mice (75% C57BL/6J, 25% SJL) were crossed with Dgat1−/− (C57BL/6J) males to generate Dgat1+/− aP2-SREBP-1c Δ36 mice (87.5% C57BL/6J, 12.5% SJL). All experiments were approved by the Committee on Animal Research, University of California, San Francisco.

Histological Analyses. See Supporting Methods.

Lipid Analyses. Livers were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, and 250 mM sucrose with complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated as described.21 TG bands were identified, scraped, and quantified spectrophotometrically.22

Adenoviruses. Cre-expressing, LacZ-expressing, and GFP-expressing adenoviruses (Vector Development Laboratory, Baylor College; 2 × 1011 particles in 0.2 mL of phosphate-buffered saline [PBS]) were injected into a jugular vein23 of Dgat1flox/flox mice (16-20 weeks old). After 4 weeks, mice were placed on a high-fat diet for 3 weeks or fasted for 20 hours.

ASOs. Control and Dgat1 ASO (Isis Pharmaceuticals) with a 2′-O-(2-methoxy)-ethyl modification at the first and last five bases24 were injected (50 mg/kg intraperitoneally) twice weekly for 5 weeks.

DGAT Activity Assays. Livers were homogenized in Buffer A (50 mM Tris-HCl, pH 7.4, 250 mM sucrose) with protease inhibitors (Roche Diagnostic). To prepare microsomes, homogenates were centrifuged three times at 4°C (600 g for 5 minutes, 10,000 g for 10 minutes, 100,000 g for 1 hour); after each centrifugation, pellets were resuspended in Buffer A. DGAT assays were performed with microsome proteins (100 μg) in mix containing 100 mM MgCl2, 1.25 mg/mL bovine serum albumin (BSA), 200 μM 1,2-dioleoyl-sn-glycerol (Sigma-Aldrich) in acetone, and 25 μM (14C)oleoyl-CoA (53.0 mCi/mMol). After 10 minutes at 37°C, lipids were extracted with chloroform:methanol (2:1, vol:vol) and separated on G-60 TLC plates with hexane:ethyl ether:acetic acid (80:20:1, vol:vol: vol). TG bands were scraped and radioactivity was measured by scintillation counting.

FA Oxidation Measurement. Hepatocytes were incubated with 3 mL of Dulbecco’s Modified Eagle Medium (DMEM) containing 200 μM [9,10-3H]oleic acid (10 μCi/mL) (Amersham) conjugated to 0.2% BSA for 2 hours. The medium was saved, cells were scraped with 1 mL of 0.1 N NaOH and 1 mL of water, and protein concentrations determined by DC protein assay (BioRad). Medium (100 μL) was placed in a microcentrifuge tube, which was placed in a 20-mL scintillation vial containing 500 μL of water and sealed and heated at 50°C for 18 hours for [3H]H2O to equilibrate. Equilibrium efficiency was determined by similar analysis of 100 μL of 0.1 μCi/μL of [3H]H2O (≈65%) or [9,10-3H]oleic acid (≈0.5%). After cooling, microcentrifuge tubes were removed, scintillation fluid (aqueous/nonaqueous) was added, and [3H]H2O was measured with a scintillation counter.

RNA Extraction and Real-time Polymerase Chain Reaction (PCR). RNA was extracted from livers with RNA STAT-60 (Tel-Test) and treated with DNase (Ambion). Complementary cDNA was synthesized from
RNA (5 μg) with Superscript II reverse transcriptase (RT) and random hexamers (Invitrogen). Real-time PCR primers (Supporting Table 2) were selected with Primer Express (v. 1.5; Applied Biosystems). Two-step RT-PCR were performed with Sybrgreen (Applied Biosystems) and an ABI 9600.

**Statistical Analyses.** Values are mean ± SEM. Means were compared by t-test or analysis of variance (ANOVA) and Student-Newman-Keuls test.

**Results**

**Hepatic DGAT1 Deficiency Protects Against High-Fat-Diet-Induced Fatty Liver.** Mice were fed a high-fat diet for 3 weeks. Dgat1+/+ livers were pale and stained positively for lipids (Fig. 1A). Lipid staining was reduced in Dgat1−/− livers, consistent with prolonged (32 weeks) high-fat feeding.17 Sirius Red staining showed similar amounts of collagen in Dgat1+/+ and Dgat1−/− livers (not shown). Hepatic TG levels were similar in chow-fed Dgat1+/+ and Dgat1−/− mice but ~80% lower in Dgat1−/− mice after high-fat feeding (Fig. 1B). All classes of FAs in TG were reduced. FA synthesis was increased in hepatocytes from Dgat1−/− mice fed a high-fat diet (Fig. 1C), consistent with reported effects,25 but reduced in hepatocytes from Dgat1−/− mice. Also reduced were mRNA levels of the lipogenic transcription factors sterol regulatory element-binding protein 1c (Srebp1c) and its targets FA synthase (Fasn) and stearoyl-CoA desaturase 1 (Scd1), and carbohydrate response element binding protein (Chrebpp) and its target liver-pyruvate kinase (L-pk) in Dgat1−/− mice.
tocytes from fat-fed

...tion was associated with increased FA oxidation in hepatic DGAT1 deficiency protects against hepatic steatosis induced by high-fat diet. (A) RT-PCR analysis of Dgat1 and Dgat2 mRNA in liver and epididymal WAT. Male Dgat1flox/flox mice received adenoviruses expressing LacZ or Cre recombinase. After 4 weeks, mice were fed a high-fat diet for 3 weeks (age 16-20 weeks, n = 5-6/genotype). *P < 0.001 versus control. (B) Microsomal hepatic DGAT activity as measured by the incorporation of [14C]-oleoyl-CoA into triglycerides (age 16-20 weeks, n = 5-6/genotype). *P < 0.001 versus control. (C, D) Livers (C) and hepatic triglyceride content (D) in mice fed high-fat diet (age 16-20 weeks, n = 5-6 per genotype). *P < 0.05 versus control.

During fasting, free FAs mobilized from the adipose tissue to liver can be esterified to form TG, which can accumulate and cause steatosis. To explore the role of DGAT1 in this process, we measured hepatic Dgat1 mRNA levels under different dietary conditions. Mice fasted for 16 hours had almost 3-fold higher hepatic Dgat1 mRNA levels than mice fed ad libitum or after refeeding (Fig. 3A). Dgat2 mRNA levels were similar during these conditions.

We therefore examined hepatic steatosis in Dgat1+/+ and Dgat1−/− mice fasted for 20 hours. Dgat1−/− livers were darker than Dgat1+/+ livers (Fig. 3B) and had ~70% lower hepatic TG content (Fig. 3C). Serum levels of the ketone ß-hydroxybutyrate, a product of FA oxidation in the liver, were slightly elevated in fasted Dgat1−/− mice, although the differences did not reach significance (0.54 ± 0.13 versus 0.32 ± 0.07 mM, n = 5/genotype, P < 0.07; 8-hour fast).

To determine if DGAT1 acts in a liver-specific manner during fasting, we examined steatosis in fasted mice. LivD1KO mice had ~75% lower hepatic Dgat1 mRNA levels than controls but similar hepatic Dgat2 mRNA levels (Fig. 3D). No apparent changes were found in the lipid oxidizing transcription factor, peroxisome proliferator-activated receptor alpha (Pparg), as well as target genes such as carnitine-palmitoyl transferase 1 (Cpt1), aldehyde dehydrogenase 3 family member A2 (Adh3a2), enoyl-coenzyme A hydratase/3-hydroxyacyl coenzyme A dehydrogenase (Ehhadh), and cytochrome P450, family 4, subfamily a, polypep-
tide 10 (Cypa10). LivD1KO mice had \( \sim80\% \) lower hepatic DGAT activity (Fig. 3E), darker livers (Fig. 3F), and \( \sim80\% \) less hepatic TG (Fig. 3G), a reduction similar to that in Dgat1\(^{-/-}\) mice. Further, Dgat1\(^{-/-}\) hepatocytes had reduced capacity to synthesize TG when FA concentrations were above 250 \( \mu \text{M} \) (Fig. 3H).

**DGAT1 Deficiency Does Not Protect Against Hepatic Steatosis Induced by Endogenous FA Synthesis.** Congenital generalized lipodystrophy is characterized by a paucity of adipose tissue, hyperinsulinemia, hyperglycemia, and hepatic steatosis. In aP2-SREBP-1c436 transgenic mice, a model of this disorder, leptin levels are reduced, leading to upregulation of FA synthesis genes and hepatic steatosis.\(^{11,12,19}\)
To determine if hepatic steatosis in this model requires DGAT1, we generated Dgat1+/H11002/H11002 aP2-SREBP-1c436 mice. Hepatic Srebp1c expression was markedly elevated in aP2-SREBP-1c436 and Dgat1+/H11002/H11002 aP2-SREBP-1c436 mice, as were mRNAs for SREBP1c targets and Dgat2 mRNA levels (Fig. 4A). In both genotypes, hepatic TG content was similarly increased to higher levels than in Dgat1+/+ and Dgat1+/− controls (Fig. 4B). Unlike livers of Dgat1+/+ and Dgat1+/− mice, livers of aP2-SREBP-1c436 and Dgat1−/− aP2-SREBP-1c436 mice were pale, enlarged, and stained positively for lipids (Fig. 4C).

To test the hypothesis that DGAT1 is not required for hepatic steatosis from increased de novo FA synthesis, we treated Dgat1+/+ and Dgat1−/− mice with an LXR agonist, T0901317, which activates FA synthesis in the liver and leads to hepatic steatosis.20 As expected, the mice had increased expression of Fasn and Scd1 (Fig. 4D). Consistent with our hypothesis, Dgat1+/+ and Dgat1−/− livers had similar degrees of steatosis after 2 weeks of treatment (Fig. 4E).

Inhibiting DGAT1 Protects Against High-Fat-Diet-Induced Fatty Liver. Next, we assessed ASO-mediated knockdown of Dgat1 as a treatment for fatty liver induced by a high-fat diet. Dgat1 ASO reduced hepatic Dgat1 mRNA levels without altering Dgat2 expression (Fig. 5A). ASO pretreatment reduced by ~40% the increase in hepatic TG content after 1 week of a high-fat diet (Fig. 5B).

Discussion

This study shows that hepatic steatosis induced in mice by a high-fat diet or fasting, which promote hepatic uptake of exogenous FAs, required hepatic DGAT1. DGAT1 was not required for hepatic steatosis induced by lipodystrophy or LXR activation, which up-regulate endogenous de novo FA synthesis. Thus, DGAT1 has a specific role in esterifying exogenous FAs. Pretreatment with
DGAT1-specific ASO reduced hepatic TG content, suggesting that pharmacologic inhibition of DGAT1 may prevent hepatic steatosis induced by a high-fat diet.

DGAT1 deficiency reduced high-fat-diet-induced hepatic steatosis, a condition involving uptake of dietary FAs and the activation of FA synthesis. On a 3-week, high-fat diet, global DGAT1 deficiency reduced hepatic TG by ~80%. Hepatic deletion of Dgat1 reduced hepatic TG by ~50%. The additional reduction with global Dgat1 deficiency may reflect loss of DGAT1 activity in tissues, such as WAT (through endocrine effects) or small intestine. Reduced FA synthesis may also have contributed to protection against steatosis. Lipogenesis is regulated by Srebp1c, an insulin-responsive transcription factor that is typically upregulated in livers of obese mice, and Dgat1−/− mice had reduced mRNA levels of Srebp1c and FA synthesis enzymes. Reduced insulin levels in Dgat1−/− mice may explain their lower hepatic levels of Srebp1c, which may have resulted from the improved insulin sensitivity associated with Dgat1 deficiency. Expression of these genes was not changed in LivD1KO mice, suggesting that the changes in lipogenesis in Dgat1−/− mice are indirect and may result from loss of DGAT1 in nonhepatic tissues.

Our results also show a role for hepatic DGAT1 in fasting. During a fast, hepatic glycogen content is depleted, and the liver switches to lipids as fuel. Lipolysis is activated in WAT, and free FAs are mobilized to the liver, where they are oxidized to yield ketones or re-esterified to TG for storage or secretion in very low density lipoprotein (VLDL). In a previous study of mice, a 16-hour fast increased hepatic Dgat1 mRNA expression ~2.7-fold, suggesting a link between DGAT1 and steatosis in fasting. We confirmed this finding and, in studies of global and liver-specific Dgat1 gene inactivation, demonstrated a functional requirement for hepatic DGAT1 during fasting because these mice were protected against steatosis. In the absence of hepatic DGAT1 expression, FAs entering the liver from WAT during fasting are likely oxidized, as suggested by the increase in circulating ketones in Dgat1−/− mice during fasting. The reduced steatosis did not appear to reflect increased hepatic VLDL secretion, because serum TG levels were lower in fasted Dgat1−/− mice (Supporting Fig. 2). Finally, in primary hepatocytes DGAT1 was required for the esterification of exogenous FA (palmitate) at high concentrations of substrate, further supporting a role for DGAT1 in esterifying exogenous FAs.

In contrast, steatosis caused by increased endogenous de novo FA synthesis was independent of DGAT1. In lipodystrophies, leptin is deficient, and FA synthesis is activated and contributes to TG accumulation in the liver. aP2-SREBP-1c mice had similar degrees of hepatic steatosis, indicating that DGAT1 was not required for steatosis in this lipodystrophy model. Similarly, in ob/ob mice, in which leptin deficiency leads to up-regulated FA synthesis, DGAT1 deficiency did not protect against hepatic TG accumulation (H. Chen, R. Farese, unpubl. obs.). Hepatic steatosis induced by LXR activation of de novo FA synthesis was also similar in wild-type and Dgat1−/− mice. Similar results were found with Dgat1 ASO treatment in mice administered LXR agonist T0901317 (Supporting Fig. 3). In these situations of activated de novo lipogenesis, FA oxidation is likely shut down, and the effects of
DGAT1 deficiency to activate this pathway may be obviated.

Our findings indicate that DGAT1 is not functionally linked to de novo FA synthesis and suggest that DGAT2 mediates TG synthesis in this situation. How the two enzymes couple to different sources of FAs is unknown. One possibility is functional compartmentalization, as DGAT2 physically associates with SCD1, which desaturates newly synthesized FAs and functions in the de novo synthesis pathway. However, DGAT2 may not be exclusively linked to de novo synthesis, because ASO knockdown of Dgat2 reduced steatosis in fat-fed mice. Evidence for compartmentalization of DGAT enzymes exists. In tung tree cells, DGAT1 and DGAT2 localize to different subdomains of the endoplasmic reticulum, where they may synthesize different pools of TG within the cell. Mammalian DGAT1 and DGAT2 also localize to distinct regions in hepatoma cells.

In contrast to our findings, Choi et al. showed that Dgat1 ASO did not block hepatic steatosis in rats fed a high-fat diet. However, the rats were treated with ASO after 3 days on a high-fat diet, whereas our mice were pretreated with ASO for 4 weeks before starting a 1-week high-fat diet. Moreover, our diet contained milk fat (57% saturated, 30% monounsaturated, and 3% polyunsaturated fat) and cholesterol, whereas theirs was rich in safflower oil (12% saturated, 13% monounsaturated, and 75% polyunsaturated fat). The composition of dietary fat can produce contrasting results in mice. For example, mice lacking liver FA-binding protein are protected against hepatic steatosis when challenged with a diet rich in saturated but not polyunsaturated fat. In db/db mice, Yamaguchi et al. showed that Dgat1 ASO reduced hepatic fibrosis, but did not protect against hepatic TG accumulation induced by a methionine choline-deficient diet, providing a role for DGAT1 in NASH. This finding is consistent with our finding that DGAT1 deficiency does not protect ob/ob (and db/db) mice against obesity, diabetes, and hepatic steatosis.

In conclusion, we showed that DGAT1 deficiency has a specific role in the development of steatosis due to exogenous FAs but not endogenous FA synthesis. Global and liver-specific inactivations of Dgat1 and knockdown of Dgat1 by ASO afforded protection from steatosis due to a high-fat diet. It remains to be determined how our findings in murine models translate to human disease. However, DGAT1 is expressed highly in human liver, and DGAT1 expression is increased in humans with NAFLD. Moreover, in a murine model of NASH treatment with Dgat1 ASO reduced hepatic fibrosis.

Because high-fat diets are common in individuals with obesity and hepatic steatosis, DGAT1 inhibition may be a useful strategy for treating hepatic steatosis.

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