Title
Differential requirements of triglyceride synthesis enzymes in the development of hepatic steatosis

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Publication Date
2007-03-28

Peer reviewed|Thesis/dissertation

Differential requirements of triglyceride synthesis enzymes in the development of hepatic steatosis

by

Claudio J. Villanueva

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I dedicate my thesis to my wife Rachel, my son Anthony, and my parents Ricardo and Maria, for their unconditional love and support.

Surviving graduate school would have been impossible without the support of my wife, Rachel. She was encouraging during the difficult moments at UCSF when I felt that there was no light at the end of the tunnel. Without her love and support none of this would have been possible.

My parents, Ricardo and Maria Villanueva are two amazing people that have encountered and overcome significant challenges in their lives, while raising four children. I would like to thank them from the bottom of my heart, they gave me the tools to persevere.
They truly are an inspiration to me.

During my childhood and adolescence, I never could have imagined that I could one day become a scientist. In fact, I did not know the meaning of being a scientist or the impact they have on our society. Fortunately in college, I encountered amazing professors that inspired me to choose the road to become one. For their encouragement and support, I would like to thank them greatly.

I thank Colleen Talbot, who was my advisor and mentor during my undergraduate studies at California State University, San Bernardino. She was a tremendous influence in guiding me towards a career in science.

I especially thank my advisor, Bob Farese for his guidance, support and encouragement throughout my graduate studies. He is a true visionary and a leader in biomedical research. He is committed to his craft and to his family, and for that I admire him tremendously. He is a brilliant role model and I hope that one day I can follow in his footsteps.

I thank my present and former lab members, particularly Ryan Streeper, Mara Monetti, Malin Levin, Michelle Shih, Hubert Chen, Meghana Rao, Ping Zhou, Sylvaine Cases, Scot Stone,Yi Guo, Charlie Harris, Suneil Koliwad, and Eric Yen. Their friendship and support made my time at UCSF unforgettable.

I thank my thesis committee, Jackie Maher, Carl Grunfeld, and Keith Yamamoto who have given me guidance and direction during my thesis work.

This work was supported by the NIH (R01-DK056084 to R.F., and F31-DK065312 to C.V.), an American Physiological Society–Porter Physiology Fellowship (to C.V.), NIH/NCRR (CO6 RR018928 to Gladstone), and the J. David Gladstone Institutes.

Contributions of Co-Authors

This dissertation is based on contributions from Mara Monetti, Michelle Shih, Ping Zhou, Steve M. Watkins, and Sanjay Bhanot who contributed to experiments, gave helpful advice, or provided reagents to this work.

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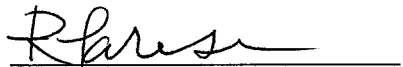

Robert V. Farese Jr., M.D.

Non-alcoholic fatty liver disease, a condition characterized by the accumulation of triacylglycerols in the liver, often accompanies obesity and can lead to steatohepatitis and cirrhosis. The synthesis of triacylglycerol is catalyzed by the acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2, and previous studies suggested different physiological roles for these enzymes. Here we investigated the role of DGAT1 in the development of hepatic steatosis. Whole animal and liver-specific deficiency of DGAT1 inhibited steatosis induced by a high-fat diet or fasting but not by conditions that increase *de novo* fatty acid synthesis in the liver, such as lipodystrophy or treatment with an LXR agonist. Instead, triglyceride synthesis coupled to fatty acid synthesis appears to be linked to DGAT2. Our studies identify mechanisms of hepatic steatosis that are dependent and independent of DGAT1 function in the liver and provide insights that may have implications for the treatment of fatty liver disease.

Chapter 1. Introduction

Non-Alcoholic Fatty Liver Disease

NAFLD (non-alcoholic fatty liver disease) is characterized by the accumulation of lipids in hepatocytes of individuals who consume little to no alcohol (Angulo 2002; Browning and Horton 2004; Clark 2006). Chronic accumulation of lipids in the liver (steatosis) may lead to steatohepatitis, which can bring about fibrotic changes that can progress to cirrhosis and liver failure (Neuschwander-Tetri and Caldwell 2003). NAFLD is the most common cause of abnormal liver enzyme tests (Daniel, Ben-Menachem et al. 1999) and, owing to the current epidemic of obesity and insulin resistance, is increasing in prevalence. Estimates are that NAFLD affects 30-60 million adults in the United States, making NAFLD the most common of all liver disorders (Angulo 2002; Ramesh and Sanyal 2005). Because of the potential impact of NAFLD on public health, a better understanding of the pathophysiology of this condition is of great importance.

NAFLD is a complex disease that's dependent on the interaction of environmental and genetic factors (Miele, Forgione et al. 2005; Merriman, Aouizerat et al. 2006). NAFLD is a progressive disorder that is thought to develop from a "two hit" hypothesis, the "first hit", hepatic steatosis, sensitizes the liver to a "second hit", often in the form of reactive oxygen species, resulting in inflammation and hepatocyte cell damage that can lead to fibrosis and cirrhosis (**Figure 1**) (Day and James 1998). The first hit develops from an imbalance in energy metabolism, where excess energy coming in to the liver, usually in the

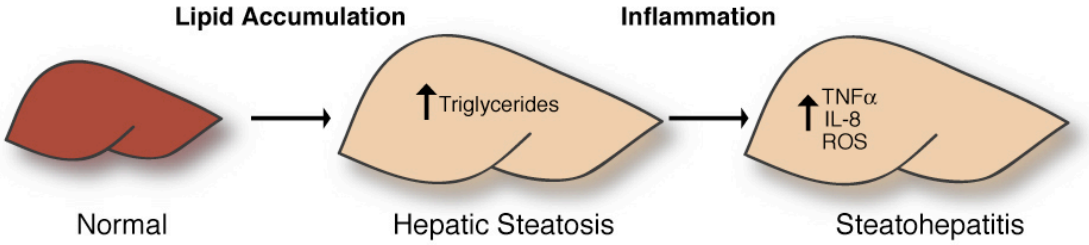

Figure 1. NAFLD is a progressive disease characterized by hepatic steatosis (triglyceride accumulation) in individuals who consume little to no alcohol. Hepatic steatosis sensitizes the liver to cell damage, often in the form of reactive oxygen species (ROS), resulting in the elevation of inflammatory mediators, such as tumor necrosis factor alpha (TNFα) and interleuking-8 (IL-8).

form of carbohydrates and fatty acids, can be stored as triglycerides (Browning and Horton 2004). Our studies will focus on understanding the role of triglyceride synthesizing enzymes in the development of the "first hit", hepatic steatosis.

Epidemiology of hepatic steatosis

Obesity, and type 2 diabetes are both conditions strongly associated with hepatic steatosis. Estimates indicate that 10-24% of general U.S. population has some form of NAFLD (Angulo 2002). However, a recent study has indicated that the prevalence may be higher, where 34% of a multiethnic population in Dallas, Texas, had excessive lipid accumulation in the liver, as measured by proton magnetic resonance spectroscopy (Browning, Szczepaniak et al. 2004; Szczepaniak, Nurenberg et al. 2005). In obese populations these numbers skyrocket, with an estimated prevalence of 75% in obese and 84-96% in morbidly obese individuals (Beymer, Kowdley et al. 2003; Clark 2006). Hepatic steatosis is also very common in individuals with type 2 diabetes. Estimates suggest that subjects with type 2 diabetes have a 49-70% prevalence of hepatic steatosis as measured by ultrasound (Gupte, Amarapurkar et al. 2004).

Diagnosis of hepatic steatosis

Most patients with hepatic steatosis do not exhibit symptoms of liver disease (Sass, Chang et al. 2005). Often hepatic steatosis is identified when circulating levels of ALT or AST are elevated (Angulo 2002). Ultrasound, nuclear magnetic resonance spectroscopy, and CT scan can also diagnose hepatic steatosis. However, liver biopsy is the gold standard in making a correct diagnosis when alcohol abuse is ruled out (Sass, Chang et al. 2005). Other causes should be ruled out as well, such as viral hepatitis, autoimmune disease, and drugs/toxins.

Hepatic steatosis and triglycerides

The major lipid found in hepatic steatosis is triacylglycerol (TG), a neutral lipid consisting of three fatty acid chains joined to one glycerol molecule. TG's provide an abundant source of energy, playing a critical role in maintaining energy homeostasis in the cell. TG's release about twice as much energy and attract less water than other energy stores such as glycogen. Therefore, our bodies can carry six times more TG mass than glycogen to generate an equivalent amount of energy (Alberts 2002). This allows organisms to carry a substantial amount of energy without having to be near an energy source, allowing travel when local resources are scarce. In mammals, most of TG's are carried in adipocytes, albeit, during times of energy excess the liver can store a substantial amount of TG.

TG's create a storage pool of essential and non-essential fatty acids. Fatty acids for TG synthesis are acquired from adipose tissue, diet or synthesized *de novo* using carbohydrates as substrate (Donnelly, Smith et al. 2005). In

hepatocytes these fatty acids can be used for phospholipid synthesis or to generate energy through fatty acid oxidation. Hepatocytes can also package triglycerides into very low-density lipoproteins, which can be secreted in the blood and utilized by tissues for use as an energy source.

Development of hepatic steatosis

Hepatic steatosis develops from an imbalance between energy input (lipid uptake and synthesis) and output (lipid oxidation and secretion). The metabolic abnormalities leading to hepatic steatosis are thought to revolve around insulin resistance and obesity (**Figure 2**) (Browning and Horton 2004). Normally insulin

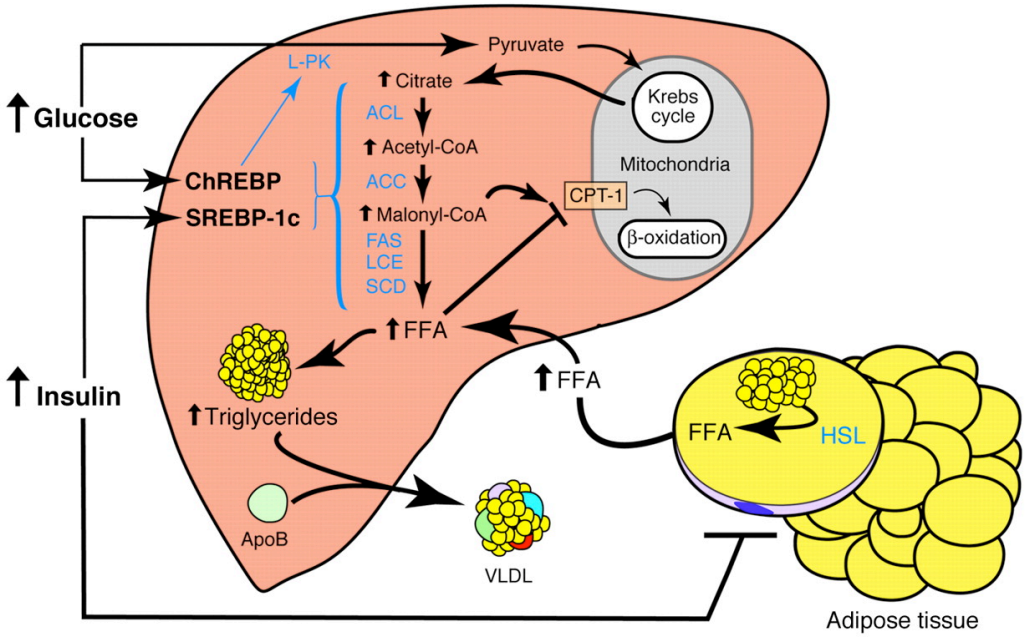

Figure 2. Hyperglycemia and hyperinsulinemia promote hepatic steatosis by activating SREBP1c and ChREBP, both transcription factors that activate fatty acid synthesis in the liver. Malonyl-CoA, an intermediate in fatty acid synthesis, also inhibits CPT-1, the rate-determining step in fatty acid oxidation. The combination of reduced insulin action in adipocytes and increased adiposity raises plasma levels of FFA's, providing substrate for triglyceride synthesis in the liver. HSL: hormone sensitive lipase, ACL: ATP-Citrate Lyase, ACC: acetyl-CoA carboxylase, FAS: fatty acid synthase, LCE: Long chain fatty acyl elongase, SCD: stearoyl-CoA desaturase, L-PK: liver pyruvate kinase, CPT-1, carnitine palmitoyl-CoA transferase. (Browning and Horton 2004).

inhibits hormone sensitive lipase (HSL) activity in the adipose tissue, however, when insulin resistance develops, suppression of HSL by insulin is attenuated (Rodbell and Jones 1966; Stralfors, Bjorgell et al. 1984). A combination of increased adiposity and increased triglyceride hydrolysis in adipose tissue leads to elevated plasma levels of free fatty acids (Donnelly, Smith et al. 2005). This can overwhelm the livers capacity to oxidize and export lipids, leading to the accumulation of triglycerides.

While adipose tissue develops insulin resistance, the liver maintains partial insulin sensitivity. Insulin's ability to activate fatty acid synthesis remains intact, while suppression of gluconeogenesis is blunted (Shimomura, Matsuda et al. 2000). Fatty acid synthesis is regulated in part by SREBP1c, a membrane bound basic helix-loop-helix-leucine zipper transcription factor that is activated by insulin (Osborne 2000; Rawson 2003). SREBP1c transcriptionally activates most of the genes necessary for fatty acid synthesis (Shimano, Horton et al. 1997; Shimano, Yahagi et al. 1999). Chronic activation of SREBP1c in the liver leads to hepatic steatosis in mouse models of leptin-deficiency (lipodystrophy and ob/ob), which are characterized by hyperglycemia and hyperinsulinemia (Kim, Sarraf et al. 1998; Shimomura, Shimano et al. 1998; Osborne 2000; Yamashita, Takenoshita et al. 2001). Increasing rates of fatty acid synthesis raise Malonyl-CoA levels, an intermediate in fatty acid synthesis that regulates fatty acid oxidation by inhibition of carnitine palmitoyl transferase 1 (CPT1) (McGarry, Mannaerts et al. 1977; Bird and Saggerson 1984). CPT1 is an enzyme that shuttles fatty acids into the mitochodria for β-oxidation. Glucose can also increase

fatty acid synthesis by activating carbohydrate response element-binding protein (ChREBP), a transcription factor that induces gene expression of pyruvate kinase, an enzyme that catalyzes an intermediate step in glycolysis (Yamashita, Takenoshita et al. 2001).

Triglyceride Synthesis and DGAT Enzymes

Triglycerides are products of the glycerol phosphate biosynthesis pathway (Coleman and Lee 2004). The final step in this pathway, the covalent joining of a fatty acyl-CoA to diacylglycerol, is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes (**Figure 3**). There are two DGAT enzymes, DGAT1 and DGAT2, members of distinct gene families that share no significant sequence homology (Cases, Smith et al. 1998; Buhman, Chen et al. 2001; Cases, Stone et al. 2001; Buhman, Smith et al. 2002). Both enzymes are membrane-associated proteins, and they have similarly broad fatty acyl CoA substrate specificities in *in vitro* assays (Cases, Stone et al. 2001). Both are ubiquitously expressed in tissues, with the highest levels of expression in tissues that play prominent roles in TG metabolism, such as intestine, adipose tissue, liver, and mammary gland (Cases, Smith et al. 1998; Cases, Stone et al. 2001).

Although DGAT enzymes share similarities, they also exhibit striking differences. In overexpression studies in intact cells, DGAT2 is a more potent enzyme than DGAT1, yielding a much larger increase in intracellular TG, which accumulated in large, centrally located cytosolic droplets (Stone, Myers et al. 2004). The enzymes also have different physiological functions, as demonstrated by the strikingly different phenotypes of targeted gene disruptions. Mice lacking

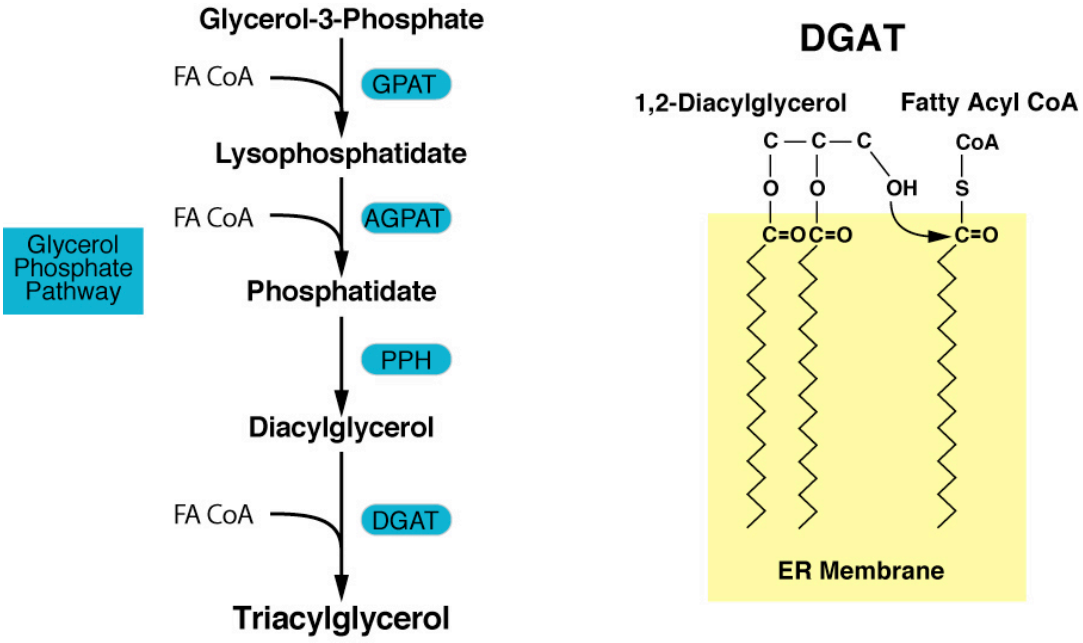

Figure 3. Triacylglycerol is the final product of the glycerolipid biosynthesis pathway. In the endoplasmic reticulum (ER), DGAT enzymes catalyze the last step in this pathway, joining 1,2-diacylglycerol to a fatty acyl-CoA molecule, generating triacylglycerol. GPAT: glycerol phosphate acyltransferase, AGPAT: acylglycerol phosphate acyltransferase, PPH, phosphatidic acid phosphatase, DGAT: diacylglycerol acyltransferase.

Dgat1 (*Dgat1*$^{-/-}$) are viable, have reduced tissue triglyceride levels, exhibit increased sensitivity to insulin and leptin, and are protected against diet-induced obesity through a mechanism that involves increased energy expenditure (Smith, Cases et al. 2000). In contrast to *Dgat1*$^{-/-}$ mice, mice lacking DGAT2 (*Dgat2*$^{-/-}$) are almost completely devoid of TG and die shortly after birth (Stone, Myers et al. 2004). These genetic models suggest that the DGAT enzymes have distinct roles in physiology.

Both DGAT1 and DGAT2 are expressed in the livers of humans and mice (Cases, Smith et al. 1998; Cases, Stone et al. 2001). In humans, *Dgat1* mRNA expression is relatively high (Farese, Cases et al. 2000), in contrast to murine liver

where *Dgat1* mRNA expression is relatively low (Cases, Smith et al. 1998). The absence of either DGAT enzyme in the livers of the respective knockout mice significantly reduced DGAT activity levels (Smith, Cases et al. 2000; Stone, Myers et al. 2004). In *Dgat2*$^{-/-}$ mice, the livers of newborn pups have almost no TG despite the presence of DGAT1, indicating that DGAT1 is unable to compensate for the absence of DGAT2 in this situation (Stone, Myers et al. 2004). Preliminary studies of *Dgat1*$^{-/-}$ mice indicated that DGAT1 might function in specific conditions that promote hepatic steatosis. These mice have reduced hepatic lipid accumulation on a high-fat diet (Smith, Cases et al. 2000) but have similar degrees of steatosis when crossed with leptin-deficient *ob*/*ob* mice (H. Chen and R. Farese, unpublished observations). These findings suggest that the two enzymes have distinct roles in TG metabolism in the liver.

Based on these observations, we hypothesized that DGAT1 functions prominently in hepatic steatosis caused by a high-fat diet or fasting, but not in steatosis caused by increased *de novo* fatty acid synthesis. To test this hypothesis, we probed several different models of murine *Dgat1* gene inactivation [whole-animal and liver-specific knockouts of *Dgat1*, and mice with hepatic knockdowns of *Dgat1* expression induced by antisense oligonucleotides (ASO)] with different conditions that promote hepatic steatosis, including a high-fat diet, fasting, genetically induced lipodystrophy (*aP2-SREBP-1c436* transgenic mice), and a pharmacologic agent that induces hepatic fatty acid synthesis (LXR agonist T0901317) (Shimomura, Hammer et al. 1998; Schultz, Tu et al. 2000). We also tested the effects of *Dgat2* ASO treatment, which reduces *Dgat2* expression in the liver and WAT (Yu, Murray et al. 2005), in combination with LXR-mediated stimulation of fatty acid synthesis.

References

Alberts, B. (2002). Molecular biology of the cell. New York, Garland Science.

Angulo, P. (2002). "Nonalcoholic fatty liver disease." N Engl J Med **346**(16): 1221-31.

Beymer, C., K. V. Kowdley, et al. (2003). "Prevalence and predictors of asymptomatic liver disease in patients undergoing gastric bypass surgery." Arch Surg **138**(11): 1240-4.

Bird, M. I. and E. D. Saggerson (1984). "Binding of malonyl-CoA to isolated mitochondria. Evidence for high- and low-affinity sites in liver and heart and relationship to inhibition of carnitine palmitoyltransferase activity." Biochem J **222**(3): 639-47.

Browning, J. D. and J. D. Horton (2004). "Molecular mediators of hepatic steatosis and liver injury." J Clin Invest **114**(2): 147-52.

Browning, J. D., L. S. Szczepaniak, et al. (2004). "Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity." Hepatology **40**(6): 1387-95.

Buhman, K. K., H. C. Chen, et al. (2001). "The enzymes of neutral lipid synthesis." J Biol Chem **276**(44): 40369-72.

Buhman, K. K., S. J. Smith, et al. (2002). "DGAT1 is not essential for intestinal triacylglycerol absorption or chylomicron synthesis." J Biol Chem **277**(28): 25474-9.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Clark, J. M. (2006). "The epidemiology of nonalcoholic fatty liver disease in adults." J Clin Gastroenterol **40**(3 Suppl 1): S5-10.

Coleman, R. A. and D. P. Lee (2004). "Enzymes of triacylglycerol synthesis and their regulation." Prog Lipid Res **43**(2): 134-76.

Daniel, S., T. Ben-Menachem, et al. (1999). "Prospective evaluation of unexplained chronic liver transaminase abnormalities in asymptomatic and symptomatic patients." Am J Gastroenterol **94**(10): 3010-4.

Day, C. P. and O. F. James (1998). "Steatohepatitis: a tale of two "hits"?" Gastroenterology **114**(4): 842-5.

Donnelly, K. L., C. I. Smith, et al. (2005). "Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease." J Clin Invest **115**(5): 1343-51.

Farese, R. V., Jr., S. Cases, et al. (2000). "Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase." Curr Opin Lipidol **11**(3): 229-34.

Gupte, P., D. Amarapurkar, et al. (2004). "Non-alcoholic steatohepatitis in type 2 diabetes mellitus." J Gastroenterol Hepatol **19**(8): 854-8.

Kim, J. B., P. Sarraf, et al. (1998). "Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1." J Clin Invest **101**(1): 1-9.

McGarry, J. D., G. P. Mannaerts, et al. (1977). "A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis." J Clin Invest **60**(1): 265-70.

Merriman, R. B., B. E. Aouizerat, et al. (2006). "Genetic influences in nonalcoholic fatty liver disease." J Clin Gastroenterol **40**(3 Suppl 1): S30-3.

Miele, L., A. Forgione, et al. (2005). "The natural history and risk factors for progression of non-alcoholic fatty liver disease and steatohepatitis." Eur Rev Med Pharmacol Sci **9**(5): 273-7.

Neuschwander-Tetri, B. A. and S. H. Caldwell (2003). "Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference." Hepatology **37**(5): 1202-19.

Osborne, T. F. (2000). "Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action." J Biol Chem **275**(42): 32379-82.

Ramesh, S. and A. J. Sanyal (2005). "Evaluation and management of non-alcoholic steatohepatitis." J Hepatol **42 Suppl**(1): S2-12.

Rawson, R. B. (2003). "The SREBP pathway--insights from Insigs and insects." Nat Rev Mol Cell Biol **4**(8): 631-40.

Rodbell, M. and A. B. Jones (1966). "Metabolism of isolated fat cells. 3. The similar inhibitory action of phospholipase C (Clostridium perfringens alpha toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline." J Biol Chem **241**(1): 140-2.

Sass, D. A., P. Chang, et al. (2005). "Nonalcoholic fatty liver disease: a clinical review." Dig Dis Sci **50**(1): 171-80.

Schultz, J. R., H. Tu, et al. (2000). "Role of LXRs in control of lipogenesis." Genes Dev **14**(22): 2831-8.

Shimano, H., J. D. Horton, et al. (1997). "Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells." J Clin Invest **99**(5): 846-54.

Shimano, H., N. Yahagi, et al. (1999). "Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes." J Biol Chem **274**(50): 35832-9.

Shimomura, I., R. E. Hammer, et al. (1998). "Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy." Genes Dev **12**(20): 3182-94.

Shimomura, I., M. Matsuda, et al. (2000). "Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice." Mol Cell **6**(1): 77-86.

Shimomura, I., H. Shimano, et al. (1998). "Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of

unsaturated fatty acid biosynthesis in transgenic mouse liver." J Biol Chem **273**(52): 35299-306.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Stone, S. J., H. M. Myers, et al. (2004). "Lipopenia and skin barrier abnormalities in DGAT2-deficient mice." J Biol Chem **279**(12): 11767-76.

Stralfors, P., P. Bjorgell, et al. (1984). "Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin." Proc Natl Acad Sci U S A **81**(11): 3317-21.

Szczepaniak, L. S., P. Nurenberg, et al. (2005). "Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population." Am J Physiol Endocrinol Metab **288**(2): E462-8.

Yamashita, H., M. Takenoshita, et al. (2001). "A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver." Proc Natl Acad Sci U S A **98**(16): 9116-21.

Yu, X. X., S. F. Murray, et al. (2005). "Antisense oligonucleotide reduction of DGAT2 expression improves hepatic steatosis and hyperlipidemia in obese mice." Hepatology **42**(2): 362-71.

Chapter 2. Mice lacking DGAT1 are protected against hepatic steatosis induced by western diet

Abstract

Non-alcoholic fatty liver disease is characterized by the accumulation of triglycerides in the liver of individuals who consume little to no alcohol. In the liver DGAT enzymes catalyze the final step in triglyceride synthesis by joining a fatty acyl-CoA molecule to diacylglycerol. To explore the role of DGAT1 in the development of hepatic steatosis, *Dgat1*$^{-/-}$ mice were placed on a high-fat diet for 3 weeks. Livers of *Dgat1*$^{-/-}$ mice had lower levels of triglycerides and cholesterol esters when compared with WT. The protection against hepatic steatosis in *Dgat1*$^{-/-}$ mice was accompanied by an increase in fatty acid oxidation and suppression in fatty acid synthesis in hepatocytes. These changes were accompanied by the activation of AMPK, a metabolic sensor that regulates hepatic fatty acid metabolism by activating fatty acid oxidation and suppressing fatty acid synthesis. Our results indicate that DGAT1 may be an important determinant in the progression of hepatic steatosis induced by high-fat feeding.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids in the liver (hepatic steatosis) of individuals who consume little to no alcohol (Angulo 2002; Clark 2006). Estimates suggest that NAFLD affects 10-24% of the adult U.S. population. Understanding the pathways that regulate lipid metabolism in the liver will provide novel targets for the treatment and prevention of NAFLD.

Triglycerides (TG) are the major lipid that accumulates in NAFLD. The liver has the capacity to store large quantities of TG. TG are synthesized through the glycerolipid biosynthetic pathway (Coleman and Lee 2004). The final step in this pathway is catalyzed by acyl-CoA diacylglycerol:acyl transferase (DGAT) enzymes, joining a fatty acyl-CoA molecule to diacylglycerol (Buhman, Chen et al. 2001; Coleman and Lee 2004). Two DGAT enzymes have been identified, DGAT1 and DGAT2. They belong to distinct gene families that share no nucleotide sequence or protein homology (Cases, Smith et al. 1998; Cases, Stone et al. 2001). Both enzymes are highly expressed in tissues involved in triglyceride metabolism, such as the adipose tissue, liver, intestine and mammary gland (Cases, Smith et al. 1998; Cases, Stone et al. 2001).

To study the physiological function of DGAT1 *in vivo*, our lab generated *Dgat1*$^{-/-}$ mice (Smith, Cases et al. 2000). *Dgat1*$^{-/-}$ mice are viable, have reduced tissue triglyceride levels, increased insulin and leptin sensitivity, and are protected against diet-induced obesity through a mechanism that involves increased energy expenditure (Smith, Cases et al. 2000; Chen, Ladha et al. 2002; Chen, Smith et al.

2002). Previous findings have shown that after a chronic high-fat diet, *Dgat1*$^{-/-}$ mice are protected against hepatic steatosis (Smith, Cases et al. 2000). However, the mechanism(s) by which DGAT1-deficiency protects against hepatic steatosis has not been elucidated. In our studies we examined two pathways, fatty acid synthesis and oxidation. Our findings suggest that both of these pathways may contribute to the protection against hepatic steatosis in *Dgat1*$^{-/-}$ mice.

Materials and Methods.

Mice. *Dgat1*$^{-/-}$ mice and wild-type mice were in a C57BL/6J background. Genotyping for *Dgat1* was performed as described earlier (Smith, Cases et al. 2000). Mice were housed in a pathogen-free barrier facility (12-hour light/12-hour dark cycle) and fed rodent chow (5053 PicoLab Diet; Purina, St. Louis, Missouri, USA). For high-fat diet experiments mice were fed a Western-type diet containing 20% milk fat by weight and 0.2% cholesterol (TD 01064 Harlan-Teklad Laboratory, Madison, Wisconsin, USA). All experiments were approved by the Committee on Animal Research of the University of California, San Francisco.

Histological Analysis. For histology mice were perfused with PBS, followed by 3% paraformaldehyde/PBS. Livers were removed and placed into 3% paraformaldehyde/PBS for 2 days at 4°C. Livers were embedded in paraffin, sectioned and stained with hematoxylin and eosin. To visualize neutral lipids, livers were snap frozen in OCT and isopentane, sectioned and stained with Oil red O.

Lipid Analysis. The lipids from plasma and liver were extracted in the presence of internal standards using chloroform:methanol (2:1 v/v) (Folch, Lees et al. 1957). Either 25 mg liver tissue or 200 μl plasma was used for each analysis. Individual lipid classes within the extract were separated by preparative thin layer chromatography (Watkins, Lin et al. 2001). Isolated lipid classes were trans esterified in 3 N methanolic HCl in a sealed vial under a nitrogen atmosphere at 100°C for 45 min. The resulting fatty acid methyl esters were extracted with

hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the hexane extracts under nitrogen.

Fatty acid methyl esters were separated and quantified by capillary gas chromatography using a gas chromatograph (Hewlett Packard model 6890, Wilmington, DE) equipped with a 30 m DB 225MS capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector (Watkins, Lin et al. 2001).

In some experiments livers were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose and complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated by thin-layer chromatography with a solvent system of hexane:diethyl ether:acetic acid (80:20:1) on Silica Gel G-60 TLC plates (Folch, Lees et al. 1957). Using triolein as a standard, triglycerides were visualized with iodine vapor. Triglycerides were identified and quantified using a spectrophotometric technique (Snyder and Stephens 1959).

Hepatocytes. Mice were anesthetized with Avertin and their livers were perfused *via* the inferior *vena cava* with 30 ml of perfusion media (Invitrogen). The perfusion media was prewarmed to 37°C and infused at a rate of ~3 ml/min using a peristaltic pump. The livers were then perfused with Liver Digest Medium containing collagenase (Invitrogen) for approximately 10 min at a flow rate of ~3 ml/min. The livers were removed from the animals and the gall bladder discarded, the hepatic capsule was stripped and the dissociated cells were dispersed by shaking, followed by filtration at 4°C through a nylon mesh (03-250/50 Lab Pak, Sefar America) into an equal volume of ice-cold DMEM (Invitrogen) containing

5% (vol/vol) FBS, 10 mM HEPES (pH 7.4), and gentamycin (0.1 mg/ml). The cells were pelleted at 50xg and washed twice at 4°C with the same medium. The cells were counted with a hemacytometer, and 1.5 x 10^6 cells were plated onto 60-mm mouse collagen IV-coated dishes (Becton Dickinson) in the above medium and incubated at 37°C in 95% air/5% CO^2 for 3 hours. The cells were then washed with Dubelccos Phosphate Buffered Saline (Invitrogen).

Fatty acid synthesis and oxidation. For fatty acid synthesis measurements, hepatocytes were incubated with 3 ml of DMEM containing 5% human lipoprotein-deficient serum, gentamycin (0.1 mg/ml), and 0.5 mM sodium [^{14}C]acetate (18 dpm/pmol). After incubating for 6 hours, the medium was removed and hepatocytes were washed with Dubelcos Phosphate Buffered Saline (Invitrogen). Hepatocyte cell media was saved and cells were harvested by scraping into 2 ml of 0.1 N sodium hydroxide. Protein concentration was determined by Dc protein assay (Biorad). Lipids were extracted and saponified, the [^{14}C]-labeled fatty acids in the cells and medium were separated by thin-layer chromatography. The free fatty acid bands were scraped and quantified by scintillation counting (Horton, Shimano et al. 1999).

For fatty acid oxidation measurements, hepatocytes were incubated with 3 ml of DMEM containing 200 mM [9,10-^{3}H]oleic Acid (10mCi/ml) (Amersham) conjugated to 0.2% BSA for 2 hours. The hepatocyte media was saved and cells were scraped with 1 ml of 0.1N sodium hydroxide and 1 ml of water, protein concentration was determined by Dc protein assay (Biorad). 100 ml of hepatocyte media was placed in an opened centrifuge tube. The opened centrifuge tube was

placed in a 20 ml scintillation vial containing 500 ml of water and sealed with a cap. The scintillation vials were then placed in a 50°C oven for approximately 18 hours to recover the [^{3}H]H_2O. To determine the equilibrium efficiency, in a separate centrifuge tube 100 ml of 0.1 mCi/ml of [^{3}H]H_2O or [9,10-^{3}H]oleic acid was added and placed in a 20 ml scintillation vial. Scintillation vials were then allowed to cool and microcentrifuge tubes were removed, scintillation fluid was then added to scintillation vial and the recovered [^{3}H]H_2O was counted using a scintillation counter. Circulating β-hydroxybutyrate levels were measured according to manufacturers specifications (β-HBA Kit 310A; Sigma).

Western blot analysis. Liver was homogenized with 50 mM Tris-HCl, 1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF and complete mini protease inhibitor (roche). Homogenate was spun at 800xg for 10 minutes to remove cell debris. 45 mg of protein were separated on a Nupage 7% Tris-Acetate gel and transferred to PVDF membrane. Specific antibodies were used to identify phosphorylation at Thr-172 of a-AMPK (Cell signaling), total a-AMPK (Cell signaling), phosphorylation at Ser-79 of acetyl-CoA carboxylase (Upstate) and LRP.

RNA extraction and real-time PCR. RNA was extracted from liver using RNA STAT-60 (Tel-Test Inc.) and traces of DNA were removed by DNase treatment (Ambion). 5 mg of RNA was used to synthesize cDNA using superscript II reverse transcriptase and random hexamers (Invitrogen). Real-time PCR primers were selected for each cDNA using Primer Express software

(version 1.5; Applied Biosystems). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with sybrgreen reagents using two-step RT-PCR reactions on Biorad iCycler or ABI 9600. 18S ribosomal RNA or cyclophilin was used to normalize gene expression. **Cyclophilin**, F:5'–TGGAAGAGCACCAAGACAGACA–3' and R: 5'–TGCCGGAGTCGACA ATGAT–3'; **Srebp1c**, F:5'–GGAGCCATGGATTGCACATT–3' and R: 5'–GGC CCGGGAAGTCACTGT–3'; **Fasn**, F:5'–GCTGCGGAAACTTCAGGAAAT–3' and R:5'–AGAGA CGTGTCACTCCTGGA CTT–3'; **Scd1**, F:5'–CCTTCCCCT TCGACTACTCTG–3' and R:5'–GCCATGCAGTCGATGAAGAA–3'; **Ppara**, F: 5'–CAGGAGAGCAGGGATTTGCA–3' and R:5'–CCTACGCTCAGCCCTCT TCAT–3'; **Cpt1,** F:5'–ACCAACGGGCTCATCTTCTAA–3' and R: 5'–CAAA ATGACCTAGCCTTCTATCGA–3'; Aox, F:5'–CTTCAGGCCCAAGTGAGT CA–3' and R: 5'–GCGAACAAGGTCGACAG AAGT–3'.

Statistical analysis. Values are reported as mean ± standard error. Statistical differences were determined by either a students t-test or an ANOVA followed by a Student-Newman-Keuls test.

Results

To fully characterize the role of DGAT1 in diet-induced hepatic steatosis, mice were placed on a 3 week high-fat diet. We found that shortly after 3 weeks of high-fat feeding, livers from wild-type (WT) mice became enlarged and developed a pale color, while livers from *Dgat1*$^{-/-}$ mice maintained a similar appearance to livers from chow fed mice (**Figure 1A**). H & E staining of liver sections showed that WT mice (**Figure 1B,i**) developed microvesicular steatosis, while livers from *Dgat1*$^{-/-}$mice (**Figure 1B,ii**) were protected. Liver sections stained with Oil Red O, demonstrated that livers from *Dgat1*$^{-/-}$ mice (**Figure 1B,iv**) had accumulated less neutral lipids when compared to WT mice (**Figure 1B,iii**). Liver mass (**Figure 2A**) was reduced in *Dgat1*$^{-/-}$ mice fed chow or high-fat diet. When liver mass was corrected for body weight, there was no longer an apparent

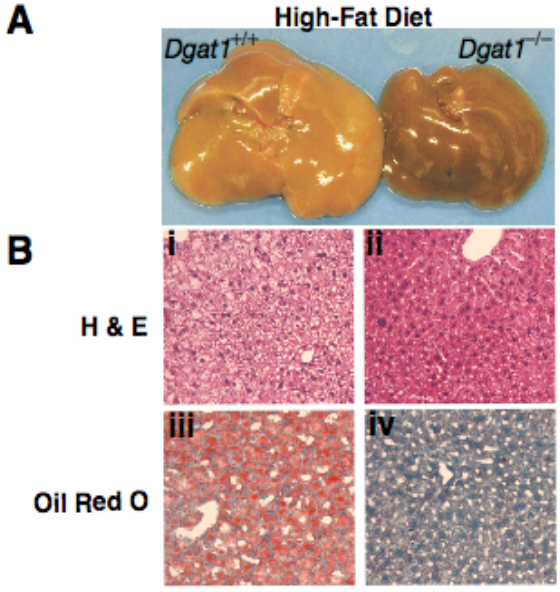

Figure 1. DGAT1-deficiency protects mice against hepatic steatosis induced by high-fat diet. (A) Gross morphology of livers from *Dgat1*$^{-/-}$ mice show reduced hepatomegaly after high-fat diet (age 12 weeks). (B) Reduced neutral lipid content in livers of *Dgat1*$^{-/-}$ mice fed high-fat diet. Histological analyses of liver sections taken from *Dgat1*$^{+/+}$ (i,iii) and *Dgat1*$^{-/-}$ (ii,iv) mice (age 12 weeks). Liver sections were stained with hematoxylin & eosin (i,ii) or Oil Red O (iii,iv). Magnification is at 20X for all.

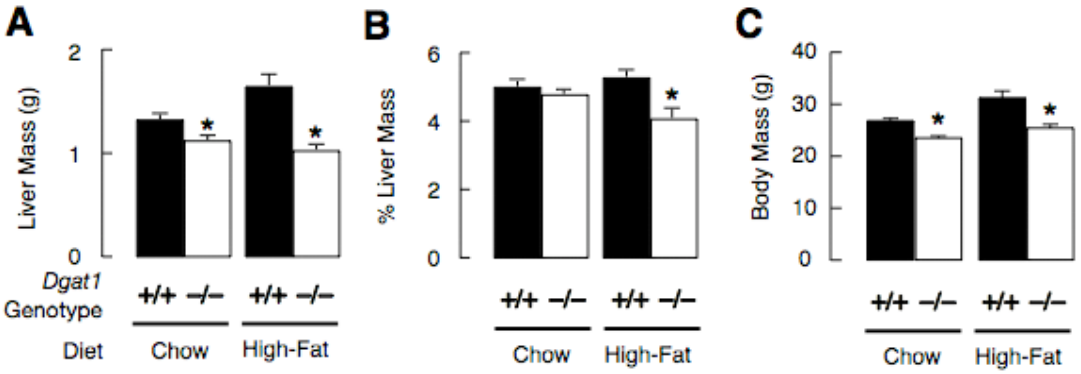

Figure 2. *Dgat1*$^{-/-}$ mice are protected against diet-induced hepatomegaly and weight-gain. (A) Reduced liver mass in *Dgat1*$^{-/-}$ mice on chow and high-fat diet. * $P < 0.05$ *versus* +/+ chow or high-fat. (B) Reduced % liver mass in *Dgat1*$^{-/-}$ mice fed high-fat diet. * $P < 0.001$ *versus* +/+ high-fat. (C) Reduced body mass on a chow and high-fat diet in *Dgat1*$^{-/-}$ mice. * $P < 0.05$ *versus* +/+ chow or high-fat. (for all, age 12 weeks, N=5).

difference between WT and *Dgat1*$^{-/-}$ mice on a chow diet (**Figure 2B**), while on a high-fat diet, *Dgat1*$^{-/-}$ mice had reduced % liver mass when compared to WT mice. On

chow and high-fat diet *Dgat1*$^{-/-}$ mice were protected against weight-gain associated with high-fat feeding (**Figure 2C**).

The morphological and histological differences between livers from WT and *Dgat1*$^{-/-}$ mice suggested that neutral lipids, such as triglycerides, would be reduced in *Dgat1*$^{-/-}$ mice. To test this hypothesis, triglycerides were quantified in livers of WT and *Dgat1*$^{-/-}$ mice fed chow or high-fat diet for 3 weeks. Triglyceride levels (**Figure 3A**) in livers of WT mice fed a standard chow diet were similar to *Dgat1*$^{-/-}$ mice. However, when mice were challenged with a high-fat diet, *Dgat1*$^{-/-}$ mice had an 81% reduction in hepatic triglyceride levels (**Figure 3A**). Since the western diet is also rich in cholesterol (0.2%), we wanted to determine whether the change in neutral lipid staining was strictly do to a change

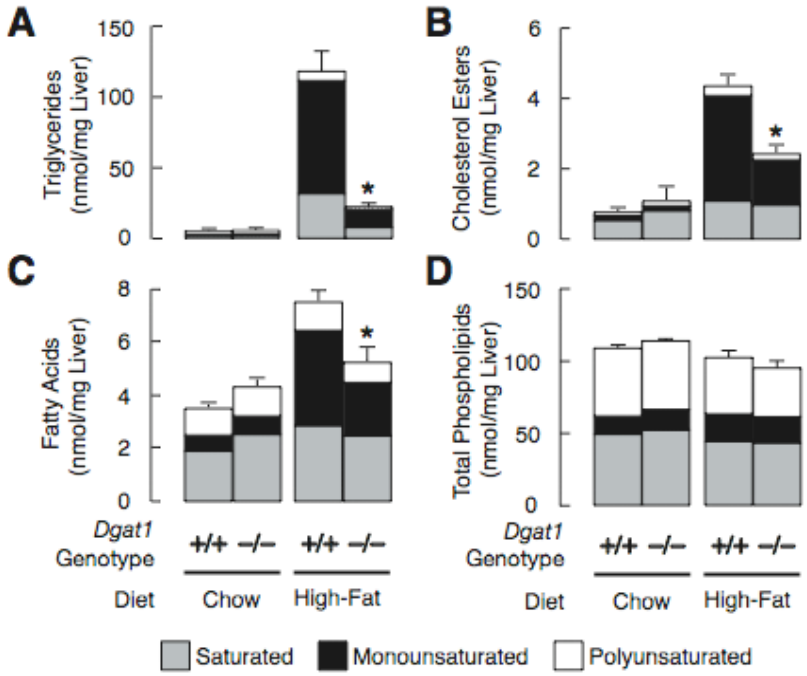

Figure 3. Lipid profile shows that *Dgat1*$^{-/-}$ mice fed a high-fat diet have reduced hepatic triglycerides, cholesterol esters and fatty acids. Saturated, monounsaturated and polyunsaturated fatty acids in triglycerides (A), cholesterol esters (B), fatty acids (C) and total phospholipids (D) were quantified as described in methods (age 8-12 weeks, N=5). Male mice were fasted for four hours before the liver was removed. Error bar represents the standard error for the total lipid class. * $P < 0.001$ *versus* +/+ high-fat. In all experiments mice were fed high-fat diet for 3 weeks.

in triglycerides or whether other neutral lipids, such as cholesterol esters could be altered. To test this hypothesis hepatic cholesterol ester levels (**Figure 3B**) were quantified. On the chow diet we found that cholesterol ester levels were similar between WT and *Dgat1*$^{-/-}$ mice. However, when mice were placed on the western diet, there was a 44% reduction in hepatic cholesterol ester levels (**Figure 3B**) in *Dgat1*$^{-/-}$ mice. Interestingly, on the high-fat diet monounsaturated fatty acids in the cholesterol ester pool (**Figure 3B**) made up the largest difference, with a 57% reduction in *Dgat1*$^{-/-}$ mice, while saturated and polyunsaturated fatty acids remained unchanged. On a chow diet, intracellular fatty acids (**Figure 3C**) in the

livers of WT and *Dgat1*$^{-/-}$ mice were similar. However, when mice were challenged with a high-fat diet, we found a 31% reduction in hepatic fatty acid levels (**Figure 3C**), of which monounsaturated fatty acids made up the largest difference (44%), while saturated and polyunsaturated fatty acids remained unaltered. In contrast to neutral lipids, on the chow and high-fat diet, total phospholipids in the liver (**Figure 3D**) were similar between WT and *Dgat1*$^{-/-}$ mice.

Since WT and *Dgat1*$^{-/-}$ mice had similar food intake, and absorbed an equivalent amount of lipids, but were still protected against hepatic steatosis on a high-fat diet, we suspected that *Dgat1*$^{-/-}$mice would have increased fatty acid oxidation in the liver (Smith, Cases et al. 2000; Buhman, Smith et al. 2002). To test this hypothesis, primary hepatocytes were isolated from high-fat fed mice, cultured on collagen-coated dishes, and labeled with [9,10]^{3}H-Oleate. We found that freshly isolated hepatocytes from *Dgat1*$^{-/-}$ mice had a ~2-fold increase in fatty acid oxidation (**Figure 4A**). In addition, circulating β-hydroxybutyrate, a product of fatty acid oxidation in the liver, was 35% higher in *Dgat1*$^{-/-}$ mice (**Figure 4B**). To determine whether the increase in fatty acid oxidation of *Dgat1*$^{-/-}$ mice was do to transcriptional changes in the liver, we compared the mRNA levels of fatty acid oxidation genes in WT and *Dgat1*$^{-/-}$ mice. We found that the expression level of hepatic fatty acid oxidation genes, such as peroxisome proliferator alpha (*Ppara*),) carnitine palmitoyl transferase 1 (*Cpt1*) and acyl-CoA oxidase (*Aox*) were similar between WT and *Dgat1*$^{-/-}$ mice on a high-fat diet (**Figure 4C**).

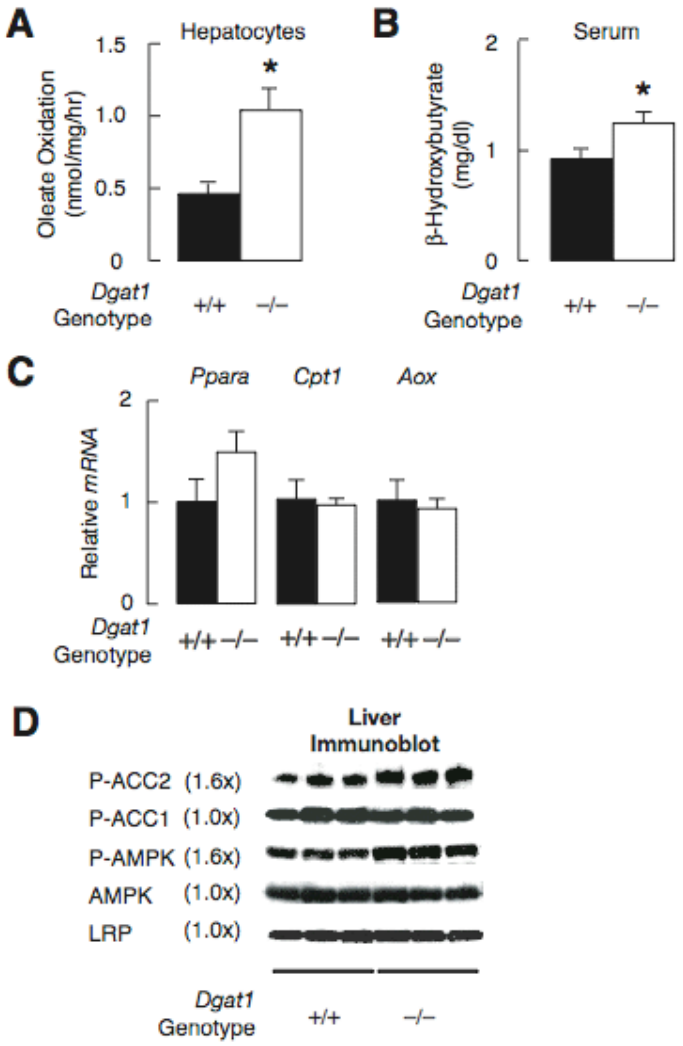

Figure 4. High-fat fed *Dgat1*$^{-/-}$ mice have increased hepatic fatty acid oxidation and AMPK signaling. (A) Increased fatty acid oxidation in freshly isolated hepatocytes from *Dgat1*$^{-/-}$ mice fed a high-fat diet. Fatty acid oxidation was determined by incubating hepatocytes with 9,10[^{3}H]-oleic acid and capturing [^{3}H]H_2O (age 11-13 weeks, N=3-4); * $P < 0.05$ *versus* +/+. (B) Increased serum levels of β-hydroxybutyrate in *Dgat1*$^{-/-}$ mice fed a high-fat diet and fasted for 4 hours (age 12 weeks, N=4-5); * $P < 0.05$ *versus* +/+. (C) No change in mRNA levels of genes involved in fatty acid oxidation in *Dgat1*$^{-/-}$ mice fed high-fat diet (age 8-12 weeks, N=5); * $P < 0.05$ *versus* +/+. (D) Increased AMPK signaling in livers of *Dgat1*$^{-/-}$ mice fed high-fat diet. Livers were isolated after mice were fasted for 4 hours. AMPK signaling was quantified by western blot analysis using antibodies specific for the phosphorylated forms of αAMPK(Thr172) and ACC(S79). Antibodies specific for LRP and total αAMPK were used as loading controls (age 12 weeks). In all experiments mice were fed high-fat diet for 3 weeks.

The lack of increase in expression of fatty acid oxidation genes in the liver

implied that fatty acid oxidation could be altered by other means. Therefore, we determined whether AMP-activated protein kinase (AMPK) signaling was elevated, which has been shown to activate fatty acid oxidation in the liver (Velasco, Geelen et al. 1997). We found that livers of high-fat fed *Dgat1*$^{-/-}$ mice had elevated phosphorylation of the alpha subunit of AMPK (**Figure 4D**). AMPK has been shown to inhibit the enzyme acetyl-CoA carboxylase (ACC) through phosphorylation (Winder, Wilson et al. 1997). ACC catalyzes the synthesis of malonyl-CoA, an intermediate in fatty acid synthesis, and suppressor of fatty acid oxidation (McGarry, Mannaerts et al. 1977). Therefore, we wanted to determine whether ACC phosphorylation was elevated. We found that livers of high-fat fed *Dgat1*$^{-/-}$ mice had elevated phosphorylation of ACC2, but not ACC1 (**Figure 4D**). These findings suggest that the loss of DGAT1 protects mice from developing hepatic steatosis by increasing fatty acid oxidation and AMPK signaling in the liver.

To determine whether DGAT1-deficiency could alter fatty acid synthesis, we isolated primary hepatocytes from WT and *Dgat1*$^{-/-}$ mice fed a high-fat diet, and measured the incorporation of ^{14}C-Acetate into fatty acids. We found that hepatocytes from *Dgat1*$^{-/-}$ mice had a ~50% reduction in the incorporation of ^{14}C-Acetate into fatty acids, when compared to WT controls, suggesting that fatty acid synthesis was reduced (**Figure 5A**) We then asked whether the decrease in fatty acid synthesis in the liver, was do to changes in the mRNA level of genes involved in fatty acid synthesis. To test this hypothesis, we isolated mRNA from livers of WT and *Dgat1*$^{-/-}$ mice fed a high-fat diet. We found that, SREBP1c, a

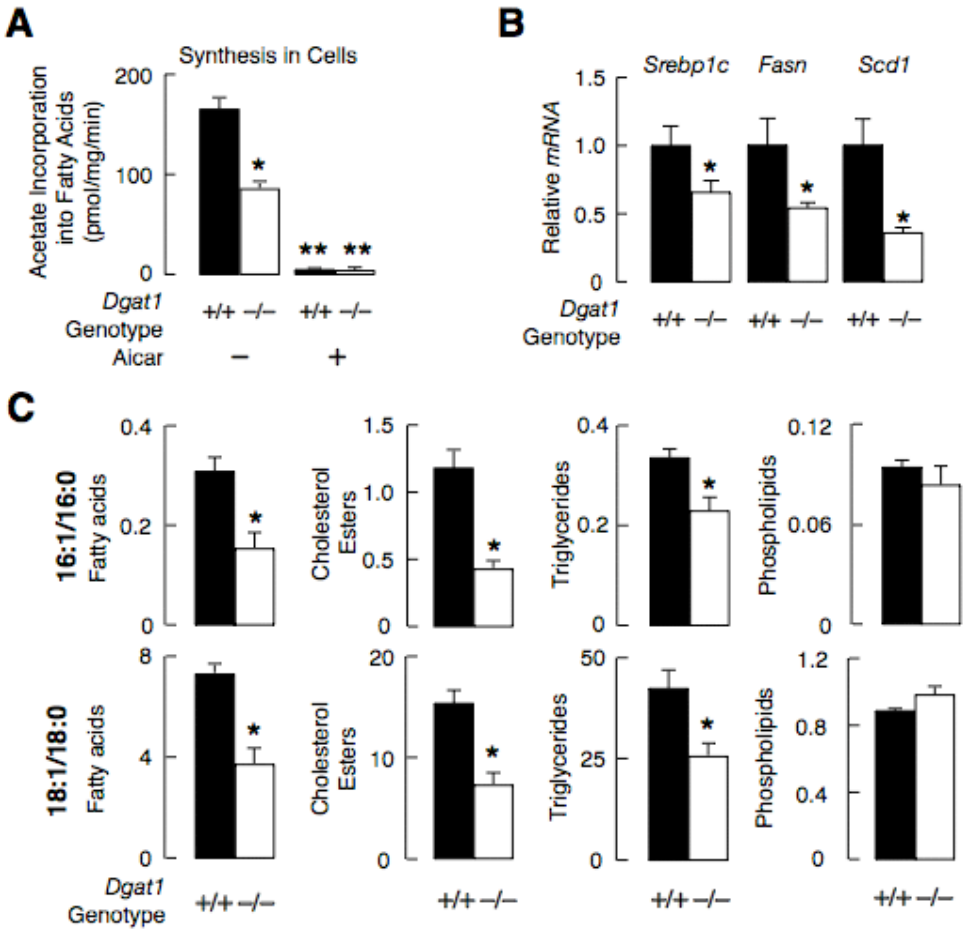

Figure 5. *Dgat1*$^{-/-}$ mice have reduced fatty acid synthesis and delta-9-desaturation. (A) Fatty acid synthesis in cells was measured by quantifying the incorporation of [^{14}C]-acetate into fatty acids. Freshly isolated hepatocytes from *Dgat1*$^{+/+}$ and *Dgat1*$^{-/-}$ mice were also treated with 2mM Aicar (age 12-16 weeks, N=3); * $P < 0.001$ *versus* +/+ control, ** $P < 0.001$ *versus* +/+ and -/- control. (B) Reduced mRNA levels of fatty acid synthesis genes in livers of *Dgat1*$^{-/-}$ mice fed high-fat diet. RNA was isolated from livers of male mice and cDNA was generated to quantify by real-time PCR. Genes were normalized to cyclophilin (age 8-12 weeks, N=5); * $P < 0.05$ *versus* +/+. (C) Reduced delta-9-desaturation index in neutral lipids of livers of *Dgat1*$^{-/-}$ mice fed high-fat diet. The delta-9-desaturation index was determined by taking the ratio of 16:1/16:0 and 18:1/18:0 in fatty acids, cholesterol esters, triglycerides and phospholipids (age 8-12 weeks, N=5); * $P < 0.001$ *versus* +/+ high-fat.

transcription factor that regulates the expression of fatty acid synthesis genes in the liver, had reduced mRNA levels by ~34% in *Dgat1*$^{-/-}$ mice (Figure 5B). This reduction in *Srebp1c* expression was accompanied by a ~54% reduction in fatty

acid synthase (*Fasn*) (Figure 5B), a multifunctional enzyme that catalyzes the synthesis of fatty acids from malonyl-CoA. Another target of SREBP1c is stearoyl-CoA desaturase 1 (*scd1*), a delta-9-desaturase that synthesizes the monounsaturated fatty acids palmitoleate (16:1) and oleate (18:1), from palmitate (16:0) and stearate (18:0), respectively. We found that livers from high-fat fed *Dgat1*$^{-/-}$ mice had a ~64% reduction in *Scd1* mRNA, as compared to WT (Figure 5B). We then asked whether the decrease in *Scd1* mRNA levels were accompanied by a decrease in the delta-9-desaturation of lipids in the liver. Delta-9-desaturation is determined by taking the ratio of 16:1/16:0 and 18:1/18:0. We found that mice lacking DGAT1 had a ~40-50% reduction in delta-9-desaturation in the fatty acid, cholesterol ester, and triglyceride pool (**Figure 5C**). In contrast, we found that hepatic delta-9-desaturation of the phospholipid pool remained similar between WT and *Dgat1*$^{-/-}$ mice (**Figure 5C**).

Discussion

The accumulation of lipids, mainly triglycerides within the liver is a hallmark of NAFLD (Angulo 2002; Clark 2006). However, the pathways involved in the progression to NAFLD have not been completely characterized. Previous findings have indicated that *Dgat1*$^{-/-}$ mice are protected against hepatic steatosis induced by chronic high-fat feeding, although the mechanism(s) has been unclear (Smith, Cases et al. 2000). Our results demonstrate that DGAT1-deficiency can reduce both hepatic triglyceride and cholesterol ester levels in mice fed a 3-week western diet. Our findings suggest that the protection against hepatic steatosis results from two possible mechanisms, the first is an increase in fatty acid oxidation, and the second is a decrease in fatty acid synthesis. These changes are accompanied by an increase in AMPK signaling, a kinase involved in sensing cellular energy stores, and replenishing them by activating catabolic pathways (fatty acid oxidation) to generate ATP, and suppressing anabolic pathways (fatty acid synthesis) that consume ATP.

The protection against hepatic steatosis in *Dgat1*$^{-/-}$ mice is likely a result of an increase in fatty acid oxidation in the liver. We found that primary hepatocytes isolated from high-fat fed *Dgat1*$^{-/-}$ mice have increased fatty acid oxidation. During fasting periods, the liver can oxidize fatty acids to generate ketone bodies, which serve as an energy source for tissues such as the brain and muscle. We found that β-hydroxybutyrate, a ketone body, is elevated in *Dgat1*$^{-/-}$ mice fed high-fat diet, supporting our hypothesis that the liver is oxidizing more fat. However, we did not find an increase in expression of genes involved in fatty

acid oxidation, therefore we suspect that post-translational changes may activate fatty acid oxidation in *Dgat1*$^{-/-}$ mice.

Protection against hepatic steatosis is often accompanied by a reduction in fatty acid synthesis in the liver. For example, Metformin, a drug used to treat type 2 diabetes, has been shown to protect against hepatic steatosis induced by high-fat feeding. Metformin action is believed to occur through AMPK signaling, which has been shown to suppress fatty acid synthesis in the liver (Zhou, Myers et al. 2001). Recent studies have indicated that AMPK, a multisubunit enzyme, can regulate fatty acid oxidation by phosphorylating and inactivating ACC, an enzyme that synthesizes malonyl-CoA, a signaling molecule that inhibits CPT1. Inhibition of ACC2, by deletion in mice or antisense oligonucleotides, increases fatty acid oxidation in the liver (Abu-Elheiga, Matzuk et al. 2001; Abu-Elheiga, Oh et al. 2003; Savage, Choi et al. 2006). Our results indicate that *Dgat1*$^{-/-}$ mice on a high-fat diet have increased phosphorylation of AMPK and ACC2. Therefore, we suspect that AMPK may be an important determinant in the regulation of fatty acid oxidation and fatty acid synthesis in *Dgat1*$^{-/-}$ mice. It will be interesting to determine whether this is true and identify the mechanisms that activate AMPK.

In the liver we found that Dgat1-deficiency reduced, not only triglyceride levels, but also cholesterol esters and fatty acids on a high-fat diet. A decrease in cholesterol esters was somewhat surprising, as DGAT1 has not been shown to synthesize cholesterol esters *in vitro*. Since mice lacking SCD1 have reduced cholesterol ester levels in the liver, we suspect that the decrease in cholesterol

ester levels is do to a suppression of *Scd1* mRNA levels (Miyazaki, Kim et al. 2000). This could explain the reduction in monounsaturated fatty acids and delta-9-desaturation (monounsaturated/saturated fatty acid ratio) of neutral lipids. Interestingly, delta-9-desaturation was unaltered in phospholipids, which may be explained by shunting of monounsaturated fatty acids toward phospholipids to maintain the correct ratio in livers of *Dgat1*$^{-/-}$ mice. Another possibility is the targeted degradation of phospholipids with specific compositions, in this case phospholipids containing saturated fatty acids.

In summary, we have shown that *Dgat1*$^{-/-}$mice are protected against hepatic steatosis by reducing both hepatic triglycerides and cholesterol ester levels. Our findings indicate that AMPK may be an important determinant in the protection against hepatic steatosis, and could contribute to the activation of fatty acid oxidation, and suppression of fatty acid synthesis in *Dgat1*$^{-/-}$mice. More importantly, our findings indicate that inhibition of DGAT1 may be a viable therapeutic target for the treatment of NAFLD.

References

Abu-Elheiga, L., M. M. Matzuk, et al. (2001). "Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2." Science **291**(5513): 2613-6.

Abu-Elheiga, L., W. Oh, et al. (2003). "Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets." Proc Natl Acad Sci U S A **100**(18): 10207-12.

Angulo, P. (2002). "Nonalcoholic fatty liver disease." N Engl J Med **346**(16): 1221-31.

Buhman, K. K., H. C. Chen, et al. (2001). "The enzymes of neutral lipid synthesis." J Biol Chem **276**(44): 40369-72.

Buhman, K. K., S. J. Smith, et al. (2002). "DGAT1 is not essential for intestinal triacylglycerol absorption or chylomicron synthesis." J Biol Chem **277**(28): 25474-9.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Chen, H. C., Z. Ladha, et al. (2002). "Deficiency of acyl coenzyme a:diacylglycerol acyltransferase 1 increases leptin sensitivity in murine obesity models." Endocrinology **143**(8): 2893-8.

Chen, H. C., S. J. Smith, et al. (2002). "Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **109**(8): 1049-55.

Clark, J. M. (2006). "The epidemiology of nonalcoholic fatty liver disease in adults." J Clin Gastroenterol **40**(3 Suppl 1): S5-10.

Coleman, R. A. and D. P. Lee (2004). "Enzymes of triacylglycerol synthesis and their regulation." Prog Lipid Res **43**(2): 134-76.

Folch, J., M. Lees, et al. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Horton, J. D., H. Shimano, et al. (1999). "Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL." J Clin Invest **103**(7): 1067-76.

McGarry, J. D., G. P. Mannaerts, et al. (1977). "A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis." J Clin Invest **60**(1): 265-70.

Miyazaki, M., Y. C. Kim, et al. (2000). "The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearoyl-CoA desaturase 1." J Biol Chem **275**(39): 30132-8.

Savage, D. B., C. S. Choi, et al. (2006). "Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide

inhibitors of acetyl-CoA carboxylases 1 and 2." J Clin Invest **116**(3): 817-24.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Snyder, F. and N. Stephens (1959). "A simplified spectrophotometric determination of ester groups in lipids." Biochim Biophys Acta **34**: 244-5.

Velasco, G., M. J. Geelen, et al. (1997). "Control of hepatic fatty acid oxidation by 5'-AMP-activated protein kinase involves a malonyl-CoA-dependent and a malonyl-CoA-independent mechanism." Arch Biochem Biophys **337**(2): 169-75.

Watkins, S. M., T. Y. Lin, et al. (2001). "Unique phospholipid metabolism in mouse heart in response to dietary docosahexaenoic or alpha-linolenic acids." Lipids **36**(3): 247-54.

Winder, W. W., H. A. Wilson, et al. (1997). "Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A." J Appl Physiol **82**(1): 219-25.

Zhou, G., R. Myers, et al. (2001). "Role of AMP-activated protein kinase in mechanism of metformin action." J Clin Invest **108**(8): 1167-74.

Chapter 3. Hepatic DGAT1 deficiency protects against hepatic steatosis induced by western diet.

Abstract

Nonalcoholic fatty liver disease is characterized by the accumulation of triglycerides in livers of individuals who consume little to no alcohol. The synthesis of triglycerides is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes. Because of the ubiquitous expression pattern of *Dgat1*, it has been unclear whether the protection against hepatic steatosis in $Dgat1^{-/-}$mice is do to cell autonomous or endocrine effects. To investigate this further, we generated a mutant mouse strain, $Dgat1^{flox/flox}$ mice, which have exons 14-17 of the *Dgat1* allele flanked by *loxP* sites. In order to generate a liver specific deletion of *Dgat1*, we infused a *Cre* expressing adenovirus through the jugular vein, reducing *Dgat1* mRNA levels and DGAT activity. Here we show that liver-specific inactivation of DGAT1 can in part protect mice against hepatic steatosis induced by high-fat diet.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common of all liver disorders affecting an estimated 14-24% of adults in the United States (Angulo 2002; Clark 2006). NAFLD is characterized by hepatic steatosis in individuals who consume little to no alcohol.

The lipid primarily found in those with NAFLD is triacylglycerol, an energy rich molecule that is synthesized by DGAT enzymes (Weiss, Kennedy et al. 1960; Coleman and Lee 2004). DGAT enzymes catalyze the last committed step in triglyceride synthesis, joining a fatty acyl-CoA molecule to diacylglycerol (Buhman, Chen et al. 2001). There are two known DGAT enzymes in the liver, DGAT1 and DGAT2. Both enzymes synthesize triglycerides in the endoplasmic reticulum with broad fatty acyl-CoA substrate specificity (Cases, Smith et al. 1998; Cases, Stone et al. 2001). DGAT1 and DGAT2 are expressed in tissues involved in triglyceride synthesis and storage, such as the small intestine, adipose tissue, mammary gland, and the liver (Cases, Smith et al. 1998; Cases, Stone et al. 2001).

In the liver DGAT2 is expressed at higher levels than DGAT1. Our findings that mice lacking DGAT1 are protected against diet-induced hepatic steatosis (Chapter 2), suggest that despite the low expression level, DGAT1 plays an important role in synthesizing triglycerides in the liver (Smith, Cases et al. 2000). To further test this hypothesis, we generated mice with exons 14-17 of the *Dgat1* allele flanked by *loxP* sites, $Dgat1^{flox/flox}$. Using a *Cre* expressing adenovirus and $Dgat1^{flox/flox}$ mice, we created mice lacking *Dgat1* specifically in the liver. We

find that the loss of DGAT1 in the liver partially protects mice against diet-induced hepatic steatosis.

Material and Methods

Mice. Mice were housed in a pathogen-free barrier facility (12-hour light/12-hour dark cycle) and fed rodent chow (5053 PicoLab Diet; Purina, St. Louis, Missouri, USA). For high-fat diet experiments, mice were fed a Western-type diet containing 20% milk fat by weight and 0.2% cholesterol (TD 01064 Harlan-Teklad Laboratory, Madison, Wisconsin, USA).

Generation of *Dgat1*$^{flox/flox}$ mice. A targeting vector was designed to flank exons 14-17 with loxP sites, a similar region deleted in *Dgat1*$^{-/-}$ mice (Smith et al., 2000). *Dgat1* genomic fragments were amplified by PCR from 129/SvJae mouse genomic DNA. The vector was constructed in the pJB1 vector (gift from Dr. Joachim Herz, University of Texas Southwestern Medical Center, Dallas, TX) by subcloning a 1.0-kilobase (kb) upstream short-arm fragment containing 5' coding sequences (sense primer 5'-cggggtaccGCTTTATTCCTACCGGGATG-3' and antisense primer 5'-aagcggccgcAAACAATGGGATAAGCACAG-3'), a 817-bp fragment containing exons 14-17 of *Dgat1* (sense primer 5'ggaattcCTGTGCTTATCCCATTGTTT-3' and antisense primer 5'-gccggtaccAAATGCCATCCCCAAGAGCA-3'), and a 7.5-kb downstream long-arm fragment containing the *Dgat1* stop codon and polyadenylation signal (sense primer 5'-ccctcgagGGCATTTGAATCTCACCACTG-3' and antisense primer 5'-ccgctcgagTCAGCTGATTGGTCTTCACAC-3'). Primer sequences (lowercase letters) were added on the primer termini to introduce *Kpn1* (short arm sense primer), *Not*I (short arm antisense primer), *Eco*RI (exons 14-17 sense primer), *Kpn*I (exons 14-17 antisense primer), and *Xho*I (long arm sense and antisense

primers) restriction enzyme sites for cloning. This targeting construct was introduced into 129/SvJae murine embryonic stem cells (line RF8)(Streeper, Koliwad et al. 2006), and clones containing targeted alleles were identified by PCR and verified by Southern blotting. Cells containing the targeted *Dgat1* allele were used to generate mice and subsequent genotyping in mice was performed by PCR. Heterozygous *Dgat1*$^{loxP:frt/+}$ mice were then crossed with Actin-FLPe transgenic mice obtained from Jackson Laboratory in a C57BL/6J genetic background to remove the neomycin resistance gene. The FLPe transgene was genotyped by PCR using sense primer 5'-CACTGATATTGTAAGTAGTTTGC-3' and antisense primer 5'-CTAGTGCGAAGTAGTGATCAGG-3'. For the southern blot, the floxed *Dgat1* allele was confirmed by hybridizing the *Eco*RI-digested genomic DNA with a ^{32}P-labelled 822-bp fragment containing exons 14-17 of *Dgat1* amplified by PCR from the genomic DNA using the sense primer 5'-CTGTGCTTATCCCATTGTTT-3' and antisense primer 5'-AAATGCCATCCCCAAGAGCA-3'. The wild-type *Dgat1* allele gave an 8.1-kb fragment and the floxed *Dgat1* allele gave a 5.8-kb fragment. A further cross was carried out with *Dgat1*$^{+/+}$ mice. Pups carrying the floxed allele of *Dgat1* and not Actin-FLPe were selected to generate *Dgat1*$^{flox/flox}$ mice for use in adenovirus experiments.

Adenovirus. *Cre* and *LacZ* expressing adenovirus were obtained from Vector Development Laboratory, Baylor College, Texas. Experiments were performed on 16-20 week-old *Dgat1*$^{flox/flox}$ mice. Mice were given an intravenous dose of $2x10^{11}$ virus particles in 0.2 ml of PBS *via* the jugular vein (Rohlmann,

Gotthardt et al. 1996). Following a 4-week recovery period, mice were placed on a high-fat diet for 3 weeks.

DGAT activity assays. Mouse liver was homogenized in buffer [Buffer A, 50 mM Tris-HCl (pH 7.4) and 250 mM sucrose] containing proteinase inhibitors (Roche Diagnostic). To prepare microsomes, the homogenates were centrifuged three times at 4°C (600×*g* for 5 min, 10,000×*g* for 10 min, and 100,000×*g* for 1 hr); after each centrifugation, the pellets were resuspended in Tris-sucrose buffer. DGAT assays were performed using microsome proteins (100 μg) in an assay mix containing Buffer A, 100 mM $MgCl_2$, 1.25 mg/ml BSA, 200 μM 1,2-dioleoyl-*sn*-glycerol (Sigma-Aldrich, St. Louis, MO) in acetone, and 25 μM [^{14}C]oleoyl-CoA (53.0 mCi/mmol). Lipids were extracted with chloroform:methanol (2:1, v:v) and separated by Silica Gel G-60 TLC plates with hexane:ethyl ether:acetic acid (80:20:1) solvent. The triglyceride bands were scraped, and radioactivity was measured by scintillation counting.

Lipid Analysis. Livers were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose and complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated by thin-layer chromatography with a solvent system of hexane:diethyl ether:acetic acid (80:20:1) on Silica Gel G-60 TLC plates (Folch, Lees et al. 1957). Using triolein as a standard, triglycerides were visualized with iodine vapor. Triglycerides were identified and quantified using a spectrophotometric technique (Snyder and Stephens 1959).

RNA extraction and real-time PCR. RNA was extracted from liver using RNA STAT-60 (Tel-Test Inc.) and traces of DNA were removed by DNase

treatment (Ambion). 5 mg of RNA was used to synthesize cDNA using superscript II reverse transcriptase and random hexamers (Invitrogen). Real-time PCR primers were selected for each cDNA using Primer Express software (version 1.5; Applied Biosystems). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with sybrgreen reagents using two-step RT-PCR reactions on ABI 9600. Cyclophilin was used to normalize for gene expression. **Cyclophilin**, F:5'–TGGAAGAGCACCAAGACAG ACA–3' and R: 5'–TGCCGGAGTCGACAATGAT–3'; **Srebp1c**, F:5'–GGAGC CATGGATTG CACATT–3' and R:5'–GGCCCGGGAAGTCACTGT–3'; **Fasn**, F:5'–GCTGCGG AAACTTCAGGAAAT–3' and R: 5'–AGAGACGTGTCACTCCTGGACTT–3'; **Scd1**, F:5'–CCTTCCCCTTCGACTACTCTG–3' and R: 5'–GCCATGCAGT CGATGAAGAA–3'.

Statistical Analysis. Values are reported as mean ± standard error. Statistical differences were determined by students t-test.

Results

Because *Dgat1* is widely expressed in murine tissues, the protection against hepatic steatosis in *Dgat1*$^{-/-}$ mice (Chapter 2) may have resulted from the loss of DGAT1 in the liver or from other tissues that affect hepatic triglyceride balance, such as small intestine, adipose tissue, or the central nervous system. We therefore examined diet-induced steatosis in mice lacking *Dgat1* specifically in the liver. For these studies, we administered intravenously an adenovirus expressing *Cre* recombinase to mice that were homozygous for a "floxed" *Dgat1* allele harboring *loxP* sites flanking exons 14–17 (*Dgat1*$^{flox/flox}$) (**Figure 1A**). Southern blot analysis (**Figure 1B**) using a probe that recognizes exons 14-17 was used to confirm genotype. In several experiments, we achieved maximal reduction of ~80% in *Dgat1* mRNA levels (**Figure 2A**) as compared to mice treated

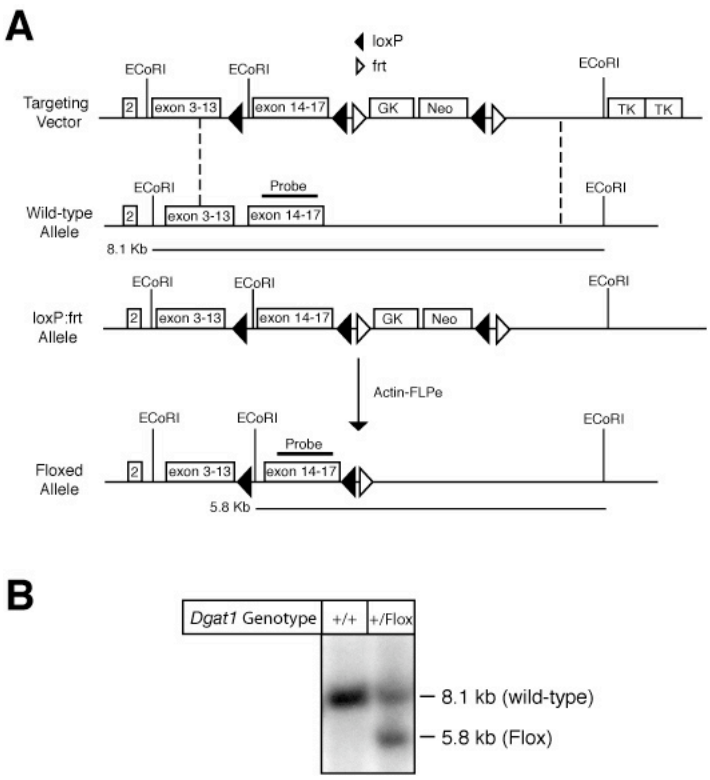

Figure 1. Targeting strategy used to generate *Dgat1*$^{flox/flox}$ mice. (A) Targeting strategy used to generate *Dgat1*$^{flox/flox}$ mice shows the targeting vector, wild-type allele, *Dgat1*$^{flox:frt}$ allele, and *Dgat1* floxed allele. (B) Southern probe on exons 14-17 indicate genotype of wild-type and *Dgat1*$^{+/flox}$ mice.

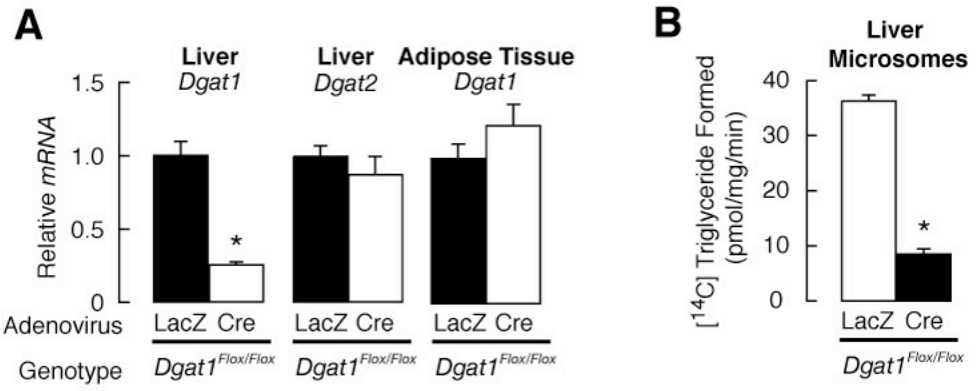

Figure 2. Reduced hepatic *Dgat1* mRNA and activity in $Dgat1^{flox/flox}$ mice treated with Cre expressing adenovirus. (A) Male $Dgat1^{flox/flox}$ mice given an intravenous dose of $2x10^{11}$ virus particles of the *LacZ* or *Cre* expressing adenovirus. $Dgat1^{flox/flox}$ mice had reduced hepatic mRNA levels of *Dgat1*, while *Dgat2* mRNA remained unchanged. *Dgat1* mRNA levels in adipose tissue were not altered in $Dgat1^{flox/flox}$ mice given the *Cre* expressing adenovirus. * $P < 0.001$ *vs LacZ.* (B) DGAT activity was reduced in $Dgat1^{flox/flox}$ mice treated with Cre expressing adenovirus. Using liver microsomes, DGAT activity was determined by quantifying the incorporation of [^{14}C]oleoyl-CoA into triglycerides. (Age 16-20 weeks, N=5-6). * $P < 0.001$ *vs LacZ.* Mice were on high-fat diet for 3 weeks.

with the control adenovirus, *LacZ.* We examined gene specificity by quantifying *Dgat2* mRNA levels in the liver, finding that *Dgat2* mRNA levels (**Figure 2A**) remained unchanged between mice treated with *LacZ* or *Cre* adenovirus. To examine tissue specificity, we quantified *Dgat1* mRNA levels (**Figure 2A**) in the adipose tissue, and found that *Dgat1* expression was similar between $Dgat1^{flox/flox}$ mice treated with *LacZ* or *Cre* adenovirus.

To determine the consequence of reducing *Dgat1* mRNA levels on DGAT activity, we prepared microsomes from $Dgat1^{flox/flox}$ mice treated with *LacZ* or *Cre* adenovirus and measured DGAT activity. In the liver, we found a 77% reduction in microsomal DGAT activity (**Figure 2B**). To determine whether the loss of DGAT1 in the liver could protect mice against diet-induced hepatic steatosis, we

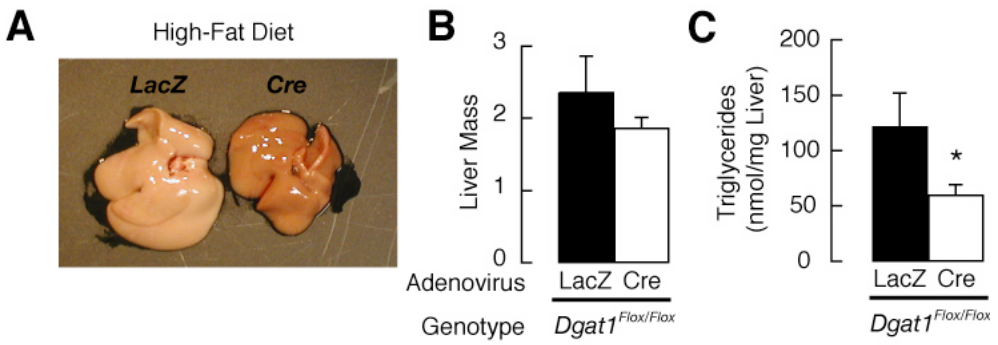

Figure 3. Protection against diet-induced hepatic steatosis in *Dgat1*$^{flox/flox}$ mice treated with *Cre* expressing adenovirus. (A) Gross morphology of livers from *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or *Cre* expressing adenovirus. (B) *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or Cre expressing adenovirus have similar liver weights. (C) Reduced hepatic triglyceride levels in *Dgat1*$^{flox/flox}$ mice treated with *Cre* adenovirus. (Age 16-20 weeks, N=5-6). * $P < 0.05$ *vs LacZ*. Mice were on high-fat diet for 3 weeks.

placed adenovirus treated *Dgat1*$^{flox/flox}$ mice on a high-fat diet for 3 weeks. By gross morphology (**Figure 3A**), mice treated with *Cre* adenovirus did not develop the usual pale color that is seen in livers from mice fed high-fat diet. However, liver mass (**Figure 3B**) was mostly unchanged between *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or *Cre* adenovirus. Furthermore, % liver mass was unchanged between *Dgat1*$^{flox/flox}$ mice treated with *LacZ* (4.36 ± 0.11 %) or *Cre* (4.42 ± 0.15 %) adenovirus. We found that *Dgat1*$^{flox/flox}$ mice treated with the *Cre* expressing adenovirus had a ~50% reduction in hepatic triglyceride levels (**Figure 3C**) as compared to *LacZ* control.

Our findings that the protection against hepatic steatosis in *Dgat1*$^{-/-}$ mice was accompanied by reduced expression of genes involved in fatty acid synthesis (Chapter 2), suggests that the lack of DGAT1 within the liver may also result in the suppression of fatty acid synthesis. To test this hypothesis, we studied the expression of genes involved in fatty acid synthesis. Our findings indicate that after a 3-week high-fat diet, *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or *Cre* expressing

adenovirus had similar expression levels of the transcription factor *Srebp1c* and its targets *Fasn* and *Scd1* (**Figure 4A**).

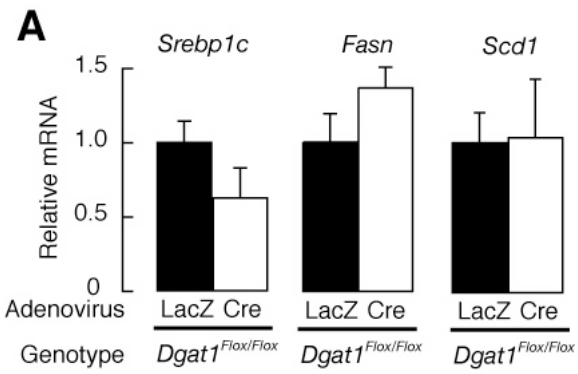

Figure 4. Similar expression levels of genes involved in fatty acid synthesis. (A) mRNA levels of *Srebp1c*, *Fasn* and *Scd1* were similar between *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or *Cre* expressing adenovirus. (Age 16-20 weeks, N=5-6). Mice were on high-fat diet for 3 weeks.

Discussion

In this study, we used *Cre/loxP* technology to study the role of hepatic-DGAT1 in diet-induced hepatic steatosis (Rohlmann, Gotthardt et al. 1996; Rossant and McMahon 1999). Using an adenovirus expressing a *Cre* recombinase, we were able to generate a liver-specific deletion of DGAT1. We found that LSD1KO mice had reduced DGAT1 expression and activity in the liver. Our findings indicate that mice lacking DGAT1 in the liver are partially protected against hepatic steatosis induced by high-fat feeding.

In *Dgat1*$^{-/-}$ mice we found an 81% reduction in hepatic triglycerides (Chapter 2). However, *Dgat1*$^{flox/flox}$ mice treated with *Cre* adenovirus have a 50% reduction in hepatic triglycerides. The difference in magnitude suggests that protection against hepatic steatosis cannot be fully explained by the loss of DGAT1 within the liver (Smith, Cases et al. 2000). We suspect that other tissues may be contributing to the protection against hepatic steatosis, such as the brain, adipose tissue, or intestine. Fat transplantation studies using *Dgat1*$^{-/-}$ fat have indicated that the effects of DGAT1-deficiency on glucose metabolism are a result of the altered endocrine function of the adipose tissue, which may also explain the protection against hepatic steatosis in DGAT1-deficient mice (Chen, Jensen et al. 2003).

Dgat1$^{-/-}$ mice on a high-fat diet have reduced expression of genes involved in fatty acid synthesis (Chapter 2). There is evidence of this in mice treated with *Dgat2* antisense oligonucleotides, which have reduced expression of genes involved in fatty acid synthesis (Yu, Murray et al. 2005). However, we

found that the loss of DGAT1 in the liver did not reduce expression of fatty acid synthesis genes, suggesting that extrahepatic tissues in *Dgat1*$^{-/-}$ mice, such as the adipose tissue, intestine, or the central nervous system could act on the liver to suppress fatty acid synthesis. For example, Leptin, a hormone released by the adipose tissue, has been shown to suppress the expression of genes involved in fatty acid synthesis in the liver by acting on the central nervous system (Shimomura, Matsuda et al. 2000; Asilmaz, Cohen et al. 2004). Therefore, reduced fatty acid synthesis in the liver may be explained by leptin sensitivity in *Dgat1*$^{-/-}$ mice (Chen, Smith et al. 2002).

In summary, by using *Dgat1*$^{flox/flox}$ mice and recombinant *Cre* adenovirus, we were able to reduce *Dgat1* mRNA levels and DGAT activity in the liver. We find that hepatic-DGAT1 is involved in the development of hepatic steatosis induced by high-fat feeding. Our findings suggest that DGAT1-specific inhibitors would be useful in treating hepatic steatosis associated with high-fat feeding.

References

Angulo, P. (2002). "Nonalcoholic fatty liver disease." N Engl J Med **346**(16): 1221-31.

Asilmaz, E., P. Cohen, et al. (2004). "Site and mechanism of leptin action in a rodent form of congenital lipodystrophy." J Clin Invest **113**(3): 414-24.

Buhman, K. K., H. C. Chen, et al. (2001). "The enzymes of neutral lipid synthesis." J Biol Chem **276**(44): 40369-72.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Chen, H. C., D. R. Jensen, et al. (2003). "Obesity resistance and enhanced glucose metabolism in mice transplanted with white adipose tissue lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **111**(11): 1715-22.

Chen, H. C., S. J. Smith, et al. (2002). "Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **109**(8): 1049-55.

Clark, J. M. (2006). "The epidemiology of nonalcoholic fatty liver disease in adults." J Clin Gastroenterol **40**(3 Suppl 1): S5-10.

Coleman, R. A. and D. P. Lee (2004). "Enzymes of triacylglycerol synthesis and their regulation." Prog Lipid Res **43**(2): 134-76.

Folch, J., M. Lees, et al. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Rohlmann, A., M. Gotthardt, et al. (1996). "Sustained somatic gene inactivation by viral transfer of Cre recombinase." Nat Biotechnol **14**(11): 1562-5.

Rossant, J. and A. McMahon (1999). ""Cre"-ating mouse mutants-a meeting review on conditional mouse genetics." Genes Dev **13**(2): 142-5.

Shimomura, I., M. Matsuda, et al. (2000). "Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice." Mol Cell **6**(1): 77-86.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Snyder, F. and N. Stephens (1959). "A simplified spectrophotometric determination of ester groups in lipids." Biochim Biophys Acta **34**: 244-5.

Streeper, R. S., S. K. Koliwad, et al. (2006). "Effects of DGAT1 deficiency on energy and glucose metabolism are independent of adiponectin." Am J Physiol Endocrinol Metab **291**(2): E388-94.

Weiss, S. B., E. P. Kennedy, et al. (1960). "The enzymatic synthesis of triglycerides." J Biol Chem **235**: 40-4.

Yu, X. X., S. F. Murray, et al. (2005). "Antisense oligonucleotide reduction of DGAT2 expression improves hepatic steatosis and hyperlipidemia in obese mice." Hepatology **42**(2): 362-71.

Chapter 4. Hepatic DGAT1 deficiency protects against hepatic steatosis induced by fasting

Abstract

During periods of prolonged fasting, several metabolic adaptations occur in order to maintain energy homeostasis, such as the release of free fatty acids from the adipose tissue to the liver. This influx of free fatty acids to the liver can result in the buildup of triglycerides, causing hepatic steatosis. DGAT1, an enzyme that catalyzes the final step in triglyceride synthesis has been implicated in synthesizing triglycerides during a prolonged fast. To test this hypothesis, *Dgat1*$^{-/-}$ mice were fasted for 20 hours, and were found to have reduced hepatic triglyceride levels. Furthermore, we found that mice that have a liver-specific deletion of DGAT1 were also protected against fasting-induced hepatic steatosis. Our findings provide evidence that hepatic DGAT1 plays an important role in maintaining triglyceride balance during times of energy deficiency.

Introduction

Mammals have evolved physiological mechanisms that allow them to deal with periods of energy deficiency. One mechanism that has evolved is the shift from using carbohydrates as a primary energy source, to using fat. During states of energy deficiency, activation of triglyceride hydrolysis in the adipose tissue elevates free fatty acid levels in plasma (Holm, Osterlund et al. 2000; Raclot 2003). Free fatty acids can then be taken up by the liver and oxidized in mitochondria to generate ATP or ketone bodies (Hegardt 1999). Ketone bodies provide a fuel source for the brain during fasting periods when glucose is scarce. Free fatty acids can also be utilized for triglyceride synthesis and stored in hepatocytes or secreted as a component of VLDL (Fukuda and Ontko 1984; Hegardt 1999). When free fatty acid influx exceeds the livers ability to oxidize or secrete VLDL, triglycerides accumulate in the liver, causing hepatic steatosis (Hashimoto, Cook et al. 2000).

The final step in triglyceride synthesis is catalyzed by acyl-CoA diacylglycerol:acyl transferase (DGAT) enzymes, which join a fatty acyl-CoA molecule to diacylglycerol (Buhman, Chen et al. 2001; Coleman and Lee 2004). Two DGAT enzymes have been identified, DGAT1 and DGAT2. They belong to distinct gene families that share no nucleotide sequence or protein homology (Cases, Smith et al. 1998; Cases, Stone et al. 2001). Both enzymes are highly expressed in tissues involved in triglyceride metabolism, such as the adipose tissue, liver, intestine and mammary gland (Cases, Smith et al. 1998; Cases, Stone et al. 2001). Studies have indicated that *Dgat1* is upregulated at the mRNA level

by fasting (Heijboer, Donga et al. 2005), suggesting that DGAT1 is involved in esterifying fatty acids during fasting.

Based on these observations, we hypothesized that DGAT1 has a role in fasting induced hepatic steatosis. To test this hypothesis, *Dgat1*$^{-/-}$ mice and mice with a liver specific deletion of DGAT1 were fasted for 20 hours. Our findings indicate that hepatic DGAT1 is involved in the adaptive response to fasting.

Materials and Methods

Mice. *Dgat1*$^{-/-}$ mice and wild-type mice were in a C57BL/6J background. Genotyping for *Dgat1* was performed as described earlier (Smith, Cases et al. 2000). Mice were housed in a pathogen-free barrier facility (12-hour light/12-hour dark cycle) and fed rodent chow (5053 PicoLab Diet; Purina, St. Louis, Missouri, USA). Mice were fasted overnight for 20 hours with free access to water. All experiments were approved by the Committee on Animal Research of the University of California, San Francisco.

Adenovirus. *Cre* and *LacZ* expressing adenovirus were obtained from Vector Development Laboratory, Baylor College, Texas. Experiments were performed on 16-20 week-old *Dgat1*$^{flox/flox}$ mice. Mice were given an intravenous dose of $2x10^{11}$ virus particles in 0.2 ml of PBS *via* the jugular vein (Rohlmann, Gotthardt et al. 1996).

Lipid Analysis. Livers were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose and complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated by thin-layer chromatography with a solvent system of hexane:diethyl ether:acetic acid (80:20:1) on Silica Gel G-60 TLC plates (Folch, Lees et al. 1957). Using triolein as a standard, triglycerides were visualized with iodine vapor. Triglycerides were identified and quantified using a spectrophotometric technique (Snyder and Stephens 1959).

RNA extraction and real-time PCR. RNA was extracted from liver using RNA STAT-60 (Tel-Test Inc.) and traces of DNA were removed by DNase treatment (Ambion). 5 mg of RNA was used to synthesize cDNA using

superscript II reverse transcriptase and random hexamers (Invitrogen). Real-time PCR primers were selected for each cDNA using Primer Express software (version 1.5; Applied Biosystems). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with sybrgreen reagents using two-step RT-PCR reactions on ABI 9600. Cyclophilin was used to normalize for gene expression. **Cyclophilin**, F:5'–TGGAAGAGCACCAAGAC AGACA–3' and R:5'–TGCCGGAGTCGACAATGAT–3'; **Fasn**, F:5'–GCTGCGGAAA CTTCAGGAAAT–3' and R:5'–AGAGACGTGTCACTCCTGGACTT–3'; **Scd1**, F:5'–CCTTCCCCTTCGACTACTCTG–3' and R:5'–GCCATGCAGTCGATGAA GAA–3'.

Statistical Analysis. Values are reported as mean ± standard error. Statistical differences were determined by students t-test.

Results

To test whether DGAT1 is involved in fasting-induced hepatic steatosis, we measured hepatic *Dgat1* mRNA levels under different dietary conditions. Hepatic *Dgat1* mRNA levels increased significantly in mice that were fasted for 16 hours compared with those in mice fed *ad libitum* or after refeeding (**Figure 1A**). In contrast, *Dgat2* mRNA levels were similar during these conditions, suggesting that DGAT1 was involved in synthesizing TG during fasting. We therefore examined hepatic steatosis in $Dgat1^{+/+}$ and $Dgat1^{-/-}$ mice that were fasted for 20 hours. Livers from $Dgat1^{-/-}$ mice were less

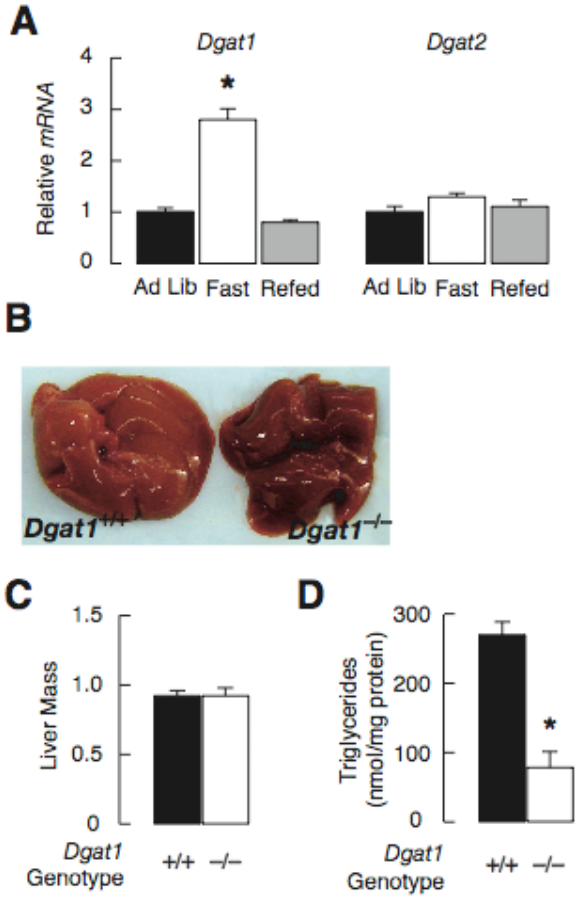

Figure 1. Protection against fasting-induced hepatic steatosis in $Dgat1^{-/-}$ mice. (A) Expression levels of *Dgat1* and *Dgat2* mRNA in the livers of male mice fed *ad libitum* (Ad Lib), fasted for 16 hours (Fast), or fasted for 24 hours and re-fed for 12 hours (Refed). mRNA levels were quantified by real-time PCR (age 13–14 weeks, n = 6–8 *per* group). **P* < 0.001 *versus* Ad Lib and Refed. (B) Gross appearance of livers showing color change in $Dgat1^{-/-}$mice (20 hour fast). (C) Liver weights in $Dgat1^{+/+}$ and $Dgat1^{-/-}$ mice (20 hour fast, age 12 weeks, n = 5 *per* genotype). (D) Decreased triglyceride content in livers of $Dgat1^{-/-}$mice (20 hour fast, age 12 weeks, n = 5 *per* genotype). **P* < 0.0002 *versus* control.

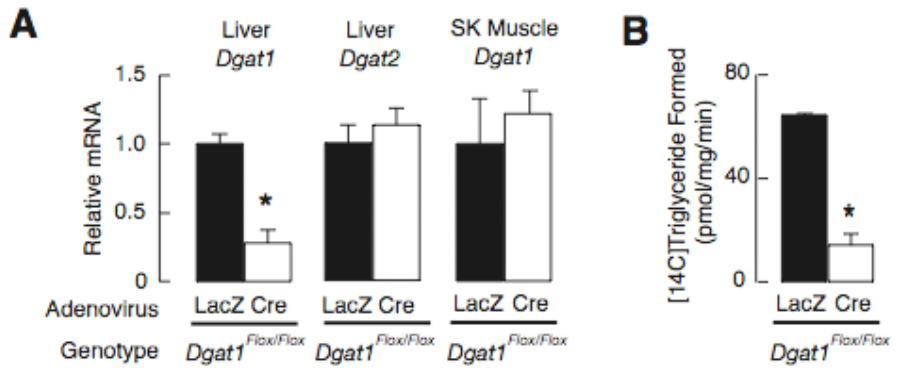

Figure 2. Reduced hepatic *Dgat1* mRNA and activity in *Dgat1*$^{flox/flox}$ mice treated with *Cre* expressing adenovirus. (A) Male *Dgat1*$^{flox/flox}$ mice given an intravenous dose of 2x10^{11} virus particles of the *LacZ* or *Cre* expressing adenovirus. *Dgat1*$^{flox/flox}$ mice had reduced hepatic mRNA levels of *Dgat1*, while *Dgat2* mRNA remained unchanged. *Dgat1* mRNA levels in gastrocnemius were not altered in *Dgat1*$^{flox/flox}$ mice infused with *Cre* expressing adenovirus. **P* < 0.002 *versus LacZ*. (B) DGAT activity was reduced in *Dgat1*$^{flox/flox}$ mice treated with *Cre* expressing adenovirus. Using liver microsomes, DGAT activity was determined by quantifying the incorporation of [^{14}C]oleoyl-CoA into triglycerides. (Age 16-20 weeks, N=5). **P* < 0.002 *versus LacZ*. Mice were fasted for 20 hours.

pale than those of *Dgat1*$^{+/+}$ mice (**Figure 1B**). Despite the change in color, liver mass (**Figure 1C**) was similar in *Dgat1*$^{+/+}$ and *Dgat1*$^{-/-}$ mice, probably because the degree of steatosis was modest. Hepatic triglyceride content in the *Dgat1*$^{-/-}$ livers was reduced by ~70% (**Figure 1D**). Serum β-hydroxybutyrate, a ketone body, was elevated in fasted *Dgat1*$^{-/-}$ mice (0.54 ± 0.13 mM *versus* 0.32 ± 0.07 mM for *Dgat1*$^{+/+}$, n = 5 per genotype, 8 hr fast), whereas serum triglyceride levels were reduced (36 ± 3 mg/dL *versus* 74 ± 7 mg/dL for *Dgat1*$^{+/+}$, n = 5-7 per genotype, 24 hr fast). Genes involved in fatty acid oxidation, such as PPAR-α and acyl-CoA oxidase, were similar between *Dgat1*$^{+/+}$ and *Dgat1*$^{-/-}$ mice (Data not shown). These findings provide evidence that DGAT1 is involved in the adaptive response to fasting in the liver.

To determine whether the protection against fasting-induced hepatic steatosis was a result of the loss of DGAT1 within the liver, and not other tissues,

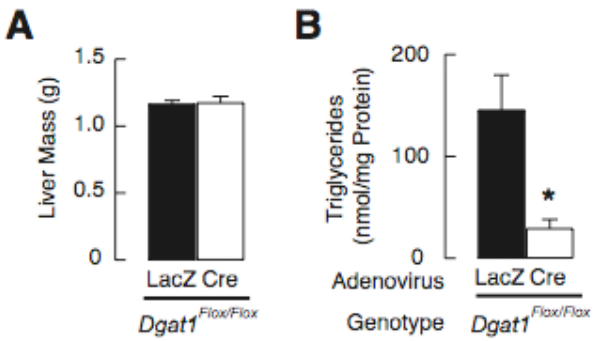

Figure 3. *Dgat1*$^{flox/flox}$ mice treated with *Cre* expressing adenovirus are protected against fasting-induced hepatic steatosis. (A) *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or Cre expressing adenovirus have similar liver weights after fasting for 20 hours. (B) *Dgat1*$^{flox/flox}$ mice treated with *Cre* adenovirus have reduced hepatic triglyceride levels after fasting for 20 hours. (Age 16-20 weeks, N=5). * $P < 0.01$ *vs LacZ.*

we generated a conditional deletion of DGAT1 in the liver. Using real-time PCR we found that mRNA levels of *Dgat1* in the liver (**Figure 2A**) were reduced by ~70% in *Dgat1*$^{flox/flox}$ mice treated with *Cre* expressing adenovirus, while mRNA levels of hepatic *Dgat2* were similar between *Dgat1*$^{flox/flox}$ mice treated with *LacZ* or *Cre* expressing adenovirus. To show that the reduction in *Dgat1* mRNA levels were specific to the liver, mRNA levels of *Dgat1* (**Figure 2A**) were quantified in the gastrocnemius, and were found to be similar in *Dgat1*$^{flox/flox}$ mice that were treated with *LacZ* or *Cre* expressing adenovirus. In comparison to the DGAT activity (**Figure 2B**) from livers of *Dgat1*$^{flox/flox}$ mice treated with *LacZ* adenovirus, *Cre* treated mice had a 78% reduction in microsomal DGAT activity. Liver mass was similar between *Dgat1*$^{flox/flox}$ mice (**Figure 3A**) infused with *LacZ* or *Cre* expressing adenovirus. We found that *Dgat1*$^{flox/flox}$ mice treated *Cre* (**Figure 3B**), had reduced hepatic triglyceride levels by 80%, which was a similar reduction found in D*gat1*$^{-/-}$ mice.

Discussion

Our results demonstrate a functional role for DGAT1 in hepatic TG balance during fasting. During a fast, hepatic glycogen content becomes depleted, and the liver switches to utilize lipids as fuel. Lipolysis is activated in WAT (Zimmermann, Strauss et al. 2004; Finn and Dice 2006), and free fatty acids are mobilized to the liver, where they can either be oxidized to generate ketones, or re-esterified to TG for storage or secretion as components of very low-density lipoproteins (Kersten, Seydoux et al. 1999; Heijboer, Donga et al. 2005). The enzymes that catalyze the synthesis of triglycerides in the liver during a fast have not been documented. Our findings indicate that DGAT1 is an important determinant in the development of fasting-induced hepatic steatosis.

A previous study of mice fasted for 16 hours showed that hepatic *Dgat1* mRNA expression was increased ~2.7-fold (Heijboer, Donga et al. 2005), suggesting a role for DGAT1 in steatosis associated with fasting. We confirmed this finding and, through our studies of global and hepatic-specific *Dgat1* gene inactivations, demonstrated a functional role for hepatic DGAT1 during a fast. In the absence of DGAT1 in the liver, fatty acids entering the liver from WAT during a fast are probably oxidized, as suggested by the increase in circulating ketones in $Dgat1^{-/-}$ mice after 8 hours of fasting. The reduced steatosis did not appear to be from increased hepatic VLDL secretion, since serum TG levels were reduced in fasted $Dgat1^{-/-}$ mice.

By treating $Dgat1^{flox/flox}$ mice with *Cre* expressing adenovirus we were able to reduce *Dgat1* mRNA levels and DGAT activity in the liver. Liver specific

deletion of DGAT1 conferred the protection against fasting induced hepatic steatosis that is seen in *Dgat1*$^{-/-}$ mice. In this study we provide evidence that hepatic-DGAT1 is an important determinant in fasting-induced hepatic steatosis and the physiological adaptation to fasting.

References

Buhman, K. K., H. C. Chen, et al. (2001). "The enzymes of neutral lipid synthesis." J Biol Chem **276**(44): 40369-72.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Coleman, R. A. and D. P. Lee (2004). "Enzymes of triacylglycerol synthesis and their regulation." Prog Lipid Res **43**(2): 134-76.

Finn, P. F. and J. F. Dice (2006). "Proteolytic and lipolytic responses to starvation." Nutrition **22**(7-8): 830-44.

Folch, J., M. Lees, et al. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Fukuda, N. and J. A. Ontko (1984). "Interactions between fatty acid synthesis, oxidation, and esterification in the production of triglyceride-rich lipoproteins by the liver." J Lipid Res **25**(8): 831-42.

Hashimoto, T., W. S. Cook, et al. (2000). "Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting." J Biol Chem **275**(37): 28918-28.

Hegardt, F. G. (1999). "Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis." Biochem J **338 (Pt 3)**: 569-82.

Heijboer, A. C., E. Donga, et al. (2005). "Sixteen hours of fasting differentially affects hepatic and muscle insulin sensitivity in mice." J Lipid Res **46**(3): 582-8.

Holm, C., T. Osterlund, et al. (2000). "Molecular mechanisms regulating hormone-sensitive lipase and lipolysis." Annu Rev Nutr **20**: 365-93.

Kersten, S., J. Seydoux, et al. (1999). "Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting." J Clin Invest **103**(11): 1489-98.

Raclot, T. (2003). "Selective mobilization of fatty acids from adipose tissue triacylglycerols." Prog Lipid Res **42**(4): 257-88.

Rohlmann, A., M. Gotthardt, et al. (1996). "Sustained somatic gene inactivation by viral transfer of Cre recombinase." Nat Biotechnol **14**(11): 1562-5.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Snyder, F. and N. Stephens (1959). "A simplified spectrophotometric determination of ester groups in lipids." Biochim Biophys Acta **34**: 244-5.

Zimmermann, R., J. G. Strauss, et al. (2004). "Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase." Science **306**(5700): 1383-6.

Chapter 5. DGAT1 deficiency does not protect against hepatic steatosis in model of lipodystrophy

Abstract

Congenital generalized lipodystrophy (CGL) is a rare disorder characterized by paucity of adipose tissue that is accompanied by hyperinsulinemia, hyperglycemia, and hepatic steatosis. *aP2-SREBP-1c436* transgenic mice, a model of CGL, express a constitutively active form of SREPB1c in adipose tissue. It has been shown that hepatic steatosis in *aP2-SREBP-1c436* transgenic mice is accompanied by the activation of *de novo* fatty acid synthesis in the liver. However, the triglyceride-synthesizing enzyme involved in the progression of hepatic steatosis has not been elucidated. Here we show that DGAT1 is not involved in the progression of hepatic steatosis in *aP2-SREBP-1c436* mice. These findings suggest that another triglyceride synthesizing enzyme, DGAT2, is involved in the progression of hepatic steatosis in this model of lipodystrophy.

Introduction

Congenital generalized lipodystrophy (CGL) is a rare disorder that is characterized by the complete or partial loss of adipose tissue and is accompanied by hyperglycemia, hyperinsulinemia, and hepatic steatosis (Garg 2000; Reitman, Arioglu et al. 2000; Agarwal, Simha et al. 2003). Transgenic mice that overexpress a nuclear form of SREBP-1c436 in the adipose tissue (*aP2-SREBP-1c436*) develop characteristics that resemble CGL. *aP2-SREBP-1c436* transgenic mice have diminished adipose tissue mass that results from a block in adipocyte differentiation, developing hyperglycemia, hyperinsulinemia, and hepatic steatosis (Shimomura, Hammer et al. 1998). Several findings have indicated that hepatic steatosis may develop through the upregulation of *Srebp1c* in the liver, a transcription factors that belongs to the basic helix-loop-helix-leucine zipper family of transcription factors (Shimomura, Matsuda et al. 2000). The genes activated by *Srebp1c* encode enzymes that catalyze the synthesis of fatty acids from glucose (Kim, Sarraf et al. 1998; Osborne 2000; Horton, Goldstein et al. 2002). The newly synthesized fatty acids can be esterified, and stored in the form of triglycerides, causing hepatic steatosis (Browning and Horton 2004).

Another hallmark of lipodystrophy is the reduction in circulating leptin, an adipocyte secreted factor that regulates food intake and energy expenditure (Shimomura, Hammer et al. 1998). Several findings have indicated that a reduction in leptin levels is the cause of the metabolic abnormalities seen in *Ap2-Srebp1c* transgenic mice and humans with CGL (Shimomura, Hammer et al. 1999; Colombo, Cutson et al. 2002; Petersen, Oral et al. 2002). Findings have

shown that recombinant leptin therapy can reverse hepatic steatosis in *aP2-SREBP-1c436* transgenic mice by suppressing Srebp1c and genes involved in fatty acid synthesis (Shimomura, Matsuda et al. 2000).

The last step in triglyceride synthesis is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes. Two DGAT enzymes have been identified, DGAT1 and DGAT2 (Cases, Smith et al. 1998; Cases, Stone et al. 2001). Mice lacking DGAT2 die shortly after birth and have diminished triglyceride levels in the liver (Stone, Myers et al. 2004). In contrast, *Dgat1*$^{-/-}$ mice are viable and have increased insulin and leptin sensitivity (Smith, Cases et al. 2000; Chen, Smith et al. 2002). *Dgat1*$^{-/-}$ mice are protected against diet-induced obesity and hepatic steatosis (Chapter 2).

In this study we examine the role of DGAT1 in mediating hepatic steatosis in mice with lipodystrophy. Our findings indicate that *aP2-SREBP-1c436* transgenic mice develop hepatic steatosis despite the lack of DGAT1, suggesting that DGAT2 may be involved in synthesizing triglycerides in *aP2-SREBP-1c436* mice.

Materials and Methods

Mice. *Dgat1*$^{-/-}$ mice and wild-type mice were in a C57BL/6J background. *Dgat1*$^{-/-}$ mice were crossed with *Ap2-nSREBP1c* transgenic mice (Jackson Laboratory) with a mixed background of 50% C57BL/6J and 50% SJL. *Dgat1*$^{+/-}$*Ap2-nSREPB1c* mice (75% C57BL/6J and 25% SJL) were crossed with *Dgat1*$^{-/-}$(C57BL/6J) males to generate *Dgat1*$^{-/-}$*Ap2-nSREBP1c* mice (87.5% C57BL/6J and 12.5% SJL).Genotyping for *Dgat1* was performed as described earlier(Smith, Cases et al. 2000). Mice were housed in a pathogen-free barrier facility (12-hour light/12-hour dark cycle) and fed rodent chow (5053 PicoLab Diet; Purina, St. Louis, Missouri, USA). All experiments were approved by the Committee on Animal Research of the University of California, San Francisco.

Histological Analysis. For histology mice were perfused with PBS, followed by 3% paraformaldehyde/PBS. Livers were removed and placed into 3% paraformaldehyde/PBS for 2 days at 4°C. Livers were embedded in paraffin, sectioned and stained with hematoxylin and eosin. To visualize neutral lipids, livers were snap frozen in OCT and isopentane, sectioned and stained with Oil red O.

Lipid Analysis. Livers were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose and complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated by thin-layer chromatography with a solvent system of hexane:diethyl ether:acetic acid (80:20:1) on Silica Gel G-60 TLC plates (Folch, Lees et al. 1957). Using triolein as a standard, triglycerides

were visualized with iodine vapor. Triglycerides were identified and quantified using a spectrophotometric technique (Snyder and Stephens 1959).

RNA extraction and real-time PCR. RNA was extracted from liver using RNA STAT-60 (Tel-Test Inc.) and traces of DNA were removed by DNase treatment (Ambion). 5 mg of RNA was used to synthesize cDNA using superscript II reverse transcriptase and random hexamers (Invitrogen). Real-time PCR primers were selected for each cDNA using Primer Express software (version 1.5; Applied Biosystems). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with sybrgreen reagents using two-step RT-PCR reactions on Biorad iCycler or ABI 9600. 18S ribosomal RNA or cyclophilin was used to normalize gene expression. **Cyclophilin**, F:5'–TGGAAGAGCACCAAGACAGACA–3' and R: 5'–TGCCGGAGTCGACA ATGAT–3'; **Srebp1c**, F:5'–GGAGCCATGGATTGCACATT–3' and R: 5'–GGCCCGGGAAGTCACTGT–3'; **Fasn**, F:5'–GCTGCGGAAACTTCAGGA AAT–3' and R:5'–AGAGACGTGTCACTCCTGGACTT–3'; **Scd1**, F:5'–CCTTCCCCTTCGACTACTCTG–3' and R:5'–GCCATGCAGTCGATGAA GAA–3'; **Dgat2**, F:5'–AGTGGCAATGCTATCATCATCGT–3' and R: 5'–AAGGAATAAGTGGGAACCCAGATCA–3'.

Statistical analysis. Values are reported as mean ± standard error. Statistical differences were determined by either a students t-test or an ANOVA followed by a Student-Newman-Keuls test.

Results

aP2-SREBP-1c436 transgenic mice expressing a truncated form of the transcription factor Srebp1c develop several features of lipodystrophy (Shimomura, Hammer et al. 1998). Because of the partial loss of adipose tissue, *aP2-SREBP-1c436* transgenic mice have diminished leptin levels, leading to hepatic steatosis and the upregulation of fatty acid synthesis genes (Shimomura, Hammer et al. 1998; Shimomura, Hammer et al. 1999; Shimomura, Matsuda et al. 2000). To test whether this distinct model of hepatic steatosis can be reversed by the lack of DGAT1, $Dgat1^{+/-}$ mice were crossed with $Dgat1^{+/-}$ *aP2-SREBP-1c436* mice to generate $Dgat1^{-/-}$*aP2-SREBP-1c436* mice. By gross morphology we found that in comparison to livers from WT and $Dgat1^{-/-}$mice, livers from *aP2-SREBP-1c436* and $Dgat1^{-/-}$*aP2-SREBP-1c436* mice developed a pale color and became enlarged (**Figure 1A**). We found that Oil Red O staining in livers from *aP2-SREBP-1c436* (**Figure 1B**) and $Dgat1^{-/-}$*aP2-SREBP-1c436* mice was

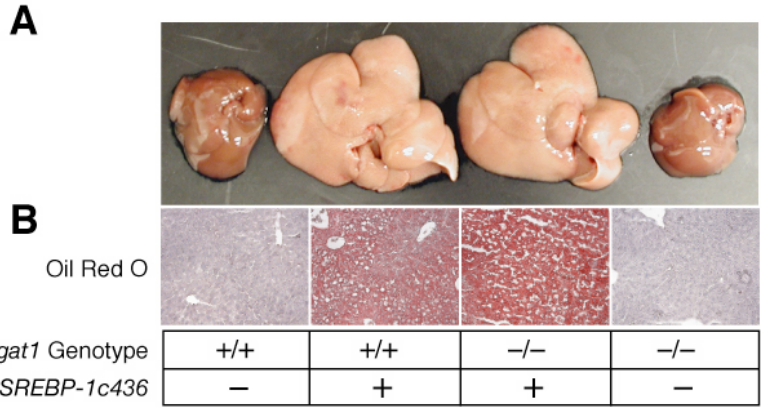

Figure 1. DGAT1 deficiency does not protect against hepatic steatosis in model of lipodystrophy. (A) Gross morphology and (B) Oil Red O staining of livers from $Dgat1^{+/+}$ *aP2-SREBP-1c436*, $Dgat1^{-/-}$*aP2-SREBP-1c436*, and $Dgat1^{-/-}$ mice show that *aP2-SREBP-1c436* transgenic mice develop hepatomegaly and hepatic steatosis despite the lack of DGAT1 (age 24 weeks). Magnification of Oil Red O is 20x for all.

elevated when compared to livers of WT and *Dgat1*$^{-/-}$ mice, suggesting that *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* had similar neutral lipid content in the liver.

In comparison to livers from WT and *Dgat1*$^{-/-}$ mice (**Figure 2A**), liver mass had increased ~3-fold in both *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice. When liver mass was corrected for body weight (**Figure 2B**), we found a similar result, where *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice had increased ~2-fold in % liver mass when compared to WT and *Dgat1*$^{-/-}$ mice. The clear morphological changes and hepatomegaly in *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice, suggested that hepatic triglycerides would be elevated dramatically in both of these genetic

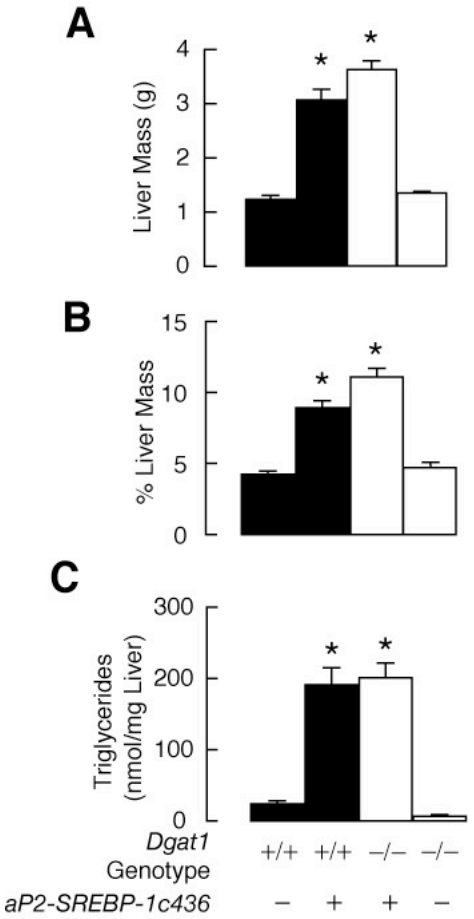

Figure 2. Hepatomegaly and increased hepatic triglycerides in lipodystrophic mice. (A) Liver mass, (B) % Liver Mass, (C) and hepatic triglycerides in *Dgat1*$^{+/+}$, *aP2-SREBP-1c436*, *Dgat1*$^{-/-}$*aP2-SREBP-1c436*, and *Dgat1*$^{-/-}$ mice show that *aP2-SREBP-1c436* transgenic mice develop hepatomegaly and hepatic steatosis despite the lack of DGAT1 (age 24 weeks, N=7-10). ** P < 0.05 versus Dgat1*$^{+/+}$ *and Dgat1*$^{-/-}$ mice.

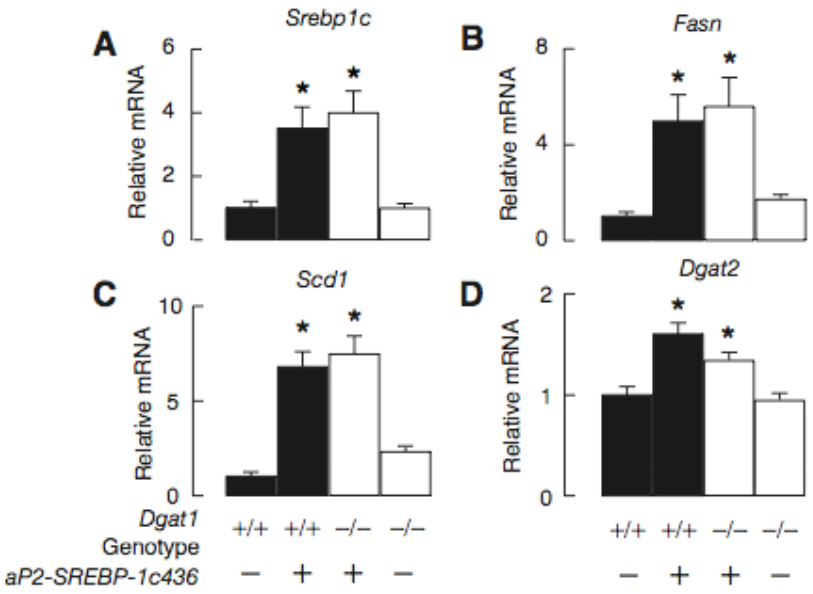

Figure 3. Increased expression of fatty acid synthesis genes in lipodystrophic mice. Liver mRNA levels of (A) sterol regulatory element binding protein 1c (*Srebp1c*), (B) fatty acid synthase (*Fasn*), (C) stearoyl-CoA desaturase 1 (*Scd1*) and acyl-CoA:diacylglycerol acylstransferase 2 (*Dgat2*) are elevated in *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$ *aP2-SREBP-1c436* mice (age 24 weeks, N=5-7). * $P < 0.001$ *versus Dgat1*$^{+/+}$ and *Dgat1*$^{-/-}$. Mice were fed a standard chow diet and fasted for 4 hours.

models. To test this hypothesis, lipids were run on a TLC plate and triglycerides were quantified. We found that hepatic triglycerides in *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice had increased more than ~8-fold, when compared to WT and *Dgat1*$^{-/-}$ mice (**Figure 2C**), indicating that DGAT1 is not necessary for the progression of hepatic steatosis in this model of lipodystrophy.

Previous findings have indicated that *aP2-SREBP-1c436* transgenic mice develop hepatic steatosis through the activation of genes involved in fatty acid synthesis. We found that expression of Srebp1c (**Figure 3A**) was dramatically elevated in both *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice, as well as targets of SREBP1c such as *Fasn* (**Figure 3B**) and *Scd1* (**Figure 3C).** We also found that *Dgat2* (**Figure 3D**) mRNA levels had increased in *aP2-SREBP-1c436*

and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice, albeit to a much lesser extent when compared to genes involved in fatty acid synthesis.

Discussion

Hepatic steatosis, the accumulation of triglycerides in the liver, is a hallmark of lipodystrophy (Garg 2000; Reitman, Arioglu et al. 2000; Agarwal, Simha et al. 2003). However, the molecular mechanisms that are involved in the progression of hepatic steatosis have not been completely elucidated. Previous findings have indicated that hepatic steatosis develops through the activation of fatty acid synthesis, resulting in excess triglycerides in the liver (Shimomura, Matsuda et al. 2000). However, the DGAT enzyme involved in catalyzing the synthesis of triglycerides has not been elucidated. Here we show that DGAT1 is not involved in the development of hepatic steatosis in lipodystrophic mice. Our findings indicate that *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$*aP2-SREBP-1c436* mice had elevated hepatic triglycerides and expression of genes involved in fatty acid synthesis. Therefore, we suspect that another triglyceride synthesizing enzyme is involved in the progression of hepatic steatosis in *aP2-SREBP-1c436* transgenic mice, possibly *Dgat2*.

Our findings are in accordance with previous findings in *ob*/*ob* mice where DGAT1-deficiency is unable to protect against any of the metabolic abnormalities found in *ob*/*ob* mice (Chen, Smith et al. 2002). *aP2-SREBP-1c436* and *ob*/*ob* mice share several phenotypic characteristics, such as hyperphagia, hyperglycemia, hyperinsulinemia, and hepatic steatosis (Shimomura, Hammer et al. 1998; Shimomura, Hammer et al. 1999). Both of these models are thought to develop hepatic steatosis through the activation of fatty acid synthesis in the liver (Shimomura, Bashmakov et al. 1999; Shimomura, Matsuda et al. 2000; Yahagi,

Shimano et al. 2002). Findings have indicated that leptin administration can normalize the expression of fatty acid synthesis genes in the liver and reverse hepatic steatosis in *aP2-SREBP-1c436* and *ob/ob* mice (Shimomura, Matsuda et al. 2000). These findings suggest that hepatic steatosis that is accompanied by the activation of *de novo* fatty acid synthesis may not require DGAT1 for triglyceride synthesis.

In summary, our findings indicate that DGAT1 is not necessary for the development of hepatic steatosis in *aP2-SREBP-1c436* transgenic mice. We suspect that another triglyceride synthesizing enzyme, DGAT2, may be involved in the progression of hepatic steatosis in this murine model of lipodystrophy.

References

Agarwal, A. K., V. Simha, et al. (2003). "Phenotypic and genetic heterogeneity in congenital generalized lipodystrophy." J Clin Endocrinol Metab **88**(10): 4840-7.

Browning, J. D. and J. D. Horton (2004). "Molecular mediators of hepatic steatosis and liver injury." J Clin Invest **114**(2): 147-52.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Chen, H. C., S. J. Smith, et al. (2002). "Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **109**(8): 1049-55.

Colombo, C., J. J. Cutson, et al. (2002). "Transplantation of adipose tissue lacking leptin is unable to reverse the metabolic abnormalities associated with lipoatrophy." Diabetes **51**(9): 2727-33.

Folch, J., M. Lees, et al. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Garg, A. (2000). "Lipodystrophies." Am J Med **108**(2): 143-52.

Horton, J. D., J. L. Goldstein, et al. (2002). "SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver." J Clin Invest **109**(9): 1125-31.

Kim, J. B., P. Sarraf, et al. (1998). "Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1." J Clin Invest **101**(1): 1-9.

Osborne, T. F. (2000). "Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action." J Biol Chem **275**(42): 32379-82.

Petersen, K. F., E. A. Oral, et al. (2002). "Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy." J Clin Invest **109**(10): 1345-50.

Reitman, M. L., E. Arioglu, et al. (2000). "Lipoatrophy revisited." Trends Endocrinol Metab **11**(10): 410-6.

Shimomura, I., Y. Bashmakov, et al. (1999). "Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus." J Biol Chem **274**(42): 30028-32.

Shimomura, I., R. E. Hammer, et al. (1999). "Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy." Nature **401**(6748): 73-6.

Shimomura, I., R. E. Hammer, et al. (1998). "Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose

tissue: model for congenital generalized lipodystrophy." Genes Dev **12**(20): 3182-94.

Shimomura, I., M. Matsuda, et al. (2000). "Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice." Mol Cell **6**(1): 77-86.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Snyder, F. and N. Stephens (1959). "A simplified spectrophotometric determination of ester groups in lipids." Biochim Biophys Acta **34**: 244-5.

Stone, S. J., H. M. Myers, et al. (2004). "Lipopenia and skin barrier abnormalities in DGAT2-deficient mice." J Biol Chem **279**(12): 11767-76.

Wang, X., R. Sato, et al. (1994). "SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis." Cell **77**(1): 53-62.

Yahagi, N., H. Shimano, et al. (2002). "Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice." J Biol Chem **277**(22): 19353-7.

Chapter 6. Liver x receptor activation and hepatic steatosis: Role of DGAT enzymes

Abstract

The liver x receptor (LXR), a ligand activated nuclear transcription factor, regulates cholesterol homeostasis, bile acid metabolism, and fatty acid synthesis. Activation of the LXR agonist using the synthetic compound T0901317 activates fatty acid synthesis in the liver and leads to hepatic steatosis. The enzymes involved in catalyzing the synthesis of triglycerides from *de novo* synthesized fatty acids had not been elucidated. In this study we investigated the role of Acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2, in the development of hepatic steatosis induced by the activation of LXR. Using *Dgat1*$^{-/-}$ mice and antisense oligonucleotides (ASO) to reduce *Dgat1* or *Dgat2* mRNA levels in the liver, and the synthetic ligand T0901317 to activate LXR, we were able to determine which enzyme, DGAT1 or DGAT2, was involved in the development of hepatic steatosis induced by LXR activation. Our findings showed that mice treated with *Dgat2* ASO had a blunted increase in hepatic triglyceride levels when treated with T0901317, while mice treated with *Dgat1* ASO were able to induce hepatic triglycerides levels to a similar extent as wild type mice treated with the LXR agonist T0901317. Taken together, our findings suggest that *Dgat2* is coupled to hepatic fatty acid synthesis induced by the activation of LXR, and that *Dgat1* is not necessary for the synthesis of triglycerides with LXR activation.

Introduction

The liver x receptor (LXR), LXRα and LXRβ, belong to a family of ligand activated nuclear transcription factors that regulate cholesterol homeostasis, bile acid metabolism, and fatty acid synthesis (Janowski, Willy et al. 1996; Peet, Turley et al. 1998; Schultz, Tu et al. 2000). Like other nuclear hormone receptors (PPAR, RAR, FXR), LXR's are obligate heterodimers with retinoid x receptor (RXR) (Issemann, Prince et al. 1993; Willy, Umesono et al. 1995; Wan, An et al. 2000). LXR's serve as sterol sensors that bind to the metabolites of cholesterol–oxysterols–activating reverse cholesterol transport in order to maintain cholesterol homeostasis in mammals (Janowski, Willy et al. 1996). The ability of LXR's to regulate cholesterol metabolism has prompted its use to treat cardiovascular disease. However, the activation of fatty acid synthesis in the liver, which leads to hepatic steatosis and hyperlipidemia, has halted its therapeutic use to treat cardiovascular disease.

The activation of fatty acid synthesis by LXR's in the liver can in large part be attributed to the upregulation of *Srebp1c*, a nuclear transcription factor that regulates the expression of genes involved in fatty acid synthesis (Joseph, Laffitte et al. 2002). By directly binding to LXR responsive elements (LRE), the LXR/RXR heterodimer can directly bind to the promoter region of *Srebp1c*, regulating the expression of *Srebp1c* in a ligand dependent manner (DeBose-Boyd, Ou et al. 2001; Yoshikawa, Shimano et al. 2001). SREBP1c belongs to the basic helix-loop-helix-leucine zipper family of transcription factors that directly bind to upstream DNA sequences that regulate the expression of target genes

(Osborne 2000). SREBP1c is synthesized in the ER and activated by proteolytic cleavage in the golgi, releasing an NH2-terminal DNA binding domain that is translocated to the nucleus where it directly binds to sterol response elements (SRE) in the promoters of genes involved in fatty acid synthesis, such as fatty acid synthase and stearoyl-CoA desaturase 1 (Wang, Sato et al. 1994; Kim, Sarraf et al. 1998; Shimomura, Shimano et al. 1998). Fatty acid synthesis, through several enzymatic steps, converts the intermediate acetyl-CoA, to long chain fatty acid molecules that can be utilized for the synthesis of triglycerides (Browning and Horton 2004).

The last step in triglyceride synthesis is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes (Farese, Cases et al. 2000). Two DGAT enzymes have been identified, DGAT1 and DGAT2 (Cases, Smith et al. 1998; Cases, Stone et al. 2001). Mice lacking DGAT2 die shortly after birth and have diminished triglyceride levels in the liver (Stone, Myers et al. 2004). In contrast, *Dgat1*$^{-/-}$ mice are viable and have increased insulin and leptin sensitivity (Smith, Cases et al. 2000; Chen, Smith et al. 2002). Our findings have indicated that *Dgat1*$^{-/-}$ mice are protected against diet-induced obesity and hepatic steatosis (Chapter 2). However, when *Dgat1*$^{-/-}$ mice were crossed into a leptin-deficient (*ob*/*ob*) background or were crossed into a model of lipodystrophy (Chapter 5), DGAT1-deficency was unable to prevent the hepatic steatosis associated with leptin-deficiency, which activates fatty acid synthesis in the liver (Shimomura, Hammer et al. 1999; Chen, Smith et al. 2002). These findings suggest that another

enzyme is involved in catalyzing the synthesis of triglycerides from *de novo* synthesized fatty acids, possibly DGAT2.

In this study we examine the role of DGAT enzymes in the development of hepatic steatosis induced by LXR activation. By using antisense oligonucleotides to reduce the mRNA levels of *Dgat1* or *Dgat2*, and the LXR agonist T0901317 to activate fatty acid synthesis, we were able to dissect a distinct role for DGAT2 in hepatic triglyceride metabolism.

Materials and Methods

Mice. C57BL/6J mice were obtained from Jackson Laboratory, housed in a pathogen-free barrier facility (12-hour light/12-hour dark cycle), and fed rodent chow (5053 PicoLab Diet; Purina, St. Louis, Missouri, USA). All experiments were approved by the Committee on Animal Research of the University of California, San Francisco.

Antisense Oligonucleotides and T0901317. Mouse Dgat1 (ASO ISIS 191761) and Dgat2 (ASO ISIS 217376) ASO were identified as described earlier, a control (ASO ISIS 141923) ASO that is not complementary to any known gene was used as a negative control. Mice were given i.p. injections containing ASO at a dose of 25 mg/kg body weight twice a week for approximately 6 weeks. After 4-weeks of treatment with ASO's, mice were treated given daily i.p. injections of T0901317 at a dose of 50 mg/kg body weight for 11 days.

Serum Metabolites. ALT and AST levels were measured by an enzymatic assay from Stanbio Laboratory, TX, USA.

Lipid Analysis. Livers were homogenized in 50 mM Tris pH 7.5, 250 mM sucrose and complete protease inhibitor (Roche). Lipids were extracted with chloroform:methanol (2:1) and separated by thin-layer chromatography with a solvent system of hexane:diethyl ether:acetic acid (80:20:1) on Silica Gel G-60 TLC plates (Folch, Lees et al. 1957). Using triolein as a standard, triglycerides were visualized with iodine vapor. Triglycerides were identified and quantified using a spectrophotometric technique (Snyder and Stephens 1959).

RNA extraction and real-time PCR. RNA was extracted from liver using

RNA STAT-60 (Tel-Test Inc.) and traces of DNA were removed by DNase treatment (Ambion). 5 mg of RNA was used to synthesize cDNA using superscript II reverse transcriptase and random hexamers (Invitrogen). Real-time PCR primers were selected for each cDNA using Primer Express software (version 1.5; Applied Biosystems). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with sybrgreen reagents using two-step RT-PCR reactions on Biorad iCycler or ABI 9600. 18S ribosomal RNA or cyclophilin was used to normalize gene expression. **Cyclophilin**, F:5'–TGGAAGAGCACCAAGACAGACA–3' and R: 5'–TGCCGGAGTCGACAATGAT–3'; **Fasn**, F:5'–GCTGCGGAAACTTCAGGAAAT–3' and R:5'–AGAGACGTGTCACTCCTGGACTT–3'; **Scd1**, F:5'–CCTTCCCCTTCGACTACTCTG–3' and R:5'–GCCATGCAGTCGATGAAGAA–3'; **Dgat1**, F: 5'–TTCCGCCTCTGGGCATT–3' and R: 5'–AGAATCGGCCCACAATCCA–3'; **Dgat2**, F: 5'–AGTGGCAATGCTATCATCATCGT–3' and R: 5'–AAGGAATAAGTGGGAACCCAGATCA–3'.

Statistical analysis. Values are reported as mean ± standard error. Statistical differences were determined by either a students t-test or an ANOVA followed by a Student-Newman-Keuls test.

Results

The liver x receptor (LXR), a ligand activated nuclear transcription factor, activates fatty acid synthesis in the liver, and leads to hepatic steatosis. Indeed, after one week of treatment, livers of both *Dgat1*$^{+/+}$ and *Dgat1*$^{-/-}$ mice developed similar levels of steatosis (triglycerides of 58±25 nmol/mg liver and 79± 23 nmol/mg liver, respectively, n = 5 per genotype, 4 hr fast), indicating DGAT1 was not required.

To directly compare the roles of DGAT1 and DGAT2, we used antisense oligonucleotides for DGAT1 and DGAT2 and the LXR agonist T0901317. To confirm that T0901317 could activate genes involved in fatty acid synthesis, we studied the expression of *Fasn* and *Scd1*, two genes that are highly regulated by LXR. Mice treated with T0901317 had a significant

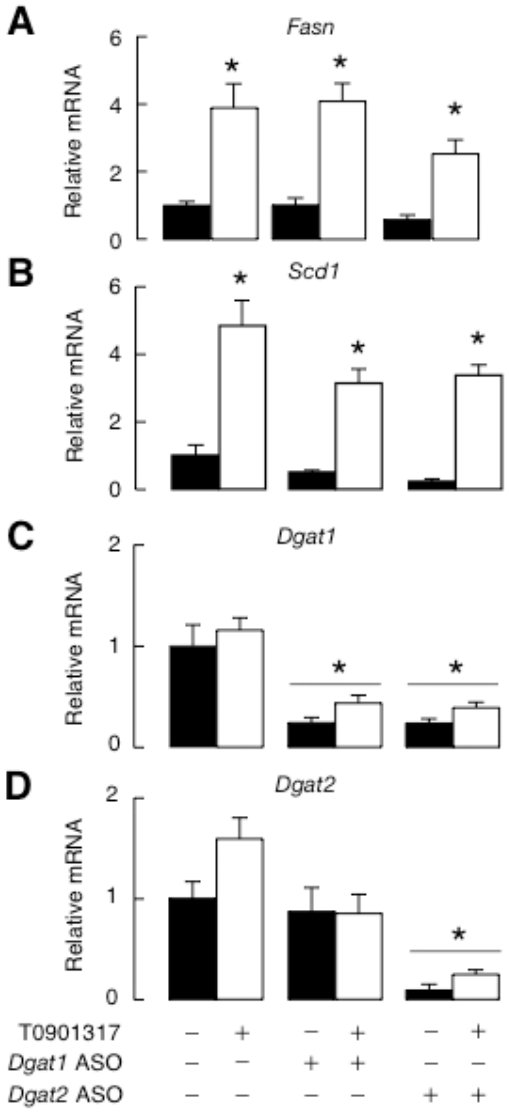

Figure 1. Use of ASO's to reduce *Dgat1* and *Dgat2* expression in the liver. Administration of LXR agonist, T0901317, increased liver mRNA levels of (A) *Fasn* and (B) *Scd1* in mice. (C) *Dgat1* mRNA levels were reduced in mice given ASO for *Dgat1* and *Dgat2*. (D) *Dgat2* mRNA levels were reduced in mice treated with *Dgat2* ASO. ASO was administered twice per week i.p. at 50 mg/kg. After 4 weeks of treatment with ASO's, mice were treated with 50 mg/kg of T0901317 or sham for 11 days (Age 15-16 weeks, N=4-5).

induction in both *Fasn* (**Figure 1A**) and *Scd1* (**Figure 1B**) expression in the liver. To confirm that administration of *Dgat1* ASO blocked expression of *Dgat1*, we quantified *Dgat1* expression (**Figure 1C**) in the liver, and found reduced *Dgat1* mRNA levels, however, mice treated with *Dgat2* ASO's also had reduced expression of *Dgat1*, possibly a secondary affect to knocking down *Dgat2* expression. To confirm that *Dgat2* expression (**Figure 1D**) was reduced in mice treated with *Dgat2* ASO, we studied the expression of *Dgat2* mRNA in the liver, and found a significant reduction in *Dgat2* mRNA levels.

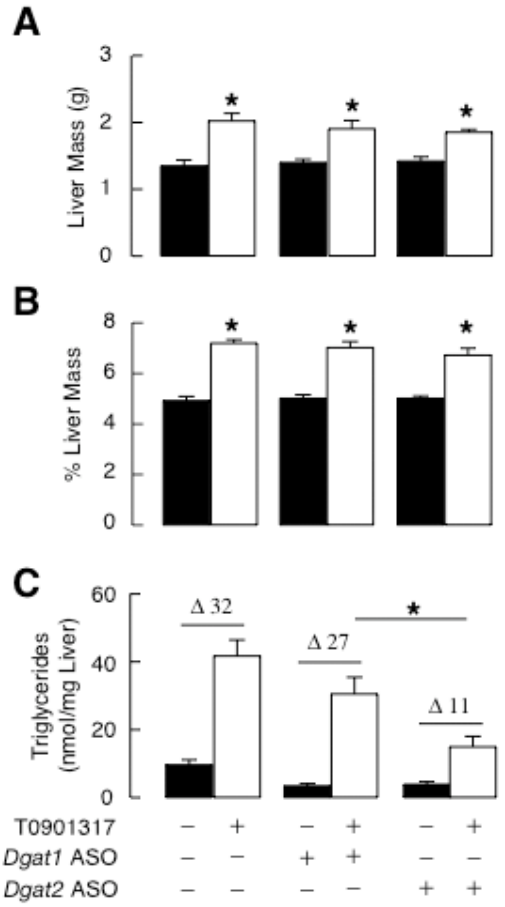

Figure 2. *Dgat2* ASO treatment blocks induction in hepatic triglycerides by T0901317. Administration of LXR agonist T0901317 increased (A) liver mass and (B) % liver mass. * $P < 0.001$ vs sham. (C) Increase in hepatic TG by T0901317 is attenuated in mice receiving *Dgat2* ASO's. * $P < 0.002$ vs *Dgat1* ASO T0901317 group. Δ refers to change from sham chontrol. ASO was administered twice per week i.p. at 50 mg/kg. After 4 weeks of treatment with ASO's, mice were treated with 50 mg/kg of T0901317 or sham for 11 days (For all, age 15-16 weeks, N=4-5 per group).

As expected, in control mice administration of T0901317 increased liver mass (**Figure 2A**), % liver mass (**Figure 2B**), and hepatic triglyceride accumulation (**Figure 2C**). Similarly, blocking *Dgat1*

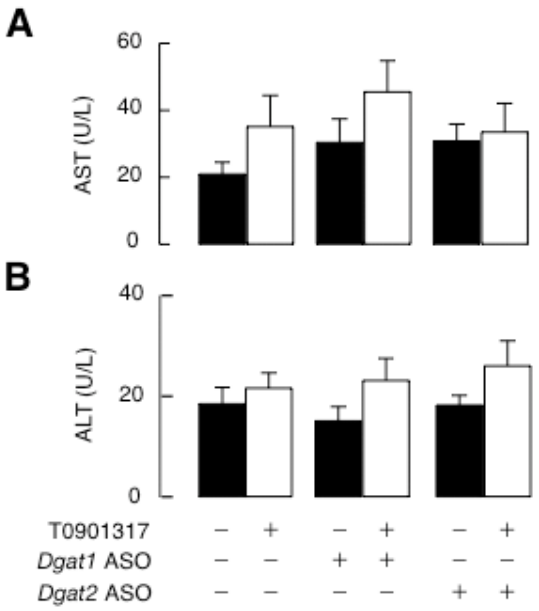

Figure 3. Normal transaminase levels with treatment of *Dgat1* and *Dgat2* ASO's. Serum levels of AST (A) and ALT (B) were normal in mice treated with T0901317 or ASO's. ASO was administered twice per week i.p. at 50 mg/kg. After 4 weeks of treatment with ASO's, mice were treated with 50 mg/kg of T0901317 or sham for 11 days (For all, age 15-16 weeks, N=4-5).

did not affect the induction in liver mass (**Figure 2A**), % liver mass (**Figure 2B**), and hepatic triglycerides (**Figure 2C**) with T0901317 treatment. However, when mice were treated with *Dgat2* ASO's, the increase in hepatic TG (**Figure 2C**) was blunted, while liver mass (**Figure 2A**) and % liver mass (**Figure 2B**) was unchanged. These findings indicate that hepatic steatosis induced by activation of fatty acid synthesis is independent of DGAT1, but dependent on DGAT2, a previously unrecognized distinction between DGAT1 and DGAT2. To address whether T0901317 or ASO administration could induce hepatotoxicity, plasma levels of ALT (**Figure 3A**) and AST (**Figure 3B**) were quantified, and were found to be normal, suggesting normal liver function.

Discussion

The accumulation of triglycerides in the liver is a hallmark of hepatic steatosis (Angulo 2002). Activation of fatty acid synthesis, where long chain fatty acids are synthesized *de novo* from acetyl-CoA, can lead to hepatic steatosis (Browning and Horton 2004). Activation of the nuclear transcription factor, LXR, activates fatty acid synthesis in the liver. To test whether DGAT1 or DGAT2 are involved in the synthesis of triglyceride from *de novo* synthesized fatty acids, we used the synthetic LXR agonist T0901317 (Schultz, Tu et al. 2000; Joseph, Laffitte et al. 2002). Our findings show that DGAT2, rather than DGAT1 is involved in the development of hepatic steatosis with activation of LXR, a previously unrecognized distinction between DGAT1 and DGAT2.

Our findings that Dgat1 ASO's did not prevent the induction in hepatic triglycerides with T0901317 treatment indicate that DGAT1 is dispensable in this model of hepatic steatosis. So why is DGAT1 dispensable and not DGAT2? One possibility is that DGAT2 is functionally linked with fatty acid synthesis, which was recently reported by Ntambi et al, where SCD1 was found to interact with DGAT2 in the ER (Chu, Miyazaki et al. 2006; Man, Miyazaki et al. 2006). However, this study did not address whether DGAT1 could interact with SCD1, further studies will be necessary to test this hypothesis. The non-overlapping roles of DGAT1 and DGAT2 may also be explained by the cellular distribution of DGAT1 and DGAT2. Recently in the tung tree, vernicia fordii, DGAT1 and DGAT2 was found in distinct regions of the ER (Shockey, Gidda et al. 2006).

In conclusion, we provide evidence suggesting that DGAT1 and DGAT2 have distinct roles in hepatic triglyceride metabolism. Hepatic steatosis induced by LXR activation involves DGAT2, and not DGAT1. These findings further our understanding of how DGAT1 and DGAT2 are involved in the development of hepatic steatosis.

References

Angulo, P. (2002). "Nonalcoholic fatty liver disease." N Engl J Med **346**(16): 1221-31.

Browning, J. D. and J. D. Horton (2004). "Molecular mediators of hepatic steatosis and liver injury." J Clin Invest **114**(2): 147-52.

Cases, S., S. J. Smith, et al. (1998). "Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis." Proc Natl Acad Sci U S A **95**(22): 13018-23.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Chen, H. C., S. J. Smith, et al. (2002). "Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **109**(8): 1049-55.

Chu, K., M. Miyazaki, et al. (2006). "Stearoyl-coenzyme A desaturase 1 deficiency protects against hypertriglyceridemia and increases plasma high-density lipoprotein cholesterol induced by liver X receptor activation." Mol Cell Biol **26**(18): 6786-98.

DeBose-Boyd, R. A., J. Ou, et al. (2001). "Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands." Proc Natl Acad Sci U S A **98**(4): 1477-82.

Farese, R. V., Jr., S. Cases, et al. (2000). "Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase." Curr Opin Lipidol **11**(3): 229-34.

Folch, J., M. Lees, et al. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**(1): 497-509.

Issemann, I., R. A. Prince, et al. (1993). "The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs." J Mol Endocrinol **11**(1): 37-47.

Janowski, B. A., P. J. Willy, et al. (1996). "An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha." Nature **383**(6602): 728-31.

Joseph, S. B., B. A. Laffitte, et al. (2002). "Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors." J Biol Chem **277**(13): 11019-25.

Kim, J. B., P. Sarraf, et al. (1998). "Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1." J Clin Invest **101**(1): 1-9.

Man, W. C., M. Miyazaki, et al. (2006). "Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis." J Lipid Res **47**(9): 1928-39.

Osborne, T. F. (2000). "Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action." J Biol Chem **275**(42): 32379-82.

Peet, D. J., S. D. Turley, et al. (1998). "Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha." Cell **93**(5): 693-704.

Schultz, J. R., H. Tu, et al. (2000). "Role of LXRs in control of lipogenesis." Genes Dev **14**(22): 2831-8.

Shimomura, I., R. E. Hammer, et al. (1999). "Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy." Nature **401**(6748): 73-6.

Shimomura, I., H. Shimano, et al. (1998). "Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of unsaturated fatty acid biosynthesis in transgenic mouse liver." J Biol Chem **273**(52): 35299-306.

Shockey, J. M., S. K. Gidda, et al. (2006). "Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum." Plant Cell **18**(9): 2294-313.

Smith, S. J., S. Cases, et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat." Nat Genet **25**(1): 87-90.

Snyder, F. and N. Stephens (1959). "A simplified spectrophotometric determination of ester groups in lipids." Biochim Biophys Acta **34**: 244-5.

Stone, S. J., H. M. Myers, et al. (2004). "Lipopenia and skin barrier abnormalities in DGAT2-deficient mice." J Biol Chem **279**(12): 11767-76.

Wan, Y. J., D. An, et al. (2000). "Hepatocyte-specific mutation establishes retinoid X receptor alpha as a heterodimeric integrator of multiple physiological processes in the liver." Mol Cell Biol **20**(12): 4436-44.

Wang, X., R. Sato, et al. (1994). "SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis." Cell **77**(1): 53-62.

Willy, P. J., K. Umesono, et al. (1995). "LXR, a nuclear receptor that defines a distinct retinoid response pathway." Genes Dev **9**(9): 1033-45.

Yoshikawa, T., H. Shimano, et al. (2001). "Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter." Mol Cell Biol **21**(9): 2991-3000.

Chapter 7. Discussion

The accumulation of TG within the liver is a hallmark of NAFLD (Day and James 1998; Angulo 2002; Clark 2006). The functional roles of DGAT enzymes in this process have not been elucidated. In these studies, we show that hepatic steatosis in mice induced by high-fat diet or fasting is dependent on hepatic DGAT1 function. In contrast, DGAT1 is not required for hepatic steatosis induced by lipodystrophy or the activation of LXR, conditions in which *de novo* fatty acid synthesis is markedly upregulated (Shimomura, Hammer et al. 1999; Schultz, Tu et al. 2000; Shimomura, Matsuda et al. 2000). Additionally, we provide evidence that hepatic steatosis in the latter situation is dependent on hepatic DGAT2 function. These findings indicate that DGAT1 and DGAT2 have distinct roles in hepatic TG metabolism.

DGAT1 deficiency reduced hepatic steatosis caused by a high-fat diet or fasting. In the high-fat diet paradigm, DGAT1 deficiency reduced hepatic TG by ~80% in the global knockout and by ~50% in liver-specific DGAT1 deficiency. The difference in magnitude most likely reflects a contribution of the loss of DGAT1 in other tissues, such as WAT or small intestine, which may contribute to the protection against diet-induced hepatic steatosis in global DGAT1 deficiency by altering endocrine mediators or delaying lipid absorption, respectively (Buhman, Smith et al. 2002; Chen, Jensen et al. 2003). Also, on a high-fat diet, the expression of genes involved in fatty acid synthesis was reduced in the global knockout but not the tissue-specific knockdown, suggesting that DGAT1 deficiency in extra-hepatic tissues caused these alterations in gene expression through hormonal or neural mechanisms. Global DGAT1 deficiency in mice is associated with increased sensitivity to leptin (Chen, Smith et al. 2002), which

may have contributed to the suppression of fatty acid synthesis genes. An alternative possibility for the discrepancy between the liver-specific and global knockouts is that degree of knockdown in the liver-specific disruption of DGAT1 was insufficient to recapitulate the effects seen with the global knockout. This seems unlikely, however, since mRNA levels were similarly reduced in liver-specific knockdown experiments in the fasting paradigm, and this degree of reduction was sufficient to recapitulate the global knockout phenotype.

Our results demonstrate a functional role for DGAT1 in hepatic TG balance during fasting. During a fast, hepatic glycogen content becomes depleted, and the liver switches to utilize lipids as fuel. Lipolysis is activated in WAT (Zimmermann, Strauss et al. 2004; Finn and Dice 2006), and free fatty acids are mobilized to the liver, where they can either be oxidized to generate ketones, or re-esterified to TG for storage or secretion as components of very low-density lipoproteins (Kersten, Seydoux et al. 1999; Heijboer, Donga et al. 2005). A previous study of mice fasted for 16 hours showed that hepatic *Dgat1* mRNA expression was increased ~2.7-fold (Heijboer, Donga et al. 2005), suggesting a role for DGAT1 in steatosis associated with fasting. We confirmed this finding and, through our studies of global and hepatic-specific *Dgat1* gene inactivations, demonstrated a functional role for hepatic DGAT1 during a fast. In the absence of DGAT1 in the liver, fatty acids entering the liver from WAT during a fast are probably oxidized, as suggested by the increase in circulating ketones in *Dgat1*$^{-/-}$ mice after 8 hours of fasting. The reduced steatosis did not appear to be from increased hepatic VLDL secretion, since serum TG levels were reduced in fasted *Dgat1*$^{-/-}$ mice.

Hepatic steatosis associated with lipodystrophy was independent of DGAT1. In lipodystrophies such as CGL, fatty acid synthesis is activated and contributes to an excessive accumulation of TG in the liver (Shimomura, Hammer et al. 1998; Shimomura, Bashmakov et al. 1999; Shimomura, Hammer et al. 1999; Shimomura, Matsuda et al. 2000). *aP2-SREBP-1c436* and *Dgat1*$^{-/-}$ *aP2-SREBP-1c436* mice had similar degrees of hepatic steatosis, indicating that DGAT1 was not required for the development of steatosis in this lipodystrophy model. These results are consistent with previous findings in *ob*/*ob* mice, another condition of leptin deficiency where DGAT1 deficiency was unable to protect against hepatic steatosis ((Chen, Smith et al. 2002) and H. Chen and R. Farese, unpublished observations). The activation of fatty acid synthesis in *ob*/*ob* mice may explain why DGAT1-deficiency is unable to protect against obesity associated with leptin-deficiency.

The lack of requirement for DGAT1 in lipodystrophy-associated steatosis suggests that DGAT2, rather than DGAT1, functions in steatosis resulting from increased *de novo* fatty acid synthesis. The studies in mice with hepatic *Dgat2* knockdown that were subsequently treated with an LXR agonist support this hypothesis. Hepatic steatosis induced by LXR activation was similar in wild-type and *Dgat1*$^{-/-}$ mice, and a knockdown of *Dgat2*, but not *Dgat1*, expression by ASO blunted the development of steatosis. These results suggest that DGAT2 is functionally coupled to *de novo* fatty acid synthesis, a conclusion proposed by a previous study examining *Dgat2* regulation (Meegalla, Billheimer et al. 2002). Also supporting this hypothesis, a study in cultured cells showed that DGAT2 co-localizes and co-immunoprecipitates with SCD1 (Man, Miyazaki et al. 2006), an enzyme that desaturates newly synthesized fatty acids (Ntambi, Miyazaki et al.

2002; Asilmaz, Cohen et al. 2004). It should be noted, however, that DGAT2 might also be important in hepatic steatosis resulting from exogenous fatty acids. A study employing *Dgat2* ASO treatment showed that DGAT2 deficiency in the liver and WAT protected against steatosis due to a high-fat diet (Yu, Murray et al. 2005).

Implications and Perspectives

DGAT1 was first identified through homology to ACAT enzymes (Chang, Huh et al. 1993; Meiner, Cases et al. 1996; Cases, Novak et al. 1998). The paralog of DGAT1 in yeast is Are2, an enzyme that catalyzes the joining of cholesterol and fatty acids to form cholesterol esters. This indicates that DGAT1 has acquired a different function through speciation. In contrast, DGAT2 belongs to a distinct gene family that includes enzymes the synthesize diacylglycerol (MGAT1,2,3) and wax esters (wax synthase) (Cases, Stone et al. 2001; Yen, Stone et al. 2002; Yen and Farese 2003). Despite their similar catalytic properties, DGAT1 and DGAT2, share no sequence homology. Therefore it's not surprising that their functions are not completely overlapping in hepatic triglyceride metabolism.

Our findings support a model in which DGAT1 functions in hepatocytes

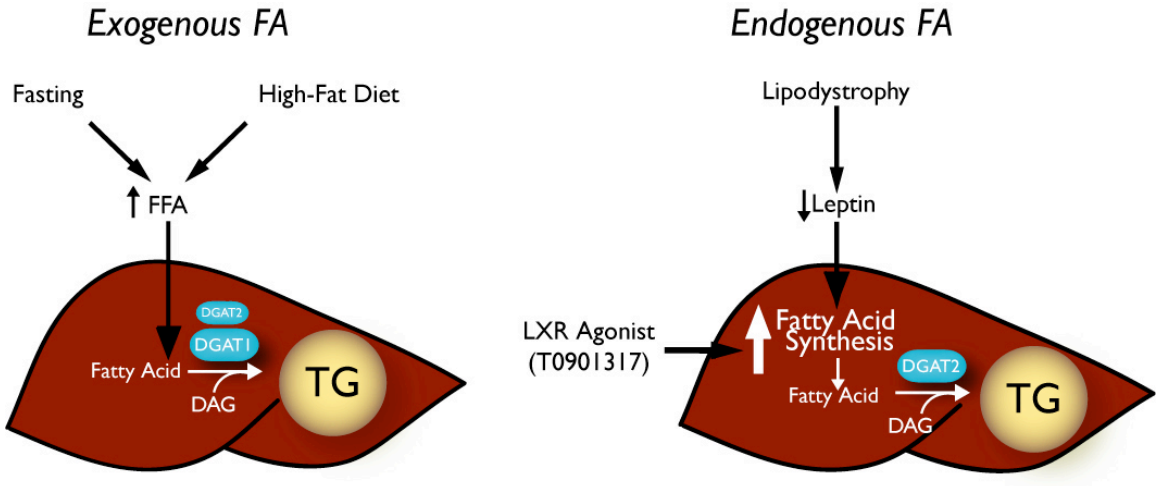

Figure 1. Model summarizing role of DGAT enzymes in the development of hepatic steatosis (See text for description).

to esterify fatty acids that are exogenously derived, and DGAT2 functions to esterify fatty acids derived from *de novo* synthesis (**Figure 1**). Hepatic steatosis associated with a high-fat diet or fasting was dependent on hepatic DGAT1, whereas steatosis resulting from the activation of fatty acid synthesis, such as in lipodystrophy or LXR activation, was independent of DGAT1 and, instead, was dependent on DGAT2. Different functions for DGAT enzymes in hepatic TG synthesis could relate to different intracellular locations (Shockey, Gidda et al. 2006), different K_m's for substrates (Cases, Stone et al. 2001), or different binding partners. These possibilities and this model for DGAT1 versus DGAT2 function require further testing.

Management strategies for the treatment of hepatic steatosis normally include diet, exercise, and treatment of associated metabolic abnormalities, such as hyperlipidemia, diabetes and obesity (Ramesh and Sanyal 2005; Sass, Chang et al. 2005; Portincasa, Grattagliano et al. 2006). Currently there are no approved agents by the FDA for the treatment of hepatic steatosis. Because hepatic steatosis is strongly associated with insulin resistance, insulin sensitizers such as thiazolodinediones (TZD) have been used with mixed success (Chao, Marcus-Samuels et al. 2000; Belfort, Harrison et al. 2006). However, TZD treatment is often associated with body weight gain. Fibrates, a class of drugs that activate PPARα, increase peroxisomal fatty acid oxidation in the liver, and have been useful in the treatment of hepatic steatosis (Issemann, Prince et al. 1993; Shiri-Sverdlov, Wouters et al. 2006).

Our findings are relevant for pharmaceutical strategies for treating hepatic steatosis. Our studies suggest that DGAT1-specific inhibitors would be useful for treating steatosis associated with obesity that is accompanied by resistance to

insulin and leptin, but not useful for steatosis associated with lipodystrophy or other conditions of leptin deficiency. While conditions where hepatic steatosis develops from the activation of fatty acid synthesis, DGAT2 inhibitors may be useful. Key questions still remain unanswered, for example, does the reduction in hepatic TG accumulation prevent the progression of hepatic steatosis to steatohepatitis, and cirrhosis? Could reducing hepatic TG by DGAT inhibition affect inflammatory mediators involved in hepatic steatosis? What are the molecules that trigger steatohepatitis and cirrhosis? Addressing these questions will be key to identifying treatments for NAFLD in the future.

References

Angulo, P. (2002). "Nonalcoholic fatty liver disease." N Engl J Med **346**(16): 1221-31.

Asilmaz, E., P. Cohen, et al. (2004). "Site and mechanism of leptin action in a rodent form of congenital lipodystrophy." J Clin Invest **113**(3): 414-24.

Belfort, R., S. A. Harrison, et al. (2006). "A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis." N Engl J Med **355**(22): 2297-307.

Buhman, K. K., S. J. Smith, et al. (2002). "DGAT1 is not essential for intestinal triacylglycerol absorption or chylomicron synthesis." J Biol Chem **277**(28): 25474-9.

Cases, S., S. Novak, et al. (1998). "ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression, and characterization." J Biol Chem **273**(41): 26755-64.

Cases, S., S. J. Stone, et al. (2001). "Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members." J Biol Chem **276**(42): 38870-6.

Chang, C. C., H. Y. Huh, et al. (1993). "Molecular cloning and functional expression of human acyl-coenzyme A:cholesterol acyltransferase cDNA in mutant Chinese hamster ovary cells." J Biol Chem **268**(28): 20747-55.

Chao, L., B. Marcus-Samuels, et al. (2000). "Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones." J Clin Invest **106**(10): 1221-8.

Chen, H. C., D. R. Jensen, et al. (2003). "Obesity resistance and enhanced glucose metabolism in mice transplanted with white adipose tissue lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **111**(11): 1715-22.

Chen, H. C., S. J. Smith, et al. (2002). "Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1." J Clin Invest **109**(8): 1049-55.

Clark, J. M. (2006). "The epidemiology of nonalcoholic fatty liver disease in adults." J Clin Gastroenterol **40**(3 Suppl 1): S5-10.

Day, C. P. and O. F. James (1998). "Steatohepatitis: a tale of two "hits"?" Gastroenterology **114**(4): 842-5.

Finn, P. F. and J. F. Dice (2006). "Proteolytic and lipolytic responses to starvation." Nutrition **22**(7-8): 830-44.

Heijboer, A. C., E. Donga, et al. (2005). "Sixteen hours of fasting differentially affects hepatic and muscle insulin sensitivity in mice." J Lipid Res **46**(3): 582-8.

Issemann, I., R. A. Prince, et al. (1993). "The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs." J Mol Endocrinol **11**(1): 37-47.

Kersten, S., J. Seydoux, et al. (1999). "Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting." J Clin Invest **103**(11): 1489-98.

Man, W. C., M. Miyazaki, et al. (2006). "Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis." J Lipid Res **47**(9): 1928-39.

Meegalla, R. L., J. T. Billheimer, et al. (2002). "Concerted elevation of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity through independent stimulation of mRNA expression of DGAT1 and DGAT2 by carbohydrate and insulin." Biochem Biophys Res Commun **298**(3): 317-23.

Meiner, V. L., S. Cases, et al. (1996). "Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals." Proc Natl Acad Sci U S A **93**(24): 14041-6.

Ntambi, J. M., M. Miyazaki, et al. (2002). "Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity." Proc Natl Acad Sci U S A **99**(17): 11482-6.

Portincasa, P., I. Grattagliano, et al. (2006). "Current pharmacological treatment of nonalcoholic fatty liver." Curr Med Chem **13**(24): 2889-900.

Ramesh, S. and A. J. Sanyal (2005). "Evaluation and management of non-alcoholic steatohepatitis." J Hepatol **42 Suppl**(1): S2-12.

Sass, D. A., P. Chang, et al. (2005). "Nonalcoholic fatty liver disease: a clinical review." Dig Dis Sci **50**(1): 171-80.

Schultz, J. R., H. Tu, et al. (2000). "Role of LXRs in control of lipogenesis." Genes Dev **14**(22): 2831-8.

Shimomura, I., Y. Bashmakov, et al. (1999). "Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus." J Biol Chem **274**(42): 30028-32.

Shimomura, I., R. E. Hammer, et al. (1999). "Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy." Nature **401**(6748): 73-6.

Shimomura, I., R. E. Hammer, et al. (1998). "Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy." Genes Dev **12**(20): 3182-94.

Shimomura, I., M. Matsuda, et al. (2000). "Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice." Mol Cell **6**(1): 77-86.

Shiri-Sverdlov, R., K. Wouters, et al. (2006). "Early diet-induced non-alcoholic steatohepatitis in APOE2 knock-in mice and its prevention by fibrates." J Hepatol **44**(4): 732-41.

Shockey, J. M., S. K. Gidda, et al. (2006). "Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum." Plant Cell **18**(9): 2294-313.

Yen, C. L. and R. V. Farese, Jr. (2003). "MGAT2, a monoacylglycerol acyltransferase expressed in the small intestine." J Biol Chem **278**(20): 18532-7.

Yen, C. L., S. J. Stone, et al. (2002). "Identification of a gene encoding MGAT1, a monoacylglycerol acyltransferase." Proc Natl Acad Sci U S A **99**(13): 8512-7.

Yu, X. X., S. F. Murray, et al. (2005). "Antisense oligonucleotide reduction of DGAT2 expression improves hepatic steatosis and hyperlipidemia in obese mice." Hepatology **42**(2): 362-71.

Zimmermann, R., J. G. Strauss, et al. (2004). "Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase." Science **306**(5700): 1383-6.

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