Suppression of Diacylglycerol Acyltransferase-2 (DGAT2), but Not DGAT1, with Antisense Oligonucleotides Reverses Diet-induced Hepatic Steatosis and Insulin Resistance

Cheol Soo Choi 1, David B. Savage 1,2, Ameya Kulkarni 3, Xing Xian Yu 4, Zhen-Xiang Liu 4, Katsutaro Morino 4, Sheene Kim 1, Alberto Distefano 5, Varman T. Samuel 1, Susanne Neschen 5, Dongyan Zhang 6, Amy Wang 7, Xian-Man Zhang 7, Mario Kahn 7, Gary W. Cline 7, Sanjay K. Pandey 9, John G. Geisler 5, Sanjay Bhanot 4, 5, Brett P. Monia 4, 5, and Gerald I. Shulman 4, 6

1 These authors contributed equally to this work.
2 Supported by the Wellcome Trust.
3 Employee of and owns stock and/or holds stock options in Isis Pharmaceuticals.
4 Received for publication, May 22, 2007. Published, JBC Papers in Press, May 27, 2007, DOI 10.1074/jbc.M704213200

From the Departments of 1Internal Medicine and 2Cellular and Molecular Physiology, 3Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510 and 4Isis Pharmaceuticals, Carlsbad, California 92008

Nonalcoholic fatty liver disease (NAFLD) is a major contributing factor to hepatic insulin resistance in type 2 diabetes. Diacylglycerol acyltransferase (Dgat), of which there are two isoforms (Dgat1 and Dgat2), catalyzes the final step in triglyceride synthesis. We evaluated the metabolic impact of pharmacological reduction of DGAT1 and -2 expression in liver and fat using antisense oligonucleotides (ASOs) in rats with diet-induced NAFLD. Dgat1 and Dgat2 ASO treatment selectively reduced DGAT1 and DGAT2 mRNA levels in liver and fat, but only Dgat2 ASO treatment significantly reduced hepatic lipids (diacylglycerol and triglyceride) and improved hepatic insulin sensitivity. Because Dgat catalyzes triglyceride synthesis from diacylglycerol, and because we have hypothesized that diacylglycerol accumulation triggers fat-induced hepatic insulin resistance through protein kinase Cε activation, we next sought to understand the paradoxical reduction in diacylglycerol in Dgat2 ASO-treated rats. Within 3 days of starting Dgat2 ASO therapy in high-fat-fed rats, plasma fatty acids increased, whereas hepatic lysophosphatidic acid and diacylglycerol levels were similar to those of control rats. These changes were associated with reduced expression of lipogenic genes (SREBP1c, ACC1, SCD1, and mtGPAT) and increased expression of oxidative/thermogenic genes (CPT1 and UCP2). Taken together, these data suggest that knocking down Dgat2 protects against fat-induced hepatic insulin resistance by paradoxically lowering hepatic diacylglycerol content and protein kinase Cε activation through decreased SREBP1c-mediated lipogenesis and increased hepatic fatty acid oxidation.

Nonalcoholic fatty liver disease (NAFLD) is the most frequent cause of abnormal liver function tests in the United States (estimated prevalence of 14–20%) (1, 2). It is caused by triglyceride (TG) accumulation within the liver and is strongly associated with insulin resistance, type 2 diabetes mellitus (T2DM), and the metabolic syndrome (3, 4). Accumulating evidence suggests that hepatic lipid accumulation causes hepatic insulin resistance. For example, increasing hepatic lipid stores in mice by overexpressing lipoprotein lipase in the liver (5) and in rats by short term high fat feeding (6) results in liver-specific fat accumulation and hepatic insulin resistance. Several strategies have been employed to reduce hepatic steatosis in rodents; these include treatment with a mitochondrial uncoupling agent (2,4-dinitrophenol) (6), antisense oligonucleotide inhibition of acetyl-coenzyme A carboxylase I and II (7), adeno viral overexpression of malonyl-CoA decarboxylase (8), and transgenic overexpression of uncoupling protein 1 (9), all of which successfully ameliorated hepatic insulin resistance. We have also shown that moderate weight loss in patients with T2DM lowers liver triglycerides and specifically improves hepatic insulin sensitivity (10). Although the above data strongly suggest that hepatic lipid accumulation causes hepatic insulin resistance, the molecular mechanisms responsible for this relationship remain uncertain. We have proposed a model in which excess diacylglycerol (DAG) activates protein kinase Cε (PKCe), a serine/threonine kinase that in turn binds to the insulin receptor and inhibits its tyrosine kinase activity (11). Until very recently, long chain acyl-coenzymes A (LCCoAs) were a favored candidate for fat-induced insulin resistance, but recent studies in mitochondrial acyl-coenzyme A (CoA):glycerol-sn-3-phosphate acyltransferase (mtGPAT) knockout mice, which accumulate LCCoAs in the liver but have reduced liver DAG con-...
tent and are protected from fat-induced hepatic insulin resistance (12), suggested that DAG may be a better candidate. DAG is also a potent activator of novel PKCs (13–15), making it an excellent candidate for this role.

Acyl-CoA:diacylglycerol acyltransferase (Dgat) catalyzes the final step in TG synthesis by facilitating the linkage of sn-1,2-diacylglycerol (DAG) with an LC-CoA. Dgat exists in two primary isoforms: Dgat1 and Dgat2 (16, 17); Dgat1 is most highly expressed in small intestine and white adipose tissue, whereas Dgat2 is primarily expressed in liver and white adipose tissue (16, 17) where its expression is insulin-responsive. There is some evidence suggesting that the two enzymes play different roles in TG metabolism. DGAT1 knock-out mice have ~50% less TG in tissues and are protected from diet-induced obesity and insulin resistance through a mechanism involving increased energy expenditure (at least partly attributable to increased physical activity) (18, 19). Study of Dgat2 has been more difficult as homozygous DGAT2 knock-out mice die shortly after birth because of severe lipopenia (~90% TG reduction in DGAT2 null carcasses) and impaired skin barrier function (20).

Here we sought to address two key questions. First, what effect does DGAT1 or DGAT2 knockdown in liver and fat have on hepatic insulin action? Other than the mtGPAT knock-out model, previous successful therapeutic strategies for NAFLD have primarily focused on increasing fat oxidation as opposed to inhibiting fat synthesis. In this study, we were therefore interested in examining the effect of inhibiting another key regulator of fat synthesis. Second, which lipid intermediates are increased or decreased by Dgat knockdown? Conventional wisdom suggests that DAG, a substrate for the enzyme activity of Dgat, might accumulate in the face of Dgat knockdown, an event which we have suggested induces insulin resistance (21–23). The present study demonstrates that Dgat2, but not Dgat1 ASO treatment, improves hepatic steatosis, insulin sensitivity, and somewhat surprisingly reduces hepatic DAG content. In addition, we provide evidence suggesting a potential mechanism to explain this paradoxical effect of DGAT2 inhibition on hepatic DAG content.

**EXPERIMENTAL PROCEDURES**

**Animals**—All rats were maintained in accordance with the Institutional Animal Care and Use Committee of Yale University School of Medicine. Healthy male Sprague-Dawley rats weighing ~200 g were obtained from Charles River Laboratories and acclimated for 1 week after arrival before initiation of the experiment. Rats received food and water ad libitum and were maintained on a 12:12 h light/dark cycle (lights on at 6:30 a.m.). They were housed individually, and food consumption and body weight were monitored. Rats received either regular rodent chow (60% carbohydrate, 10% fat, 30% protein calories) or a high fat diet (26% carbohydrate, 59% fat, 15% protein calories). Safflower oil was the major constituent of the high fat diet (Dyets Inc.). We have shown previously that this diet produces hepatic steatosis and hepatic insulin resistance within 3 days (6). Intraperitoneal ASO therapy was initiated 3 days after commencing the high fat diet. All ASOs (control, Dgat1, and Dgat2) were prepared in normal saline, and the solutions were sterilized through a 0.2-µm filter. Rats were given ASO solutions or saline twice per week via intraperitoneal injection at a dose of 50 or 75 mg/kg/week for 4 weeks. ASO control rats were treated at the higher dose (75 mg/kg/week). During the treatment period, body weight and food intake were measured twice weekly. The Yale Animal Care and Use Committee approved all protocols.

**Selection of Rat Dgat ASOs**—To identify rat Dgat1 and Dgat2 ASO inhibitors, rapid throughput screens were performed in vitro as described previously (24). In brief, 80 ASOs were designed to the rat DGAT1 and DGAT2 mRNAs sequences, respectively, and initial screens identified several potent and specific ASOs, all of which targeted a binding site within the coding region of the DGAT1 and DGAT2 mRNAs. After extensive dose-response characterization of the most potent ASOs from the screen, two lead ASOs, ISIS-327822 (sequence CTCCTGCGGCGCACCACA) for Dgat1 and ISIS-369235 (sequence GCCATACCCTCCATTCTTT) for Dgat2, were chosen. The control ASO, ISIS-141923, has the following sequence, 5’-CCTTCCCTGAGTTCCTCC-3’, and does not have perfect complementarity to any known gene in public data bases. The first five bases and last five bases of chimeric ASOs have a 2’-O-(2-methoxy)-ethyl modification, and the ASOs also have a phosphorothioate backbone. This chimeric design has been shown to provide both increased nuclease resistance and mRNA affinity, while maintaining the robust RNase H terminating mechanism utilized by these types of ASOs (25). These benefits result in an attractive in vivo pharmacological and toxicological profile for 2’-O-(2-methoxy)-ethyl chimeric ASOs.

**Determination of DGAT Activity in Liver Homogenates and Fatty Acid Oxidation and Triglyceride Synthesis in Transfected Rat Hepatocytes in Vitro**—Total DGAT activity was measured in liver tissue homogenates from ASO-treated rats as described previously (26). Primary rat hepatocytes were isolated as described previously and plated onto collagen-coated 25-cm² flasks for fatty acid oxidation measurement or 60-mm plates for triglyceride synthesis measurement (27). Hepatocytes were treated with ASO (150 nM) and Lipofectin® (Invitrogen) mixture for 4 h in serum-free William’s E media (Invitrogen). ASO and Lipofectin were mixed in a ratio of 3 µg of Lipofectin® for every 1 ml of 100 nM ASO concentration. After 4 h, ASO reaction mixture was replaced with normal maintenance media (William’s E media with 10% fetal bovine serum and 10 nM insulin). The cells were incubated under normal conditions for 20–24 h, and then fatty acid ([14C]oleate) oxidation and triglyceride synthesis (incorporation of [3H]glycerol into triglyceride) were measured as described previously (27).

**Hyperinsulinemic–Euglycemic Clamp Studies**—Seven days prior to the hyperinsulinemic-euglycemic clamp studies, indwelling catheters were placed into the right internal jugular vein extending to the right atrium, and the left carotid artery extending to the aortic arch. After an overnight fast, [3-3H]glucose (HPLC-purified; PerkinElmer Life Sciences) was infused at a rate of 0.15 µCi/min for 3 h to assess the basal glucose turnover. Following the basal period, the hyperinsulinemic-euglycemic clamp was conducted for 135 min with a primed/continuous infusion of human insulin (60 milliunits/kg prime, 4 milliunits/kg/min infusion) (Novo Nordisk) and a variable infusion of 20%...
Suppressing DGAT2 Reverses Hepatic Insulin Resistance

dextrose to maintain euglycemia (~100 mg/dl). [3-3H]Glucose was infused at a rate of 0.4 µCi/min throughout the clamps. A 25-µCi bolus of 2-deoxy-o-[1-14C]glucose (PerkinElmer Life Sciences) was injected at the 90th min of the clamp to estimate the rate of insulin-stimulated tissue glucose uptake. Additional blood samples (20–60 µl) were taken at 0, 70, and 135 min for the determination of plasma FFA and/or insulin concentrations. At the end of the clamp, rats were anesthetized with pentobarbital sodium injection (150 mg/kg), and all tissues were taken within 4 min, frozen immediately using liquid N2-cooled aluminum tongs, and stored at −80 °C for subsequent analysis.

Biochemical Analysis and Calculations—Plasma glucose was analyzed during the clamps using 10 µl of plasma by a glucose oxidase method on a Beckman glucose analyzer II (Beckman Coulter). Plasma insulin, adiponectin, and leptin were measured by radioimmunoassay using kits from Linco Research Inc. (Pfizer, Chicago) as described by Crooke et al. (32). The adiponectin kit was primarily designed to measure mouse adiponectin, but it shows significant cross-reactivity with rat adiponectin. Plasma fatty acid concentrations were determined using an acyl-CoA oxidase-based colorimetric kit (Wako Pure Chemical Industries Ltd.). Plasma lipoprotein and cholesterol profiling in pooled samples of each group was performed with a Beckman System Gold 126 HPLC system, 507e refrigerated autosampler, 126-photodiode array detector (Beckman Instruments, Fullerton, CA) and a Superose 6 HR 10/30 column (Pfizer, Chicago) as described by Crooke et al. (28). For the determination of plasma [3H]glucose, plasma was deproteinized with ZnSO4 and Ba(OH)2, dried to remove 3H2O, resuspended in water, and counted in scintillation fluid (Ultima Gold, PerkinElmer Life Sciences) on a Beckman scintillation counter. Rates of basal and insulin-stimulated whole body glucose turnover were determined as the ratio of the [3-3H]glucose infusion rate (disintegrations per min (dpm)) to the specific activity of plasma glucose (dpm per mg) at the end of the basal period and during the final 30 min of the clamp experiment, respectively. Hepatic glucose production was determined by subtracting the glucose infusion rate from the rate of total glucose appearance. The plasma concentration of [3H]2O was determined by the difference between [3H] counts without and with drying, and whole body glycolysis was calculated from the rate of increase in plasma [3H]2O concentration, determined by linear regression of the measurements at 100, 105, 115, 125, and 135 min (29). Whole body glycolysis synthesis was estimated by subtracting whole body glycolysis from whole body glucose uptake, assuming that glycolysis and glycerone synthesis account for the majority of insulin-stimulated glucose uptake (30). Glucose uptake and glycerone synthesis in individual muscles were calculated from muscle 2-[14C]deoxyglucose 6-phosphate (2-DG-6-P) content and [3H] incorporation into muscle glycerone as described previously (29). For the determination of muscle 2-DG-6-P content, muscle samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously (31). The radioactivity of [3H] in muscle glycerone was determined by digesting muscle samples in KOH and precipitating glycerone with EtOH as described previously (30).

Tissue Lipid Measurement—The solid-phase extraction and purification of medium, long chain, and very long chain fatty acyl-CoAs from liver have been described previously (32, 33). After purification, fatty acyl-CoA fractions were dissolved in methanol/H2O (1:1, v/v) and subjected to lipid chromatography/tandem mass spectrometry analysis. A turbo ion spray source was interfaced with an API 3000 tandem mass spectrometer (Applied Biosystems) in conjunction with two 200 series micro pumps and a 200 series autosampler (PerkinElmer Life Sciences). The DAG extraction and analysis were performed as described previously (12, 15). Total DAG content is expressed as the sum of individual species. Tissue TG was extracted using the method of Bligh and Dyer (32) and measured using a DCL triglyceride reagent (Diagnostic Chemicals Ltd.). Tissue lysophosphatidic acid (LPA) content was measured as described previously (12). In short, ~100 mg of liver tissue was homogenized in chloroform/methanol (1:1 v/v) with 1 nmol of C17-lysophosphatidic acid as an internal standard. After phase separation with H2O, samples were vortexed and centrifuged, and the methanol/water phase was collected. The supernatant was applied to conditioned Waters Oasis HLB extraction cartridges (Waters), and after a washing step LPA was eluted using methanol. Individual LPA derivatives were measured using lipid chromatography/tandem mass spectrometry analysis, and total LPA content was expressed as the sum of individual species.

Insulin Signaling—IRS2-associated PI3K and Akt2 activity were assessed in protein extracts from livers harvested after short term insulin stimulation. PI3K and Akt2 assays were performed according to methods previously described (6, 34–36). Primary antibodies used for experiments were rabbit polyclonal IgG. Antibodies for PI3K and Akt2 were obtained from Upstate (Charlottesville, VA).

For PKCe membrane translocation, 50 µg of crude membrane and cytosol protein extracts were resolved by SDS-PAGE using 8% gel and electrophoresed onto polyvinylidene difluoride membrane (DuPont) using a semidy-transfer cell (Bio-Rad). The membrane was then blocked for 2 h at room temperature in phosphate-buffered saline/Tween (PBS-T:10 mmol/liter NaH2PO4, 80 mmol/liter Na2HPO4, 0.145 mol/liter NaCl, and 0.1% Tween 20, pH 7.4) containing 5% (w/v) nonfat dried milk, washed twice, and then incubated overnight with rabbit anti-peptide antibody against PKCe (Santa Cruz Biotechnology) diluted 1:100 in rinsing solution. After further washings, membranes were incubated with horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG (Bio-Rad), diluted 1:5000 in PBS-T, for 2 h. PKCe translocation was expressed as the ratio of membrane bands over cytosol bands (arbitrary units).

Total RNA Preparation, Real Time Quantitative RT-PCR Analysis, and Immunoblotting Analysis—Total RNA was extracted using total RNA isolation reagent (BL-10500, Biotecx Laboratories Inc.) according to manufacturer’s instructions. Real time quantitative RT-PCR was performed using custom-made RT-PCR enzymes and reagents kit (Invitrogen.) and ABI Prism 7700 sequence detector (Applied Biosciences). Primers and probes for analysis of the expression of different genes (supplemental Table 1) were designed using Primer Express Software (Applied Biosciences). For the analysis, 100 ng of total RNA was used. Each RNA sample was run in duplicate, and the
mean value was used to calculate the gene expression level. For Western protein analysis, 200 μl of homogenization buffer (160 NaCl, 20 Trizma (Tris base), 1 EDTA, 1 EGTA, 1 dithiothreitol, 1% Triton X-100, 0.1% SDS, 1 sodium orthovanadate, 1 sodium fluoride, 1 phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A) was added to 20 mg of powdered tissue. After homogenization for 15 min on ice, the samples were transferred to microcentrifuge tubes and rotated on a rocking platform for 30 min in the cold room. After centrifugation for 60 min at 20,500 relative centrifugal fields in a refrigerated centrifuge at 4 °C, insoluble debris was removed. Forty micrograms of tissue lysates were then separated on 4–12% SDS-PAGE and immunoblotted with SREBP1 (Santa Cruz Biotechnology, catalog number 13551), ACC1 (Cell Signaling, catalog number 36620), SCD1 (Santa Cruz Biotechnology, catalog number 14720), or UCP2 (Santa Cruz Biotechnology, catalog number 6526) antibodies.

**Fat Absorption**—During the treatment period, fat absorption was measured by placing a sucrose polybehenate marker in a safflower oil diet and measuring the percent discarded in collected stool samples (37).

**Statistical Analysis**—Data are expressed as means ± S.E. The significance of the differences in mean values among the three groups was evaluated using the one-way analysis of variance, followed by ad hoc analysis using the (Duncan’s test). Statistical analyses for the two groups were made using a two-tailed Student’s t test, and p < 0.05 was considered statistically significant.

**RESULTS**

**Dgat ASOs Reduce DGAT Expression**—After 3 days on a high fat diet, an interval that allows for significant fat accumulation in the liver (6), rats were treated with control (ASOctrl), Dgat1, or Dgat2 ASOs at 25 (for Dgat1) or 37.5 (for Dgat2 and ASOctrl) mg per kg body weight twice a week for 4 weeks. In comparison with ASOctrl, Dgat1 and Dgat2 ASO treatment selectively reduced DGAT1 mRNA (Fig. 1A) and DGAT2 mRNA (Fig. 1B) levels by 84 and 70%, respectively, in the liver. Both ASOs significantly reduced total Dgat activity in liver homogenates (Fig. 1C). Dgat1 ASO had a greater effect on total Dgat activity than Dgat2 ASO because assay conditions for total Dgat activity with 20 mM MgCl₂ are optimized for DGAT1 rather than DGAT2 activity (16, 17). To exclude off-target effects of Dgat2 ASO, mRNA expression of acyl-coenzyme A:monoacylglycerol acyltransferase (Mgat) 1, 2, and 3 were tested in liver because they catalyze the synthesis of DAG from monoacylglycerol and share sequence homology with DGAT2 (38–41). In addition, they have some Dgat activity (38–41). MGAT1 mRNA expression is unaltered in the liver (ASOctrl versus Dgat2 ASO; 0.09 ± 0.01 versus 0.11 ± 0.02 arbitrary units, not significant). MGAT2 is expressed at very low levels in rat liver (barely detectable using RT-PCR), and MGAT3 is suggested to be specific to the intestine in rodents (40). Target gene expression was reduced to a similar extent in adipose tissue (90 and 87% for DGAT1 and DGAT2, respectively), but no significant changes were seen in DGAT expression in muscle (Fig. 1, D and E). There was no compensatory increase in the untargeted isoform. None of the ASOs appeared to be associated with overt toxicity as assessed by hepatic transaminase levels (Table 1) and food intake.
Suppressing DGAT2 Reverses Hepatic Insulin Resistance

Table 1

Metabolic parameters during fasting and hyperinsulinemic-euglycemic clamp periods

Fasting refers to an overnight fast.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (Fasting n = 8)</th>
<th>Clamp (Fasting n = 11)</th>
<th>Dgat1 (Fasting n = 7)</th>
<th>Clamp (Fasting n = 11)</th>
<th>Dgat2 (Fasting n = 7)</th>
<th>Clamp (Fasting n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>422 ± 7</td>
<td>401 ± 8</td>
<td>415 ± 11</td>
<td>412 ± 11</td>
<td>407 ± 13</td>
<td>370 ± 8*</td>
</tr>
<tr>
<td>Epididymal fat mass (g)</td>
<td>5.9 ± 1.0</td>
<td>ND</td>
<td>9.8 ± 2.2</td>
<td>ND</td>
<td>2.9 ± 0.4*</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>116 ± 3.0</td>
<td>111 ± 2.5</td>
<td>125 ± 3.0</td>
<td>98.5 ± 2.2</td>
<td>125 ± 6.7</td>
<td>114 ± 2.7</td>
</tr>
<tr>
<td>Insulin (microunits/liter)</td>
<td>7.3 ± 1.6</td>
<td>96.1 ± 7.0</td>
<td>13.6 ± 5.4</td>
<td>99.1 ± 7.7</td>
<td>3.4 ± 0.7*</td>
<td>97.7 ± 4.8</td>
</tr>
<tr>
<td>FA (meq/liter)</td>
<td>0.84 ± 0.06</td>
<td>0.43 ± 0.03</td>
<td>0.82 ± 0.08</td>
<td>0.49 ± 0.06</td>
<td>0.64 ± 0.05</td>
<td>0.23 ± 0.04*</td>
</tr>
<tr>
<td>Plasma TG (mg/dl)</td>
<td>36.0 ± 2.2</td>
<td>ND</td>
<td>34.9 ± 1.7</td>
<td>ND</td>
<td>26.1 ± 1.3*</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>40.3 ± 2.2</td>
<td>ND</td>
<td>50.1 ± 6.2</td>
<td>ND</td>
<td>32.7 ± 2.3*</td>
<td>ND</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.2 ± 0.3</td>
<td>ND</td>
<td>3.2 ± 0.46</td>
<td>ND</td>
<td>2.6 ± 0.36</td>
<td>ND</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>3.2 ± 0.4</td>
<td>ND</td>
<td>41 ± 3.0</td>
<td>ND</td>
<td>42 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>ALT (units/liter)</td>
<td>38 ± 2.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*p < 0.05 Dgat2 (clamp) versus control and Dgat1 (clamp).
*p < 0.05 Dgat2 (fasting) versus control (fasting).
*p < 0.05 Dgat2 (fasting) versus control and Dgat1 (fasting).
*ALT is alanine aminotransferase; ND is not determined.

Food Intake and Body Weight Response—Dgat2 ASO therapy was associated with a small decrease in body weight and epididymal fat pad mass (Table 1). As caloric intake (AS0ctrl versus Dgat2 AS0; 98.8 ± 2.3 versus 97.0 ± 8.2 kcal/day) and intestinal fat absorption (AS0ctrl versus Dgat2; 99.1 ± 0.4% versus 99.4 ± 0.4%) were similar in control and Dgat2 treated rats, these data suggest that energy expenditure was slightly increased in the Dgat2 ASO treatment group.

Dgat2 ASO Reduces Liver and Muscle Steatosis and Plasma Triglycerides—Hepatic TGs were markedly reduced by Dgat2 ASO (50% reduction, p < 0.05) but not by Dgat1 ASO treatment (Fig. 2, A and B). DAG levels were also substantially decreased by 4 weeks of Dgat2 ASO therapy (53% reduction, p < 0.05) (Fig. 2C), whereas LCCoAs were unchanged (Fig. 2D). Fasting plasma TGs and total cholesterol fell by 28 and 19%, respectively, in the Dgat2 ASO group, whereas plasma fatty acids were unchanged in fasting rats after 4 weeks of treatment (Table 1). Very low density lipoprotein and high density lipoprotein cholesterol were both substantially reduced in Dgat2 ASO-treated rats (Fig. 2E). In vitro studies in primary rat hepatocytes suggest that reduced TG synthesis is likely to be the primary mechanism for the observed reduction in liver lipids (Fig. 2, F–H), although fat oxidation was also significantly increased. In addition, muscle TG content was reduced by 33% in the Dgat2 ASO group (AS0ctrl versus Dgat2; 5.67 ± 0.51 versus 3.80 ± 0.69 mg/g tissue, p < 0.05).

Dgat2 ASO Treatment Improves Hepatic Insulin Sensitivity—Fasting plasma glucose and fatty acid concentrations were similar in both groups after 4 weeks of ASO therapy, whereas plasma insulin concentrations were significantly reduced in Dgat2 ASO-treated rats (Table 1). Leptin levels were also lower.
in Dgat2 ASO-treated rats, but adiponectin levels were unchanged. To independently assess hepatic and peripheral (predominantly muscle) insulin sensitivity, we performed hyperinsulinemic-euglycemic clamps (including radioisotope labeled glucose infusions) in ASO-treated rats. Glucose infusion rates were significantly higher in Dgat2 ASO but not in Dgat1 ASO-treated high fat fed rats than in the ASOctrl group (Fig. 3A). The ability of insulin to suppress endogenous glucose production was significantly increased in the Dgat2 ASO group (Fig. 3B) as was insulin-stimulated peripheral glucose turnover (47% increase, see Fig. 3C). Dgat2 ASO treatment did not change whole body glycolysis (Fig. 3D) but significantly increased glycogen synthesis rates by 71% as compared with ASOctrl therapy (Fig. 3E). The increase in insulin-stimulated glucose uptake was predominantly accounted for by a 35 and 230% increase in insulin-stimulated glucose uptake in skeletal muscle (gastrocnemius) and white adipose tissue (epididymal) (Fig. 3F and G). The ability of insulin to suppress peripheral lipolysis and decrease fatty acid concentration also serves as an indicator of adipose tissue insulin sensitivity. In comparison with the control and Dgat1 ASO groups, Dgat2 ASO treatment increased insulin-mediated suppression of lipolysis, demonstrated by significantly lower fatty acid levels during the clamp (Table 1).

Effect of Dgat2 ASO Treatment on Hepatic Insulin Signaling—
We have previously shown that hepatic steatosis (6) in rats fed a high fat diet for 3 days is specifically associated with activation of PKCε (as reflected by an increase in the plasma membrane: cytosol ratio of PKCε), an event that we believe may be causally related to the development of hepatic insulin resistance. Dgat2

Suppressing DGAT2 Reverses Hepatic Insulin Resistance

FIGURE 3. Dgat2 ASO treatment significantly improved hepatic and peripheral insulin sensitivity in high fat-fed rats. Peripheral and hepatic insulin sensitivities were assessed by means of hyperinsulinemic-euglycemic clamps. A, glucose infusion rates; B, suppression of hepatic glucose production (HGP) during hyperinsulinemic-euglycemic clamps; C, peripheral glucose disposal (Rd); D, whole body glycolysis; E, whole body glycogen synthesis; F, skeletal muscle (gastrocnemius) glucose uptake; and G, epididymal white adipose tissue (WAT) glucose uptake. Data are expressed as mean values ± S.E. for 9–11 rats per treatment group; error bars indicate S.E., * p < 0.05 versus ASOctrl and Dgat1 ASO treatment groups.
ASO therapy significantly reduced hepatic glucose production during the hyperinsulinemic phase of the hyperinsulinemic-euglycemic clamp (Fig. 4, A and B) and significantly reduced PKCζ membrane translocation (Fig. 4C). These changes were associated with increased insulin-stimulated PI3K (Fig. 4D) and Akt2 activity (Fig. 4E).

Why Don’t DAG Levels Increase in Dgat2 ASO-treated Rats?—As shown in Fig. 2, 4 weeks of Dgat2 ASO therapy significantly reduced liver DAGs and TGs, whereas liver LCCoAs were unchanged. To better understand this paradoxical observation, concentrations of plasma fatty acids and hepatic lipid intermediates in the TG synthesis pathway were measured at an early time point (3 days) after initiation of ASO therapy (Fig. 5). It is important to note that although maximum target gene suppression requires several doses of the antisense oligonucleotides, DGAT2 mRNA was significantly reduced within 3 days of commencing ASO therapy (Fig. 5A). At this stage, plasma fatty acids (Fig. 5B) were significantly increased, and hepatic LCCoAs tended to be increased \( p = 0.11 \) (Fig. 5C), whereas LPA (Fig. 5D) and DAG levels (Fig. 5E) were similar to those in control rats. This metabolite pattern is similar to that observed in mtGPAT knock-out mice (12). Gene expression analysis confirmed that mtGPAT mRNA expression was reduced (Fig. 6A). This may in turn be due to the fatty acid-induced inhibition of sterol regulatory element-binding protein-1c (Srebp1c) activity (42, 43). SREBP1c mRNA and protein levels were also reduced at early time points as were levels of other lipogenic SREBP1c target genes, stearoyl-CoA desaturase-1 (SCD1) and ACC1 (acetyl-CoA carboxylase 1) (Fig. 6, A and D). Importantly, plasma insulin levels were similar in Dgat2 and control ASO-treated rats at early time points (ASOctrl versus Dgat2 ASO: 6.0 ± 1.07 versus 4.4 ± 0.68 microunits/liter), so we do not believe that insulin was responsible for the early changes in lipogenic gene expression. Further evidence favoring increase in intracellular fatty acid rather than insulin as the signaling mediator of early changes in hepatic gene expression in this model comes from the fact that fatty acids might also be expected to activate PPARα and increase PPARα target gene expression. In this regard, PPARα mRNA levels were unchanged, but CPT1 mRNA and UCP2 (uncoupling protein 2) mRNA and protein levels were increased, as were plasma ketones \( (2.23 ± 0.12 \text{ mmol/liter versus } 1.92 ± 0.06 \text{ mmol/liter in control rats}) \), an indirect marker of hepatic fatty acid oxidation (Fig. 6, B–D).

DISCUSSION

Hepatic steatosis is consistently associated with the development of hepatic insulin resistance in both rodent models and humans. Fat accumulation in the liver can occur as a result of increased fat delivery, increased fat synthesis, reduced fat oxidation, and/or reduced fat export in the form of lipoproteins (23). In humans, we have shown that reducing energy intake in association with modest weight loss reduces hepatic steatosis and improves hepatic insulin sensitivity in patients with T2DM (10). Unfortunately, lifestyle modification is notoriously difficult to sustain, necessitating the
development of additional strategies for the treatment of NAFLD and hepatic insulin resistance. To date, the majority of studies has targeted fat oxidation with few attempts to reduce fat synthesis in the liver. Here we assessed the impact of anti-sense oligonucleotide-mediated reduction of Dgat1 or Dgat2 in rats with diet-induced NAFLD, an intervention that primarily influences TG synthesis. The results show that DGAT1 and DGAT2 were significantly reduced in an isoform- and tissue-selective (liver and adipose tissue) manner, and that there was no compensatory increase in the untargeted isoform. DGAT1 knockdown was surprisingly ineffective in improving NAFLD in this model, whereas reducing DGAT2 expression significantly improved hepatic steatosis. Although DGAT1 null mice are resistant to diet-induced obesity and insulin resistance, this phenotype is dominated by an as yet incompletely understood increase in energy expenditure and weight loss (44). Interestingly, when adipose tissue was transplanted from DGAT1 null mice to wild type mice, the latter lost weight, leading the authors to postulate that DGAT1 knock-out adipocytes produce a circulating factor that promotes energy expenditure (45). Recent work by the same group suggests that this putative factor is not adiponectin (46). Adiponectin levels were similar in all of our treatment groups. Our model differs from whole body knock-out studies in so far as DGAT1 or DGAT2 were selectively reduced in liver and adipose tissue (antisense oligonucleotides also reduce target gene expression in the kidney tubules but have no effect on muscle, bowel, or skin). The Dgat1 ASO does, however, reduce DGAT1 expression in adipose tissue without significantly altering body weight. We did see a small reduction in weight gain in the Dgt2 ASO-treated group, a finding that we believe results from a small increase in energy expenditure as food intake and intestinal fat absorption were similar in ASOctrl and Dgt2 ASO-treated rats. The DGAT2 null mouse is almost totally devoid of fat and dies shortly after birth, suggesting that Dgat2 may be more critical to TG synthesis than Dgat1 (20). This is in keeping with our data and with the reported efficacy of a Dgat2 ASO in mice with fatty liver (27). Dgat2 is also the predominant Dgat isoform in the liver (17) with almost no expression in stellate cells in mouse liver, whereas the expression of DGAT1 is about 1.6-fold higher in stellate cells than in hepatocytes (17). These findings may help explain the lack of effect of the Dgat1 ASO on hepatic triglyceride content.

Although ectopic fat accumulation in tissues such as liver and skeletal muscle is clearly associated with the development of insulin resistance, previous studies by our group have dissociated increase in intracellular TG from insulin resistance in skeletal muscle (15, 23). Early studies (3, 5, 47, 48) seemed to suggest that LCCoAs might be directly responsible for insulin resistance. However, few models have selectively altered LCCoAs without modifying DAG. In the mtGPAT null mouse liver, LCCoAs were significantly increased, whereas DAG and TG contents were reduced, and hepatic insulin sensitivity was improved, thereby dissociating elevated LCCoAs and insulin resistance (12). Intriguingly, Dgat2 knockdown also lowered liver DAG and TG content and improved hepatic insulin sensitivity despite similar hepatic LCCoA content compared with ASOctrl. The failure of intrahepatocellular DAG, a Dgat substrate, to increase following treatment with the Dgt2 ASO was

somewhat paradoxical and led to additional studies focused on elucidating the mechanism underlying this observation. Gene and protein expression analysis suggested that the lipogenic pathway was inhibited shortly after commencing Dgat2 ASO therapy. Fatty acids are well known to inhibit Srebp1c activity and expression (42, 43) and can also reduce transcriptional activation of lipogenic target genes by carbohydrate-response element-binding protein (49). The fact that mtGPAT mRNA expression was reduced in Dgat2 ASO-treated rats might explain, at least in part, the absence of an increase in LPA, DAG, and TG in the presence of increased plasma fatty acids, which are thought to be freely transported into the liver, and is con-

Suppressing DGAT2 Reverses Hepatic Insulin Resistance
Suppressing DGAT2 Reverses Hepatic Insulin Resistance

In summary, our study suggests that in this rat model of diet-induced NAFLD, targeting Dgat2, but not Dgat1, with antisense oligonucleotides successfully improves hepatic steatosis, hepatic insulin signaling, and in vivo hepatic insulin sensitivity. We also proposed a novel explanation for the paradoxical decrease in diacylglycerol concentrations in Dgat2 ASO-treated rats and provided further support for the notion that diacylglycerol accumulation and PKCe activation cause insulin resistance in NAFLD. It will be of great interest to see if alternative strategies for lowering hepatic diacylglycerol levels yield similar therapeutic results in patients with NAFLD and T2DM.

Acknowledgments—We thank Jianying Dong and Richard Lee for expert technical assistance with the studies and Aida Groszmann for performing the hormone assays.

REFERENCES


Schematic illustration of key elements of the triglyceride synthesis pathway and their modulation following Dgat 2 ASO therapy. Initially knocking down Dgat2 (step 1) increases plasma (and liver) fatty acids at early time points (step 2). As fatty acids, especially polyunsaturated fatty acids (PUFAs), are well known to inhibit liver X receptor α-mediated expression of SREBP1c (step 3), expression of SREBP1c and its target genes, mtGPAT, SCD1, and ACC1 was decreased (step 4), leading to reduced DAG content. Fatty acid accumulation also promotes fat oxidation by activating PPARα, a key transcriptional regulator of hepatic fat oxidation, CPT1, and UCP2 in rodents (5). This potentially accounts for the observed increase in genes involved in fat oxidation and the early increase in plasma ketones. PA, phosphatidic acid; LPA, lysophosphatidic acid; acyl-CoA, long chain acyl CoA; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; AGPAT, acyl-CoA:glycerol-sn-3-phosphate acyltransferase; GPAT, acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT2, acyl-CoA:diacylglycerol acyltransferase2; LXRs, liver X receptor α; CHREBP, carbohydrate-response element-binding protein, SCD1, stearoyl-CoA desaturase; ACC1, acetyl-CoA carboxylase 1; CPT1, carnitine palmitoyl transferase 1; UCP2, uncoupling protein 2.

SREBP1c
LXRs
Saturated FFA
Malonyl-CoA
ACSS
MUFAP
LPA
PA
PAP
DGAT2
SCD1
AGPAT
Acetyl-CoA
DGAT2
TG
VLDDL
G3-p
lysophosphatidic acid;
(PPUAs), are well known to inhibit liver X receptor...
Suppression of Diacylglycerol Acyltransferase-2 (DGAT2), but Not DGAT1, with Antisense Oligonucleotides Reverses Diet-induced Hepatic Steatosis and Insulin Resistance


doi: 10.1074/jbc.M704213200 originally published online May 27, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M704213200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 22 of which can be accessed free at http://www.jbc.org/content/282/31/22678.full.html#ref-list-1