Novel liver-specific TORC2 siRNA corrects hyperglycemia in rodent models of type 2 diabetes

Maziyar Saberi,1 David Bjelica,1 Simon Schenk,1 Takeshi Imamura,1 Gautam Bandyopadhyay,1 Pingping Li,1 Vasant Jadhar,2 Chandra Vargeese,2 Weimin Wang,2 Keith Bowman,2 Ye Zhang,2 Barry Polisky,2 and Jerrold M. Olefsky1

1Department of Medicine, University of California-San Diego, La Jolla; and 2Research and Development, Sirna Therapeutics, Inc., San Francisco, California

Submitted 12 March 2009; accepted in final form 18 August 2009


IN PATIENTS WITH TYPE 2 DIABETES, increased glucose output from the liver is a major cause of fasting hyperglycemia (5, 15, 20, 22, 26), and, consistent with this idea, the degree of fasting hyperglycemia is positively correlated to the magnitude of the increase in hepatic glucose production (HGP) (5, 15, 20, 33). Hepatic gluconeogenesis is a key component of HGP (30), and studies in fasted individuals using magnetic resonance spectroscopy and isotope dilution-based techniques suggest that increased gluconeogenesis is the major contributor to fasting hyperglycemia in type 2 diabetes (5, 22, 33). Excessive glucose output from the liver can also contribute to postprandial hyperglycemia (22, 30), which is due to a reduced ability of insulin to suppress HGP as well as impaired insulin-stimulated glucose uptake in insulin sensitive tissues (15, 26).

The cAMP-responsive element-binding protein (CREB) is a major positive transcriptional regulator of the gluconeogenic gene program (11). The ability of CREB to induce gluconeogenesis is dependent on the recently identified CREB coactivator transducer of regulated CREB activity 2 [TORC2, also known as CREB-regulated transcription coactivator 2 (CRTC2)] (3, 7, 8, 16). When hepatic CAMP levels are increased (e.g., by increased glucagon), TORC2 becomes dephosphorylated and translocates to the nucleus, where it coactivates CREB to induce transcription of the major gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (3, 7, 8, 16). When TORC2 is phosphorylated, it is localized to the cytosol, where it is transcriptionally inactive. Consistent with an important role for TORC2 in mediating gluconeogenesis, overexpression of hepatic TORC2 in mice leads to fasting hyperglycemia (16). Conversely, administration of an adenovirus expressing a short hairpin (sh)RNA against TORC2 leads to reduced expression of gluconeogenic genes (8, 16) and decreased plasma glucose levels.

Identification of therapeutic strategies to reduce gluconeogenesis and HGP could have importance in the treatment of type 2 diabetes. Small interfering (si)RNA technology is an attractive approach for treating liver-related abnormalities such as increased HGP, as siRNAs are naturally directed to the liver because of high rates of blood flow and the fenestrated endothelium within hepatic sinusoids (1, 28). Nonetheless, although gene silencing by siRNA interference has been a powerful tool for studying gene function over a broad range of in vitro applications, the use of siRNA technology to achieve systemic effects in vivo has been quite limited (1, 29). Reasons for these limitations include the relatively short biological half-life of siRNAs (they are rapidly degraded by RNAses once inside the cell) as well as the low uptake efficacy and lack of tissue-specific delivery (1, 29). To circumvent these issues, we recently designed a novel delivery system using chemically modified siRNAs in a lipid nanoparticle formulation (23). The modified siRNA/lipid nanoparticle is more stable in the circulation and results in more efficient transduction into targeted cells, where the chemically modified siRNA is relatively protected from degradation, yielding a prolonged biological half-life (23).

Accordingly, in the present study we exploited the known role of TORC2 as a major regulator of hepatic gluconeogenesis and coupled this with our recently described modified siRNA/lipid nanoparticle system to determine whether adequate and...
sustained TORC2 knockdown could be achieved by siRNA administration and whether this could correct hyperglycemia in high-fat diet (HFD)-fed, insulin-resistant mice and Zucker diabetic fatty (ZDF) rats. The results reveal that intravenous (iv) delivery of the TORC2 siRNA causes sustained knockdown of TORC2 for at least 11 days, in parallel with reduced expression of TORC2 gluconeogenic target genes, decreased HGP, reduced hyperglycemia, and improved hepatic and peripheral insulin sensitivity.

MATERIALS AND METHODS

Animals, Tissue Collection, and siRNA Administration

Eight-week-old male C57Bl6 mice were purchased from The Jackson Laboratory, and 10-wk-old ZDF rats were purchased from Charles River Laboratories. All animals were housed on a 12:12-h light-dark cycle. ZDF rats were fed a standard rodent chow (no. 5008; LabDiet, PMI Nutrition International). Mice were fed either standard rodent chow (5001; LabDiet, PMI Nutrition International) or an HFD with 60% of calories from fat (D12492, Research Diets). For all in vivo studies, mice and rats were injected once daily on three consecutive days (3 days in a row) with either siRNA against TORC2 (siTORC2, 3mg/kg) or a scrambled/control siRNA (siCON). Details on the formulation of the siRNA are presented below. Unless otherwise described, protein and qPCR measurements were performed on gastrocnemius muscle (mixed), adipose tissue (epidydimal), liver, and kidney from fasted (6 h) mice that had been on an HFD for 12 wk. Tissues were collected 3 days after the initial injection with siCON or siTORC2 (i.e., day 4). Tissue used for measurement of liver and skeletal muscle triglyceride and/or glycogen concentration was collected from fasted (6 h) mice 48 h after completion of the euglycemic-hyperinsulinemic clamp studies (i.e., 6 days after initial injection with siCON or siTORC2). For tissue collection, mice were anesthetized via intramuscular injection with a ketamine (80 mg/kg), acepromazine (0.5 mg/kg), and xylazine (16 mg/kg) cocktail. After excision, tissues were briefly rinsed in sterile 0.9% saline, blotted dry, frozen in liquid nitrogen, and stored at −80°C for later analysis. All experimental procedures were approved by the Animal Subjects Committee at UCSD according to NIH guidelines.

Oligonucleotide Synthesis and Characterization

All RNAs were synthesized at IRNA Therapeutics by standard procedures (34). Complementary strands were annealed in PBS, desalted, and lyophilized. The sequences and modified chemistry of the control siRNA (siCON) and siRNA against TORC2 (siTORC2) were as follows: siCON passenger strand: 5’-GcaGacGacGacGacGaCAACATTT-3’, siCON guide strand: 5’-ccuaGAGAGAguAAAGA-GAUU-3’; siTORC2 passenger strand: 5’-BgcCAnuGuaAACCAGuGGcGGuu-3’, siTORC2 guide strand: 5’-AAUcuGGuuAAcAuuGGGcUuU-3’. In the sequences above, lower-case characters indicate 2’ flumodified nucleotides, upper-case characters indicate deoxynucleotides, underlined characters indicate 2’-ome-modified nucleotides, and characters in boldface indicate ribonucleotides. The letter B indicates an inverted abasic residue.

Formulation of the siRNA

Lipid nanoparticles were made using the cationic lipid [2-(5-[3β-cholest-5-en-3yloxy]-3-oxapentan-1-oxyl)-3-dimethylamino-1-(cis, cis-9’,12’-octadecadienoyloxy)propane] (CplINDMA) or 2-[5’-[(chol5-en-3β-oxy)butyloxy]-3-dimethylamino-1-(cis,cis-9’,12’-octadecadienoyloxy)propane (CLINDMA), phospholipid [1,2-distearyl-sn-glycero-3-phosphocholine (DSPC)], cholesterol, peg lip [3,4-ditetracetoxybenzyl-α-methyl-polyleylene glycol ether (PEGDMG)], and linoleyl alcohol in 43.5:36.2:9.5:3.9:6.9 molar ratio. The siRNAs were incorporated in the lipid nanoparticles with high encapsulation efficiency by mixing siRNA in buffer into an alcoholic solution of the lipid mixture, followed by a stepwise dialfiltration process. DSPC and cholesterol were purchased from Northern Lipids. CINDMA and PEG-DMG were synthesized by siRNA Therapeutics. The encapsulation efficiency was determined using a size exclusion HPLC assay. The particle size and charge density measurements were performed using a Brookhaven ZetaPall particle sizer. The mean siRNA encapsulation efficiency was found to be 85 ± 2% for siTORC2 and siCON lipid nanoparticles. The mean particle size was 170 ± 10 and 167 ± 10 nm for the siTORC2 and siCON lipid nanoparticles, respectively, with polydispersity of 0.15 ± 0.05. All lipid nanoparticles had a positive surface charge density of 30 ± 2 mV.

5’ RACE Assay of Target RNA Cleavage

Ten-week-old C57Bl6 mice fed standard rodent chow were injected (3 mg/kg iv) once daily on three consecutive days with siCON or siTORC2. Livers were harvested from fasted (6 h) mice 3 days after the first injection (i.e., day 4). RACE (5’ rapid amplification of cDNA ends) analysis was done according to the GeneRacer Kit (Invitrogen) protocol, except without prior treatment of total RNA. Total liver RNA (5 μg) was ligated to the GeneRacer adaptor molecule, and ligated RNA was reverse transcribed using a TORC2 gene-specific primer (GSP1: 5’-cagacGCTGCGGGGAGGAGGAGAT) in a PCR amplification using primers complementary to the adaptor (GRS’ 5’-cGACTGCGAGGCGAGACACTGCA and TORC2 (GSP2: 5’-cGACGTCTGGGGGAGGAGGAGAGAT). The size of the cleaved product (142 bp) was further confirmed by nested PCR using primers (GRS’ nested 5’-cGACGTGCAATGGCGACGAGAATC and (GSP2: 5’-cGACTGCGAGGCGAGGAGGAGAT) and electrophoresis on native PAGE. The amplified product of 142 bp was gel purified, cloned, and sequenced to reveal the site of siRNA cleavage.

Transduction on siCON and siTORC2 into Cultured Hepatocytes

Sixty to seventy percent confluent mouse hepatocytes were incubated with siCON or siTORC2 (1 μg/ml siRNA against 5 × 10⁶ cells) for 16 h. After this, cells were washed three times in PBS and then incubated in MEM (Cellgro) for 72 h. Cells were then washed twice with PBS and were lysed, as previously described (14), for RNA or protein measurements.

Quantitative PCR

Total RNA from cultured mouse hepatocytes or livers was prepared using an RNA purification kit (RNeasy Plus, QIAGEN), according to the specifications of the manufacturer. First-strand cDNA was synthesized by SuperScript III reverse transcriptase and random hexamers (Invitrogen) and subjected to PCR amplification with gene-specific primers in the presence of SYBR Green (iTaq SYBR Green Supermix With ROX, Bio-Rad). The quantitative qPCR reaction was monitored and quantified using Opticon 3 software (Chromo4 Real-Time PCR Detection System, Bio-Rad). Relative abundance of mRNA was calculated after normalization to 18S ribosomal RNA. The following primer sequences were used for qPCR: G-6-Pase: forward 5’-cGauGAGAGAGaaGGGAAAT-3’; TORC2 (GSP1: 5’-cGACTGCGGGGAGGAGGAGAT) and (GSP2: 5’-cGACTGCGGGGAGGAGGAGAT). This was followed by PCR amplification using primers complementary to the adaptor (GRS’ 5’-cGACTGCGAGGCGAGACACTGCA and TORC2 (GSP2: 5’-cGACTGCGGGGAGGAGGAGAT). The size of the cleaved product (142 bp) was further confirmed by nested PCR using primers (GRS’ nested 5’-cGACGTGCAATGGCGACGAGAATC and (GSP2: 5’-cGACTGCGGGGAGGAGGAGAT) and electrophoresis on native PAGE. The amplified product of 142 bp was gel purified, cloned, and sequenced to reveal the site of siRNA cleavage.

Western blotting

Total cell lysates from livers or cultured mouse hepatocytes were analyzed for TORC2 protein (a gift from Dr. Marc Montminy, The Salk Institute) or β-tubulin (Santa Cruz Biotechnology) by SDS-PAGE and immunoblotting, as previously described (14).
Liver and Muscle Substrates

Tissue glycogen concentration was determined according to the method of Seifert et al. (31). Glycogen concentration was calculated using a pure glycogen standard as reference. Tissue triglyceride concentration was determined as previously described (2).

Metabolic Studies

Fed blood glucose concentration, insulin, glucose, and pyruvate tolerance tests, and hyperinsulinemic-euglycemic clamp studies. Fed blood glucose was measured at the same time each day. Intraperitoneal glucose (GTG: 1 g/kg ip), insulin (ITT: 0.5 U/kg ip), and pyruvate (PTT: 1 g/kg) tolerance tests were performed on 6-h-fasted mice. Hyperinsulinemic-euglycemic clamps were performed using 6 mU·kg⁻¹·min⁻¹, as previously described (12).

Metabolic studies in ZDF rats. For the rats studies the siCON and siTORC2 was administered iv via a chronically cannulated jugular vein, as previously described (21). After a 5-day recovery, rats were iv injected once daily with siCON on three consecutive days (days 3 to 5). On day 0, the same rats were then iv injected with siTORC2 (3 mg/kg). Fed blood glucose concentration was measured daily after beginning the study. On days 0 (3 days after the first siCON injection, and before injection with siTORC2), 4, 8, and 12, rats were fasted (10 h), and blood glucose concentration was measured. A blood sample (~75 μl) was also collected. These samples were centrifuged at 13,000 rpm for 5 min, and the plasma was stored at −80°C for measurement of plasma insulin (ELISA, ALPCO Diagnostics), fatty acid (colorimetric assay, Wako Chemicals), and lactate (glucose oxidize method, Yellow Springs Instruments) concentrations.

Statistics and Calculations

Hepatic glucose production was calculated as described previously (32). In the basal (i.e., fasted) state, the glucose disposal rate (GDR) equals HGP. During the insulin-stimulated (clamp), GDR is equal to the sum of HGP plus glucose infusion rate (GINF). Insulin-stimulated (IS-GDR) is equal to GDR minus basal HGP or the increase in glucose utilization in response to the clamp. Statistical differences in mRNA and protein expression, liver and skeletal muscle triglyceride and glycogen concentration, and percent suppression of HGP, GINF, and IS-GDR were detected by one-way ANOVA. Differences between siCON and siTORC2 in HGP during the basal and clamp periods and blood glucose, plasma insulin, plasma fatty acids, and plasma lactate concentrations during the ITT, GTT, and PTT or in temporal measurements were detected using two-way repeated-measures (treatment × time) ANOVA, with Tukey’s post hoc analysis where appropriate. Statistical analyses were performed using SigmaStat for Windows (v. 3.0.1a; Systat Software). Statistical significance was defined as P < 0.05. All results are presented as means ± SE.

RESULTS

RISC-Mediated Cleavage of TORC2 RNA by Modified siRNA

The specific modifications used to optimize the use of siRNA technology for in vivo applications have been described previously (23). Nevertheless, to directly demonstrate that our novel, chemically modified siRNA works through the proposed RNAi mechanism, we used the RACE method to detect RISC-mediated cleavage of the TORC2 RNA. RISC-mediated target cleavage is expected to occur precisely between the 10th and 11th position when measured from the 5’ end of the guide strand. Since the cleaved target fragment contains a 5-phosphate, it can be ligated with an adaptor sequence and amplified by adaptor and TORC2 specific primers. The size of amplified product depends on the location of PCR primers and target cleavage site. With the primers that we used, the expected size of the amplified product was 142 bp. Balb/C animals were injected (3 mg/kg iv) with the control siRNA (siCON) or a siRNA against TORC2 (siTORC2), which was encapsulated in our previously described lipid nanoparticle formulation (23). As shown in Fig. 1A, by use of RACE analysis on total liver RNA, the expected amplification product was observed in the siTORC2-treated mice, but not the siCON mice. PCR products were then subcloned and sequenced, confirming the correct junction between the adaptor sequence and the predicted cleavage site of the TORC2 siRNA (Fig. 1B). These results demonstrate that the siTORC2 works through an RNAi pathway by engaging to RISC to mediate specific RNA cleavage.

Chemically Modified siTORC2 Efficiently Knocks Down TORC2 and Decreases Gluconeogenic Enzyme Expression In Vitro and In Vivo

Treatment of primary C57bl6 mouse hepatocytes with siTORC2 resulted in an ~80% decrease in TORC2 protein (Fig. 2A) and a 70–80% decrease in G-6-Pase and PEPCK mRNA expression (Fig. 2B). Similarly, in vivo, iv siTORC2 administration to C57Bl6 mice reduced hepatic TORC2 mRNA and protein expression by 80% (Fig. 2C), and this decrease was sustained for at least 11 days (Fig. 2D). Hepatic
PEPCK and G-6-Pase mRNA expression was also reduced in siTORC2-treated mice by ~70% (Fig. 2E). In contrast, no decrease in TORC2, PEPCK, and G-6-Pase protein or mRNA was observed in the kidney, which can also provide systemic glucose via gluconeogenesis (Fig. 2F).

**TORC2 Knockdown Improves Glucose Homeostasis and Decreases In Vivo Gluconeogenic Capacity**

To investigate the effects of siTORC2 on systemic glucose metabolism, we placed C57Bl6 mice on an HFD for 12 wk, which resulted in fasting (Fig. 3A) and postprandial (Fig. 3B) hyperglycemia. siTORC2 treatment reduced blood glucose concentrations by ~50% under both fasted and postprandial conditions (Fig. 3, A and B), whereas siCON was without effect.

We next performed a PTT to determine whether the normalization of glucose concentration in siTORC2-treated mice was due to changes in gluconeogenic capacity. Indeed, HFD-fed mice treated with siTORC2 had a marked reduction in the glucose excursion during the PTT compared with siCON mice on HFD, confirming reduced in vivo gluconeogenic capacity (Fig. 3C).

We confirmed the effectiveness of siTORC2 treatment by testing a second independent siRNA against TORC2. Similarly to the original siTORC2, the siTORC2B decreased TORC2 protein expression in the liver by ~80% (data not shown) and resulted in a comparable reduction in fasting blood glucose concentration in HFD-fed mice (Fig. 3D).

The effects of the siTORC2 were specific to the liver, as we found no decrease in TORC2, PEPCK, or G-6-Pase protein or mRNA expression in kidney (Fig. 2F), skeletal muscle, or adipose tissue (data not shown).

**siTORC2 Does Not Cause Hepatotoxicity or Inflammation**

One concern when unmodified siRNAs are being used in vivo is induction of inflammation and hepatotoxicity (1, 23, 29). Previous work has shown that treatment of mice with these modified siRNA preparations did not increase serum IFNα, or the inflammatory cytokines IL-6 and TNFα (23). Compared with HFD controls, we also found no increases in serum bilirubin (0.33 ± 0.06 μM), alkaline phosphatase (30.2 ± 2 IU), aspartate aminotransferase (114 ± 26 IU), or alanine aminotransferase levels (20.5 ± 2.5 IU), and lymphopenia, thrombocytopenia, or piloerection were not observed. Consistent with these observations, histopathology analysis revealed no evidence of hepatocyte vacuolization, necrosis, or elevation in neutrophil cell content.

**Glucose Homeostasis and Insulin Sensitivity in TORC2 siRNA-Treated Mice**

To assess systemic glucose homeostasis we performed GTTs in the control and treated HFD mice. Whole body glucose metabolism was improved by siTORC2 treatment, as evidenced by the reduced glucose (Fig. 3E) and insulin (Fig. 3F) responses during the GTT. These changes in glucose homeostasis were sustained throughout the 11-day study period and were not due to changes in body weight, as neither siTORC2 or siCON treatment affected body weight (Fig. 3G).

We also administered siTORC2 to chow fed mice, and, as can
be seen in Fig. 3, H and I, this led to a significant lowering of glycemic excursions during both GTTs and PTTs. Thus, even in the absence of insulin resistance and abnormal glucose tolerance, as in the HFD mice, siTORC2 still showed glucose lowering effects, although not nearly as great as in the HFD mice.

To gain insight into the effects of siTORC2 treatment on HGP and hepatic insulin sensitivity, we performed hyperinsulinemic-euglycemic clamp experiments. In the fasted state (6 h), basal glucose (109 ± 10 vs. 209 ± 10 mg/dl, siTORC2 vs. siCON, P < 0.05) and insulin (1.6 ± 0.4 vs. 3.2 ± 0.6 ng/ml, siTORC2 vs. siCON, P < 0.05) concentrations were lower in siTORC2 compared with siCON mice, whereas body weight (6-h-fasted) was the same between the two groups (39.9 ± 0.1 vs. 39.9 ± 0.01 g, siCON vs. siTORC2, P > 0.05). Importantly, the decrease in fasting blood glucose concentration was associated with a marked reduction (~45%) in HGP (Fig. 4A). In human type 2 diabetes, there is a well-established direct correlation between basal HGP and the magnitude of the fasting hyperglycemia (9, 10, 15, 30). Similarly, we observed a highly significant positive correlation between basal HGP and fasting blood glucose levels in siCON- and siTORC2-treated mice (R² = 0.61, P < 0.05; Fig. 4B). This indicates that the siTORC2-mediated decrease in hepatic gluconeogenesis...
resulted in reduced blood glucose concentrations via a proportionate reduction in HGP. To our knowledge, these are the first direct in vivo results demonstrating that knockdown of TORC2 leads to a decrease in HGP.

During the hyperinsulinemic-euglycemic clamp study, the steady-state insulin concentrations (11.6 ± 1.5 vs. 14.8 ± 2.7 ng/ml, siCON vs. siTORC2, P > 0.05) were not different between the two groups. The exogenous GINF required to maintain euglycemia was ~25% higher in siTORC2 mice, indicating enhanced whole body insulin sensitivity (P < 0.05; Fig. 4C). This improvement in GINF was due, in part, to an increase in hepatic insulin sensitivity, as indicated by the lower HGP during the clamp (P < 0.05; Fig. 4A) and the greater insulin-mediated suppression of HGP in siTORC2 mice (P < 0.001; Fig. 4D). This finding indicates that not only was basal gluconeogenesis reduced in the HFD-fed siTORC2 mice, but hepatic insulin sensitivity was also improved in these mice. Hepatic steatosis is a characteristic feature of HFD-fed mice and other insulin-resistant/diabetic states (13, 24, 25), and, this abnormality was prevented in HFD-fed siTORC2 mice (Fig. 4E). The reduction in hepatic triglyceride concentration could have contributed to the improved hepatic insulin sensitivity, and it is possible that decreased gluconeogenesis in siTORC2 mice resulted in an increased reliance on hepatic triglyceride stores for energy production.

Liver glycogen concentration was also lower in siTORC2 mice (Fig. 4F), perhaps due to a greater reliance on liver glycogen to maintain HGP, in the face of reduced gluconeogenesis. Importantly, this lower glycogen store and reduction in gluconeogenic capacity did not compromise the ability of these mice to increase HGP when blood glucose levels were rapidly decreased. This is demonstrated by the fact that the siTORC2 mice did not become markedly hypoglycemic during an ITT and displayed a normal increase in blood glucose after an insulin injection (Fig. 4G). Another potential adverse effect of inhibiting gluconeogenesis is that this might have led to
lactate accumulation as a result of diverting intrahepatic carbon flow from glucose to lactate. However, we found that fasting plasma lactate concentrations were significantly lower in siTORC2 vs. siCON mice (0.85 ± 0.52 vs. 1.54 ± 0.63 mmol/l, siTORC2 vs. siCON, P < 0.05), perhaps due to increased utilization of lactate for hepatic energy production via the TCA cycle (Fig. 4H).

**Peripheral Insulin Sensitivity**

IS-GDR, a measure of skeletal muscle insulin sensitivity, was also significantly greater in the siTORC2 mice (P < 0.05; Fig. 5A).

Complementing the improvement in skeletal muscle insulin action, intracellular triglyceride concentration was lower in skeletal muscle of siTORC2 mice (P < 0.05; Fig. 5B). Since skeletal muscle TORC2 was not reduced in siTORC2 mice (data not shown), the enhanced skeletal muscle insulin action was most likely an indirect effect. It is well known that hyperglycemia and elevated fatty acid levels can cause insulin resistance (27); therefore, the normalization of these factors in the TORC2 knockdown mice would be expected to secondarily improve peripheral insulin sensitivity.

**Hyperglycemia in the ZDF Rat Is Improved by siTORC2**

The ZDF rat is a well-described model of type 2 diabetes that develops chronic fasting and postprandial hyperglycemia (4). When these rats were treated with siTORC2, the fed (Fig. 6A) and fasted (Fig. 6B) blood glucose concentrations were significantly reduced by ~50% (P < 0.05), and this reduction was maintained for the 23-day study period (Fig. 6A). When ZDF rats develop hyperglycemia, a consistent feature is pancreatic β-cell failure. Whereas fasting insulin levels were unchanged by siTORC2 treatment (Fig. 6C), plasma insulin concentrations in the fed state were increased (Fig. 6C). This is consistent with improved β-cell function, which might be due to a decrease in glucotoxicity and/or lipotoxicity. siTORC2 treatment in ZDF rats also resulted in a significant reduction in plasma fatty acid levels in both the fasted and fed states, suggesting improved adipose tissue insulin sensitivity (P < 0.05; Fig. 6D).
DISCUSSION

Since increased HGP, due to uncontrolled gluconeogenesis, is a primary contributor to chronic hyperglycemia in type 2 diabetes (5, 15, 22, 33), we utilized a novel lipid nanoparticle system to deliver a chemically modified siRNA against TORC2, a co-activator that regulates gluconeogenesis (3, 7, 8, 16). The major advance in the present studies is to harness previously established molecular knowledge about TORC2 transcriptional control of gluconeogenesis (3, 7, 8, 11, 16) to the emerging technology of chemically modified siRNAs in order to achieve a sustained, liver-targeted intervention in rodent models of diabetes. To our knowledge, this is the first successful use of this strategy in this important metabolic disorder. Our results demonstrate that the lipid nanoparticle system for delivering the siTORC2 is highly effective and specific at depleting hepatic TORC2, both in vitro and in vivo, resulting in a dramatic decrease in the gluconeogenic target genes PEPCK and G-6-Pase for up to 10–21 days. Corresponding to these changes, siRNA treatment of HFD-fed mice resulted in a remarkable reduction in fasting and fed glucose levels, improved glucose tolerance, and enhanced hepatic insulin sensitivity. Consistent with previous human and animal studies (9, 10, 15, 30), the reduction in fasting hyperglycemia in siTORC2 mice was highly correlated with a reduction in basal HGP. Interestingly, siTORC2 treatment of ZDF rats also caused a large reduction in fasting and fed glucose concentrations and appeared to preserve pancreatic β-cell function.

Recently, the transcription factor TORC2 has been demonstrated as a key control point for hepatic gluconeogenesis (3, 7, 8, 11, 16). TORC2 regulates the expression of the key gluconeogenic enzymes G-6-Pase and PEPCK (3, 7, 8, 11, 16), and in vivo studies in mice have demonstrated that adenoviral-mediated knockdown of TORC2 results in a significant reduction in the expression of G-6-Pase and PEPCK (7, 8, 17). As expected, our TORC-siRNA led to highly efficient TORC2 knockdown in a liver-specific manner. In contrast to previous studies, however, the current system allows for sustained knockdown of TORC2, for as long as 11 days in mice and 21 days in rats, after initial treatment. This is a much longer duration of knockdown of a target gene compared with traditional siRNA treatments using naked siRNA, in which knockdown of a target gene is sustained for a matter of hours (1, 23, 29). Treatment with our siRNA did not require repeated injections and used significantly lower doses to sustain physiologically relevant knockdown. The primary reason for this prolonged efficiency is related to the structure of the modified siRNA, which renders it relatively resistant to intracellular degradation (23). The siRNA is encapsulated in a stable nucleic acid-lipid particle (SNALP) consisting of a lipid bilayer that contains a mixture of cationic and fusogenic lipids, resulting in more efficient cellular uptake and endosomal release of the particle’s siRNA payload (23). The SNALP is also coated with a diffusible polyethylene glycol-lipid conjugate that provides a neutral, hydrophilic exterior and stabilizes the particle during formulation (23). Last, the surface coating shields the cationic bilayer in vivo, preventing rapid systemic clearance (23). All together, we demonstrate that a lipid nanoparticle delivery system can be used to deliver a chemically modified siRNA in a liver-specific manner, resulting in large reductions in systemic hyperglycemia. Given the beneficial effects of siTORC2 on glycemia, it is possible to envision future therapeutic applications of this approach. However, with current methodologies, these siRNA preparations can only be administered intravenously or intraperitoneally, and this is not a practical route of delivery for treatment of a chronic disease such as type 2 diabetes. To achieve clinical applicability, it will most likely be necessary to develop a delivery method that allows subcutaneous or even oral administration. Given the long biological half-life of the TORC2 siRNA, a subcutaneous route of delivery could quite feasible, since periodic injections readily translate to the clinical environment.

Given the importance of gluconeogenesis in contributing to HGP, one potential pitfall of TORC2 knockdown could be the development of overt hypoglycemia. Interestingly, however, we found that a 6-h fast of sTORC2-treated animals (comparable to ~20 h fast in humans) did not result in overt hypoglycemia. Also, fasted sTORC2-treated mice did not develop marked hypoglycemia during the ITT and showed an adequate rebound of glucose values to normal after the insulin injection. This is an important observation, because it indicates that the counterregulatory mechanisms responsible for maintaining euglycemia were not compromised in sTORC2-treated mice. Although the reasons for the lack of hypoglycemia in sTORC2 animals are not completely clear, several explanations are possible. First, sTORC2 treatment did not completely deplete TORC2, and it is possible that the remaining TORC2, G-6-Pase, and PEPCK were sufficient to provide enough glucose via gluconeogenesis to prevent hypoglycemia. In addition, other transcription factors, such as FOX01 and hepatocyte nuclear factor 1a regulate gluconeogenesis and might compensate for TORC2 deficiency under hypoglycemic conditions. It is also possible that there was increased contribution of extrahepatic renal glucose production in sTORC2 mice. Last, it is possible that the liver relied more on lactate for intrahepatic carbohydrate metabolism, conserving glycogen for HGP, since lactate levels were significantly reduced in sTORC2 mice. It should be noted that, during prolonged bouts of physical exercise, when glycogen stores become depleted, hepatic gluconeogenesis becomes an important factor in maintaining plasma glucose levels. Therefore, in the context of prolonged physical exercise, it is possible that inhibition of gluconeogenesis could lead to an unwanted and exaggerated decline in blood glucose levels. Thus, it will be of importance to assess the interaction between inhibition of gluconeogenesis and physical exercise regimens in animals and, eventually, in humans.

The improvement in peripheral insulin sensitivity is also noteworthy. Thus, TORC2 knockdown resulted in a significant increase in liver, skeletal muscle, and adipose tissue insulin sensitivity. Given that the biodistribution of TORC2 siRNA exclusively targeted the liver and was without effect on TORC2 levels in adipose tissue or skeletal muscle, it is likely that the enhanced peripheral insulin sensitivity represents an indirect effect, secondary to the hepatic effects of TORC2 knockdown, to improve overall glucose homeostasis. For example, it is well known that hyperglycemia and elevated fatty acid levels can exacerbate insulin resistance (18, 27). Therefore, the normalization of glycemia and fatty acid levels in the TORC2 knockdown mice could attenuate peripheral insulin resistance by alleviating gluco toxicity and lipotoxicity. Consistent with the improved skeletal muscle...
insulin sensitivity, we also found that the HFD-induced increase in skeletal muscle triglyceride content, which has been associated with insulin resistance (19), was reduced in the TORC2 knockout group. Similarly, accumulation of liver triglycerides is associated with hepatic insulin resistance (6), and liver triglyceride concentration was lower in TORC-siRNA-treated mice.

In summary, in type 2 diabetes, increased hepatic gluconeogenesis is the major cause of fasting hyperglycemia and is also an important contributor to postprandial hyperglycemia. Accordingly, inhibition of hepatic gluconeogenesis has been a goal of antidiabetic therapies, albeit an elusive one. Here, we show that intravenous delivery of a novel, chemically modified siRNA directed against TORC2, a major positive regulator of the gluconeogenic program, leads to a marked and relatively long-lasting reduction in in vivo gluconeogenesis. This results in decreased hepatic glucose production rates with a subsequent striking improvement in hyperglycemia and hepatic insulin resistance in rodent models of type 2 diabetes/insulin resistance. If translatable to human disease, these results hold promise for the therapeutic use of siRNAs to treat hyperglycemia and type 2 diabetes.

ACKNOWLEDGMENTS

We thank Carlos Castorena, Anh-Khoi Nguyen, and Arezou Amidi from UCSD for assistance with animal maintenance and ITT, GTT, and PTT measurements.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants DK-033651 (J. M. Olefsky) and DK-063491 (J. M. Olefsky), and a grant from Sirna Therapeutics (J. M. Olefsky). M. Saberi was supported through an NIDDK Training Grant (5 T32 DK-007494-24), and S. Schenk is supported by a Mentor-Based Postdoctoral Fellowship from the American Diabetes Association.

REFERENCES

2. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM. Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects is associated with insulin resistance (19), was reduced in the TORC-siRNA-treated mice.
7. Huang J, Usui I, Satoh H, Bevery J, Olefsky JM.
16. Magnusson I, Rothman DL, Katz LD, Shulman RG, Shulman GI.
26. Olefsky JM, Courtney CH.
28. Poitout V, Robertson RP.
30. Rondinone CM.
32. Rondinone CM.


