Cell Metabolism
Short Article

Prevention of Hepatic Steatosis and Hepatic Insulin Resistance by Knockdown of cAMP Response Element-Binding Protein

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SUMMARY

In patients with poorly controlled type 2 diabetes mellitus (T2DM), hepatic insulin resistance and increased gluconeogenesis contribute to fasting and postprandial hyperglycemia. Since cAMP response element-binding protein (CREB) is a key regulator of gluconeogenic gene expression, we hypothesized that decreasing hepatic CREB expression would reduce fasting hyperglycemia in rodent models of T2DM. In order to test this hypothesis, we used a CREB-specific antisense oligonucleotide (ASO) to knock down CREB expression in liver. CREB ASO treatment dramatically reduced fasting plasma glucose concentrations in ZDF rats, *ob/ob* mice, and an STZ-treated, high-fat-fed rat model of T2DM. Surprisingly, CREB ASO treatment also decreased plasma cholesterol and triglyceride concentrations, as well as hepatic triglyceride content, due to decreases in hepatic lipogenesis. These results suggest that CREB is an attractive therapeutic target for correcting both hepatic insulin resistance and dyslipidemia associated with nonalcoholic fatty liver disease (NAFLD) and T2DM.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is strongly associated with hepatic insulin resistance in patients with poorly controlled type 2 diabetes mellitus (T2DM) (Petersen et al., 2005; Yki-Jarvinen, 2005). In contrast to our understanding of the mechanism of fat-induced hepatic insulin resistance, the pathogenesis of increased hepatic gluconeogenesis in T2DM is less well understood. Progressive declines in insulin secretion as well as inappropriate increases in glucagon secretion have both been viewed as critical factors responsible for increased rates of hepatic gluconeogenesis (Cherrington et al., 1987; Reaven et al., 1987; Unger and Orci, 1977). Glucagon signals through a canonical G protein-coupled receptor, leading to the activation of cAMP-dependent protein kinase A (PKA) (Jelinek et al., 1993). Consequently, PKA phosphorylates Ser133 on cAMP response element-binding protein (CREB) and causes subsequent translocation to the nucleus (Gonzalez and Montminy, 1989). CREB is a leucine BH/zipper transcription factor that promotes gene transcription by binding to conserved sequences known as a cAMP-responsive element (CRE) (Mayr and Montminy, 2001). CREB is a well-known activator of gluconeogenic gene transcription through CREB-binding sites located on promoter regions of glucose-6-phosphatase (G6Pase), FBPase, and phosphoenolpyruvate carboxykinase (PEPCK). Insulin antagonizes the induction of gluconeogenic enzymes by phosphorylating CREB-binding protein (CBP) (He et al., 2009; Zhou et al., 2004) and transducer of regulated CREB activity 2 (TORC2) (Koo et al., 2005). Thus, CREB is a key nexus where insulin and glucagon signaling converge on the regulation of hepatic gluconeogenesis.

In the present study, we examined the hypothesis that knockdown of CREB would blunt the transcriptional actions of glucagon signaling and improve hepatic glucose metabolism in diabetic rodent models associated with NAFLD and hepatic insulin resistance. In order to assess this hypothesis, we knocked down the expression of CREB in liver and adipose tissue of four different insulin-resistant rodent models using an antisense oligonucleotide (ASO) (Samuel et al., 2009). The major advantage of this approach is that it is possible to decrease specific gene expression in a normal adult animal and avoid the confounding compensatory developmental effects often associated with gene-knockout mouse models.

RESULTS

CREB ASO Decreases Plasma Insulin and Glucose

To achieve a model of mild diabetes, we used an initial streptozotocin (STZ) injection coadministered with a low dose of nicotinamide. The rats were fed a high-fat diet to induce insulin resistance. This rat model mimics the pathophysiology of hyperglycemia seen in patients with T2DM (Samuel et al., 2009).

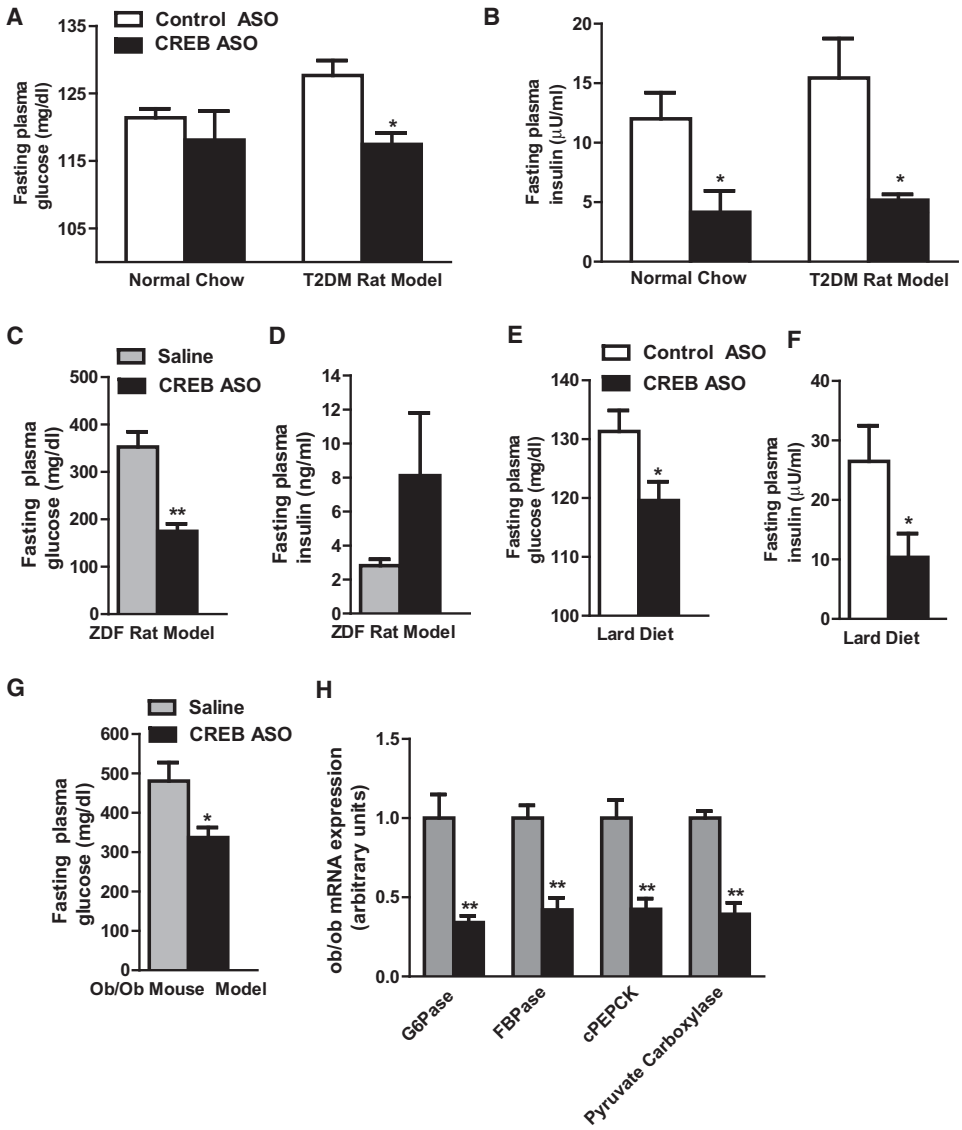

Figure 1. CREB ASO Improves Metabolic Parameters in Three Other Diabetic Models

(A–H) Fasting plasma glucose (A) and insulin (B) concentrations (n = 8–21) in the normal chow and T2DM rat models. Fasting plasma glucose concentrations (C) were decreased with CREB ASO in the ZDF rat model (n = 4–5). Due to the pancreas-saving effect of lower glucose values, plasma insulin values tended to be increased (D) (n = 4–5). In the lard-diet-based model, CREB ASO reduced fasting plasma glucose (E) and insulin (F) concentrations (n = 7–9). Lastly, CREB ASO also reduced fasting plasma glucose in an *ob/ob* mouse model (G) due to reductions in gluconeogenic genes (H) (n = 4–5). *$p < 0.05$, **$p < 0.005$. All values are expressed as the average SEM.

Fasting (T2DM: 129 ± 4 mg/dl versus normal: 111 ± 3 mg/dl, $p < 0.05$) and peak (T2DM: 547 ± 24 mg/dl versus normal: 321 ± 75 mg/dl, $p < 0.01$) glucose concentrations during an intravenous glucose tolerance test were increased in the T2DM rat model compared to normal rats fed a standard rodent chow. Despite their marked hyperglycemia following the intravenous glucose tolerance test, there was no compensatory hyperinsulinemia as compared to the normal rats (T2DM: 2626 ± 833 μU-min/ml versus normal: 2280 ± 372 μU-min/ml, p = NS), which is similar to what is observed in patients with T2DM (Samuel et al., 2009).

CREB ASO reduced both CREB mRNA and protein levels in liver (Figures S1A and S1B) and WAT but not muscle (Table S2). In normal rats fed a standard rodent chow, CREB ASO treatment had no effect on fasting plasma glucose concentrations (Figure 1A) but increased whole-body insulin sensitivity, as reflected by a 65% reduction in fasting plasma insulin concentrations (Figure 1B). In T2DM rats, CREB ASO treatment decreased fasting plasma glucose concentrations compared to the control ASO (Figure 1A). Consistent with our findings in normal rats, CREB ASO treatment also improved whole-body insulin sensitivity in this T2DM rat model, as reflected by a 66% reduction in fasting plasma insulin concentrations (Figure 1B). Fasting plasma glucagon concentrations were similar in both ASO-treated groups (Table S1). Among the gluconeogenic enzymes and their key cofactors—cytosolic PEPCK, mitochondrial PEPCK,

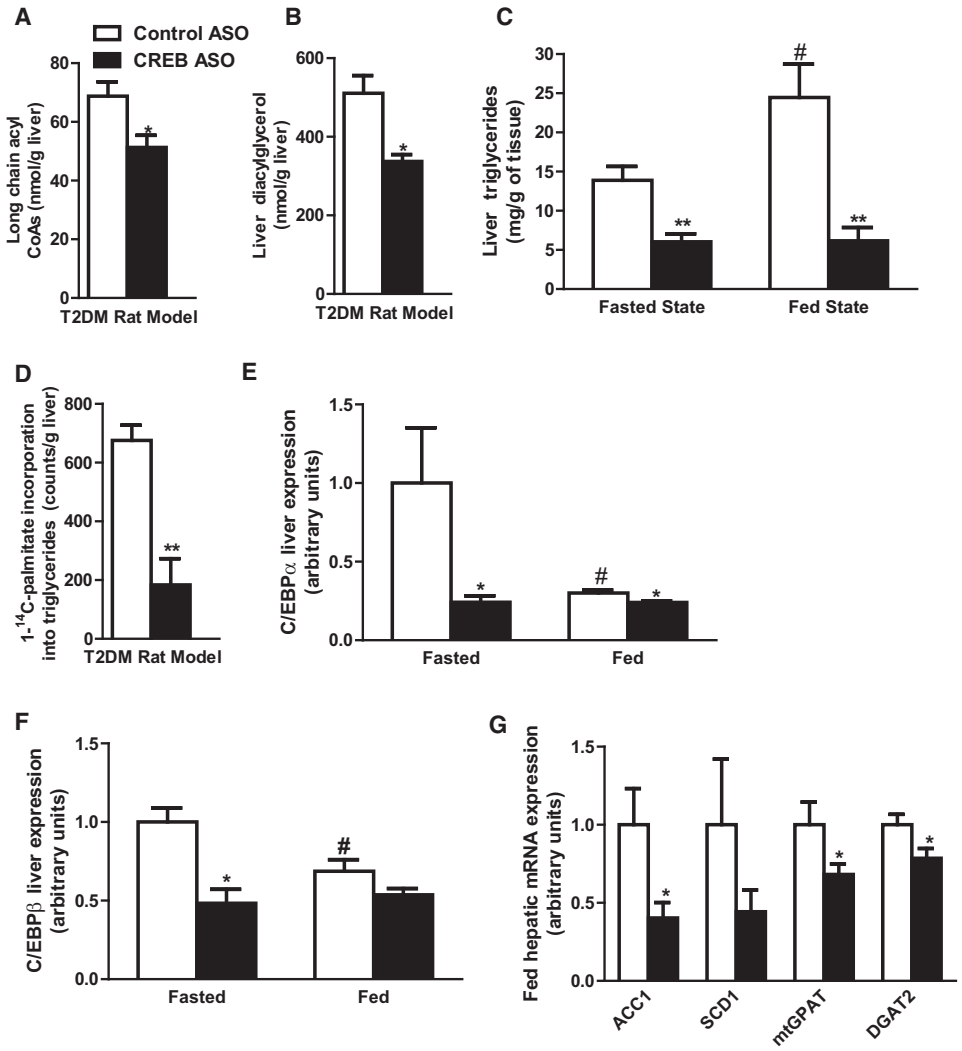

Figure 2. CREB ASO Treatment Reduces Hepatic Lipid Content

(A–G) Long-chain acyl-CoAs (A) and liver DAGs (B) were decreased with CREB ASO treatment (n = 8–10). Liver triglycerides were reduced in the fasted and fed states with CREB ASO (C). In vivo lipogenesis was reduced with CREB ASO, as assessed by the incorporation of [1-^{14}C]palmitate into triglycerides (n = 4 per group) (D). C/EBPα (E), C/EBPβ (F), and key lipogenic genes (G) were assessed by RT-PCR (n = 4–7). *p < 0.05, **p < 0.005 for control ASO versus CREB ASO; # p < 0.05 for control ASO-fasted versus control ASO-fed. All values are expressed as the average SEM.

and the transcriptional coactivator peroxisomal proliferator-activated receptor gamma coactivator-1α (PGC-1α)—mRNA were decreased by 43%, 55%, and 54%, respectively, in the liver of the CREB ASO group (Table S2). Interestingly, there was no observed difference in catalytic G6Pase and hepatocyte nuclear factor-4α (HNF-4α), even though the promoter sequences encoding these genes contain known CREB-binding sites (Table S2). These data suggest that other transcriptional regulatory factors independently regulate G6Pase expression.

To verify the results found with CREB ASO in the T2DM rat model, we used three other models of diabetes. CREB ASO reduced fasting glucose concentrations by 178 mg/dl in Zucker Diabetic Fatty (ZDF) rats (Figure 1C), with significant reductions of CREB in liver but not WAT (Figures S1C and S1D). Plasma insulin values tended to be increased due to pancreatic sparing from lower plasma glucose values (Figure 1D) (Harmon et al., 2001). To complement the studies in our T2DM rat model, we substituted the safflower oil with a lard-based diet that is high in hydrogenated fatty acids. Consistent with the other insulin-resistant rodent models, CREB ASO reversed insulin resistance in these lard-fed rats, as reflected by decreased fasting plasma glucose (Figure 1E) and insulin concentrations (Figure 1F). In addition to the above-mentioned rat models, CREB ASO also dramatically reduced plasma glucose concentrations by 144 mg/dl in *ob/ob* mice (Figure 1G), which was associated with ~60% reductions in G6Pase, FBPase, cPEPCK, and pyruvate carboxylase mRNA expression (Figure 1H).

CREB ASO Reduces Hepatic Lipid Content in the T2DM Rat Model

Intrahepatic long-chain coenzymes A (CoAs) and diacylglycerols (DAGs) were both lowered with CREB ASO treatment (Figures 2A

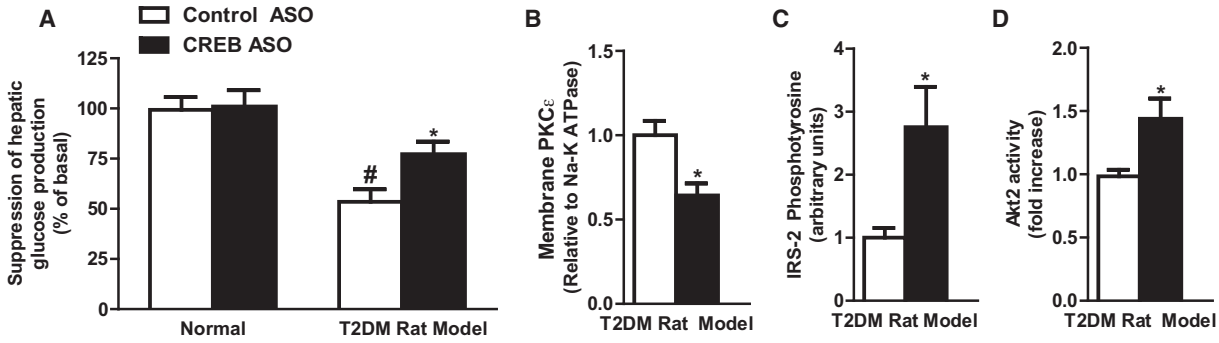

Figure 3. CREB ASO Treatment Improves Hepatic Insulin Sensitivity in T2DM Rats

(A–D) Suppression of hepatic glucose production (A) was assessed using the hyperinsulinemic-euglycemic clamp (n = 4–14 per treatment group). In the T2DM rat model, CREB ASO reduced membrane PKCε (B) (n = 4 per treatment group), increased IRS-2 tyrosine phosphorylation (C) (n = 9 per treatment group), and improved Akt2 activity (D) (n = 5 per treatment group). *p < 0.05 for CREB ASO versus control ASO; # p < 0.05 for T2DM control ASO versus normal chow control ASO. All values are expressed as the average SEM.

and 2B). CREB ASO reduced liver triglyceride content by 57% in the fasted state. After a meal enriched with fat, liver triglycerides increased by 76% in the control ASO-treated rats compared to the fasted state, but did not change in the CREB ASO group (Figure 2C). To test if in vivo lipogenesis was decreased, we gavaged control and CREB ASO T2DM rats with whipping cream enriched with [1-^{14}C]palmitate to examine the rate of incorporation of labeled fatty acids into triglycerides. There was a 73% reduction in the radioactive counts found in hepatic triglycerides with CREB ASO, reflecting decreased hepatic lipogenesis, specifically re-esterification (Figure 2D). Both C/EBPα and C/EBPβ have been implicated in the regulation of lipogenesis (Matsusue et al., 2004; Pedersen et al., 2007; Qiao et al., 2006; Rahman et al., 2007; Schroeder-Gloeckler et al., 2007) and have CREB-binding sites when we analyzed the promoter sequences of these genes (Niehof et al., 1997). We examined hepatic expression of both of these proteins and found them to be dramatically reduced with CREB ASO (Figures 2E and 2F). Consistent with these observations, DGAT2 and mtGPAT mRNA expression were also decreased, which could explain the decreased hepatic lipogenesis (Figure 2G).

CREB ASO Increases Hepatic Insulin Sensitivity and Insulin Signaling

We assessed the effects of decreased liver and adipose CREB expression on hepatic and peripheral insulin sensitivity using hyperinsulinemic-euglycemic clamps (Samuel et al., 2007). Basal hepatic glucose production (Figure S1E) and insulin-stimulated peripheral glucose uptake rates (Figure S1F) were similar in both rat models and ASO groups. In contrast, hepatic insulin responsiveness was markedly increased in the CREB ASO-treated T2DM rat model, as reflected by a 45% increase in suppression of hepatic glucose production during the clamp compared to the control ASO-treated rats (Figure 3A). Additionally, CREB ASO increased hepatic responsiveness in the lard-fed rats (Figure S1G). We have previously shown that NAFLD is associated with accumulation of intracellular DAG content, leading to increased activation of PKCε and subsequent reduction in insulin signaling at the level of insulin receptor substrate 2 (IRS-2) tyrosine phosphorylation (Samuel et al., 2004, 2007). Consistent with these studies, we found that CREB ASO treatment reduced hepatic DAG content, which was associated with lower PKCε membrane translocation in liver (Figure 3B). These changes were associated with improved insulin signaling, as reflected by increased insulin-stimulated IRS-2 tyrosine phosphorylation (Figure 3C) and Akt2 activity (Figure 3D). Our results are in contrast to previous studies that used a dominant-negative polypeptide A-CREB or Ad-CREB RNAi to knock down the expression of CREB in primary rat hepatocytes (Canettieri et al., 2005). While these studies found that it resulted in reduced expression of IRS-2 (Canettieri et al., 2005), we found no differences in IRS-2 expression (Table S2) in the CREB ASO-treated rats compared to the control ASO-treated rats. Regulation of IRS-2 transcription is complicated and dependent on the fed and fasted state and multiple transcription factors, which may explain these discrepancies.

CREB ASO Lowered Plasma Triglycerides and Cholesterol

CREB ASO treatment decreased plasma triglycerides by 24% in the T2DM rats (Figure 4A). Total plasma cholesterol and HDL cholesterol were also decreased in rats treated with CREB ASO (Table S1). FPLC analysis revealed that the decreases in total plasma cholesterol could be attributed to significant decreases in VLDL, LDL, and HDL cholesterol (Figure 4B). Hepatic free cholesterol was unaltered (Figure 4C), but CREB ASO decreased hepatic cholesterol esters, as assessed by nuclear magnetic resonance (NMR) (Figure 4D). Genes relating to cholesterol synthesis, sterol regulatory element-binding protein 2 (SREBP-2), and hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) were increased by 50%–60% with CREB ASO treatment (Table S2). We measured the rate of sterol synthesis in primary rat hepatocytes and found no effect of CREB ASO treatment on the incorporation rate of [1-^{14}C]acetic acid into cholesterol (Figure 4E). Additionally, expression of the low-density lipoprotein receptor (LDLR) and scavenger receptor class B type 1 (SR-B1) were unaltered with CREB ASO treatment, suggesting that alterations in cholesterol uptake were not the cause of reduced plasma cholesterol concentrations (Table S2). Lastly, we looked at the rate of bile acid synthesis by quantifying the expression of

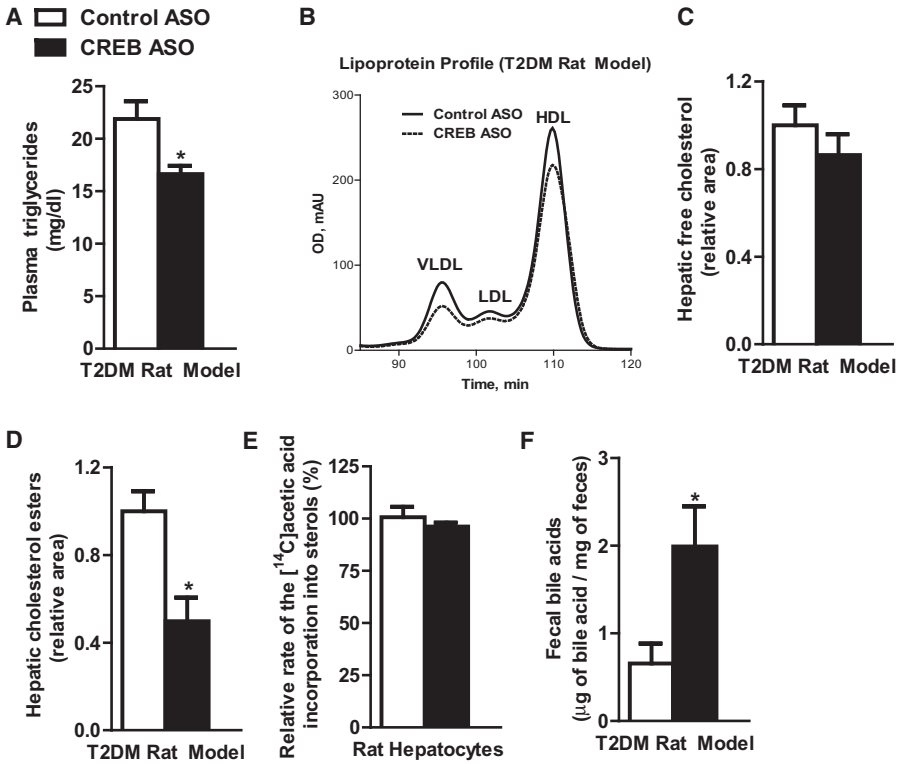

Figure 4. CREB ASO Therapy Decreases Plasma Triglycerides and Cholesterol

(A–F) Plasma triglycerides were decreased with CREB ASO (n = 10–12) (A). The lipoprotein profile showed significant reductions in VLDL, LDL, and HDL with CREB ASO (B). Free hepatic cholesterol was unchanged with CREB ASO (n = 4 per treatment group) (C), but CREB ASO decreased the concentration of hepatic cholesterol esters (D), as assessed by NMR. Synthesis of cholesterol in rat hepatocytes was assessed by relative [1-^{14}C]acetic acid incorporation into sterols (E) (n = 3–7 per treatment group). Amount of bile acids extracted from feces during a 12 hr fast is shown in (F) (n = 7 per treatment group). *$p < 0.05$ for control ASO versus CREB ASO. All values are expressed as the average SEM.

cholesterol 7 alpha-hydroxylase (Cyp7A1). CREB ASO treatment resulted in an approximately 2-fold increase in Cyp7A1 expression (Table S2). Consistent with the increased expression of Cyp7A1, fecal bile content was approximately 3-fold higher compared to control ASO-treated rats (Figure 4F). This suggests that increased cholesterol export into fecal bile is the most likely explanation for the reduced plasma cholesterol concentrations in the CREB ASO-treated rats.

DISCUSSION

These studies found that decreasing hepatic expression of CREB dramatically improved fasting plasma glucose and insulin concentrations in three different insulin-resistant rodent models. In addition, CREB inhibition improved hepatic insulin sensitivity and reduced plasma glucose concentrations in a T2DM rat model associated with fatty liver and hepatic insulin resistance. We found that decreased CREB expression improves hepatic insulin responsiveness by two mechanisms. First, decreased expression of CREB led to reduced intrahepatic DAG content and decreased PKCε activation (Samuel et al., 2007). Previous studies have demonstrated that PKCε binds to the insulin receptor, leading to reduced insulin receptor-β kinase activity. Moreover, decreasing expression of PKCε, using a similar ASO approach, protects rats from fat-induced hepatic insulin resistance (Samuel et al., 2007). In addition, knockdown of CREB resulted in reduction of PEPCK and transcriptional gene cofactors involved in the regulation of hepatic gluconeogenesis. It is likely that a combination of these two mechanisms contributed to the decreased glucose production observed in the CREB ASO-treated rats during the hyperinsulinemic-euglycemic clamp.

Surprisingly, CREB ASO treatment also decreased hepatic triglyceride content in the T2DM rat model, which we found could mostly be ascribed to decreased hepatic lipogenesis. These findings are in contrast to a previous study, which showed that dominant-negative CREB-expressing adenovirus indirectly increased expression of peroxisome proliferator-activated receptor gamma (PPARγ) and increased hepatic triglyceride content independent of SREBP by decreasing expression of hairy and enhancer of split 1 (HES-1), a transcriptional repressor of PPARγ (Herzig et al., 2003). In our model, we observed no increase in the level of PPARγ expression, possibly reflecting differences in the models used or the method of knockdown. We also saw reductions in lipid synthesis, as assessed by incorporation of [1-^{14}C]palmitate into hepatic triglycerides independent of differences in ChREBP or SREBP1c. Here, we observed decreased C/EBPα and C/EBPβ in the fasted and fed states with CREB ASO. C/EBPβ has been thought to reduce lipogenesis through PPARγ2 (Schroeder-Gloeckler et al., 2007); however,

in our study, we saw only a tendency for decreased PPARγ2 expression. Previous reports have indicated that decreases in C/EBPα are also associated with reductions in lipogenesis through potentially direct binding to the promoters of key lipogenic genes and through controlling upstream lipogenic transcription factors (Matsusue et al., 2004; Qiao et al., 2006). It is likely that both of these proteins contributed to the decreased lipogenesis observed with CREB ASO. Immunoneutralization of glucagon and the glucagon receptor (GCGR) ASO have both been shown to decrease triglycerides in certain rodent models (Sloop et al., 2004; Sørensen et al., 2006). The reductions of lipids in these models could potentially be mediated via reductions in CREB signaling, since glucagon is upstream of CREB. A recent study showed that CREB increased expression of adiponectin through ATF3, resulting in decreased hepatic lipids (Qi et al., 2009). However, in our study, we found no differences in plasma adiponectin concentrations. Taken together, these data suggest that the reduction in hepatic lipogenesis and improvement in hepatic insulin responsiveness that we observed in the CREB ASO-treated animals is most likely due to decreased hepatic expression of CREB and not to alterations in adiponectin secretion secondary to reductions in CREB expression in adipocytes. This hypothesis is further supported by our studies in the ZDF T2DM rat model, where we found no reductions in adipose tissue CREB expression but dramatic reductions in fasting plasma glucose concentrations.

In summary, this study demonstrates that reduction of CREB expression in liver and fat lowers plasma triglyceride and cholesterol concentrations in a T2DM rat model and protects against fat-induced hepatic steatosis and hepatic insulin resistance by reductions in PEPCK expression and hepatic diacylglycerols. Consistent with these results, CREB ASO proved to lower fasting plasma glucose concentrations in four different rodent models of T2DM and hepatic insulin resistance. This paper is in contrast to previous literature arguing that hepatic CREB would be a poor target for pharmacological inhibition (Canettieri et al., 2005; Herzig et al., 2003). Since T2DM is often associated with hypertriglyceridemia, hypercholesterolemia associated with NAFLD, and hepatic insulin resistance, CREB is a potentially attractive therapeutic target for T2DM.

EXPERIMENTAL PROCEDURES

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine. Male Sprague-Dawley rats, ZDF rats, or *ob/ob* mice were received from Charles River Laboratories (Wilmington, MA) and given 3 days to acclimate. For the T2DM rat model, rats were then given a 175 mg/kg dose of nicotinamide by intraperitoneal injection; after 15 min, rats were then dosed with a 65 mg/kg dose of STZ (Masiello et al., 1998). Rats were given 4 days of a recovery period and randomized for blood glucose values prior to the first ASO injection. Rats with overt diabetes (defined by a glucose value greater than 200 mg/dl) were excluded from the study. Rats were housed individually on a 12:12 hr light/dark cycle and received food and water ad libitum. Body weight and food consumption were monitored weekly. The food consumed consisted of regular rodent chow (60% carbohydrate, 10% fat, 30% protein calories); a high-fat diet (26% carbohydrate, 59% fat, 15% protein calories), in which the major constitute is safflower oil; a high-fat lard diet (24% carbohydrate, 55% fat, 21% protein), in which the major constitute is hydrogenated vegetable shortening; or, in the case of the ZDF rats, the Purina Lab Diet 5008.

In Vivo Lipogenesis

Heavy whipping cream (1.8 ml), sucrose (0.6 g; Sigma Aldrich; St. Louis), normal saline (0.3 ml), and [1-^{14}C]potassium palmitate (30 μCi) dissolved in 0.3 ml EtOH (PerkinElmer; Waltham, MA) were mixed together. The solution was gavaged into fasted ~380 g rats treated with either control ASO or CREB ASO. Plasma blood samples were taken at 0, 60, 180, and 360 min for free fatty acid enrichment. Rats were anesthetized with pentobarbital sodium injection (150 mg/kg), and all tissues were extracted and frozen immediately with the use of liquid N_2-cooled aluminum tongs. Ground liver (100 mg) was used for triglyceride enrichment and extracted using the method from Bligh and Dyer (Bligh and Dyer, 1959). The organic layer was evaporated to dryness and redissolved in 0.5 ml chloroform. Twenty-five microliters of the lipid extract solution was plated on a Silica Gel 60 plate and ran with a 80:20:1 solution of hexane:diethylether:acetic acid. The plate was dried and developed with 0.005% primuline in 80% acetone in water. The triglyceride spots were scraped off the plate and eluted with diethylether in a glass Pasteur pipette. The diethylether was evaporated to dryness, and the remaining residue was dissolved in 0.5 ml hexanes. Fifty microliters of the hexane mixture was measured using a radioactive scintillation counter to determine total counts.

Hyperinsulinemic-Euglycemic Clamp Studies

Hyperinsulinemic-euglycemic clamps were performed as previously described (Erion et al., 2009).

Biochemical Analysis and Calculations

Plasma insulin, glucagon, leptin, and adiponectin concentrations were determined using RIA kits (Linco; Billerica, MA). For FPLC analysis, samples from control ASO and CREB ASO were pooled together and injected on to an Amersham AKTA FPLC (Amersham Pharmacia Biotech/GE Healthcare; Piscataway, NJ) and eluted at a constant flow rate of 0.5 ml/min FPLC buffer (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA [pH 7.5]).

Tissue Lipid Measurement

The purification and measurements of DAGs and long-chain fatty acyl-CoAs from liver were performed as previously described (Bligh and Dyer, 1959; Neschen et al., 2002).

Liver Insulin Signaling

To test the effect of CREB treatment on insulin signaling, rats were treated exactly the same as described for the T2DM rat model. These rats underwent a 20 min hyperinsulinemic-euglycemic clamp, and immediately following the 20 min period, the liver was harvested and subsequently frozen with the use of N_2-cooled aluminum tongs. The activity of AKT was assessed by measuring the incorporation of ^{32}P onto a synthetic AKT substrate as previously described (Choi et al., 2007). PKCε and IRS-2 blots were performed as previously described (Choi et al., 2007). Blots were quantified as amount of PKCε relative to Na-K ATPase located in the membrane fraction (arbitrary units).

Total RNA Preparation, Real-Time Quantitative RT-PCR Analysis, and Immunoblotting Analysis

PCR was performed as previously described (Erion et al., 2009). For western blot analysis, 20 mg of powdered tissue was homogenized in 200 μl of homogenization buffer (50 mM Tris-HCl buffer [pH 7.5 at 4°C], 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol [DTT], 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride [PMSF], glycerol [10% v/v], Triton X-100 [1% v/v]). After centrifugation for 15 min at 10,000 *g*, 40 μg crude protein was added to an equal portion of loading buffer for 5 min of 95°C heat and then separated on a 4%–12% gradient polyacrylamide gel in MOPS buffer system (Invitrogen; Carlsbad, CA). Subsequently, membranes were transferred to nitrocellulose membranes, and membranes were incubated in blocking buffer (5% milk) for 1 hr and immunoblotted with anti-CREB antibody (Cell Signaling; Danvers, MA) overnight. The secondary antibody detection was performed as previously described (Erion et al., 2009).

Statistical Analysis

All values are expressed as the average SEM. The significance between the mean values for each study was evaluated by two-tailed unpaired Student's

t test or one-way ANOVA followed by post hoc analysis using the Tukey's Honestly Significant Differences (HSD) test.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and one figure and can be found online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00306-4.

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